The Assessment of Human Islets for Transplantation and The Effect of the Immunosuppressant Rapamycin on MIN-6 Cells, Rat and Human Islets

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Abstract

Human islet transplantation is currently successfully establishing a role in the management of certain patients with type 1 diabetes. For islet transplantation to be successful there must be an adequate supply of high quality islets, which are damaged neither by host immunological defences nor the immunosuppressants used.

In an attempt to characterise human islet quality several tests were developed. The static stimulation test assesses β-cell function and responsiveness to glucose by measuring insulin release. To assess the degree of exocrine contamination of the islet preparation, the amylase content of both the whole pancreas and islet preparation were measured. Measuring the insulin content of both the whole pancreas and islet preparation allowed us to quantify the increase in insulin concentration as a consequence of the islet isolation procedure. The mean exocrine contamination (EC) was 2.5 ± 0.7%, mean insulin enrichment (IE fold increase) 180 ± 37 and mean static stimulation index (SSI) was 1.47 ± 0.08 suggesting that the islet preparations have low levels of exocrine contamination, have significant insulin enrichment and release insulin in response to glucose. Thus the islet isolation procedure is reasonably successful at separating the endocrine from the exocrine pancreas. The curative potential of each islet material was then determined by transplanting representative islet samples to non-obese diabetic mice with severe combined immunodeficiency disease (NOD-SCID) as the biological endpoint in a standardised system. In the first two weeks following transplant, the degree of EC of the islet preparation may have some role in predicting the in-vivo function of islets, with those preparations of highest EC taking longer to restore normoglycaemia than the other groups. However, beyond 14 days post-transplant, EC is of no value in predicting the in-vivo function of islets. Similarly the degree of IE and the SSI have no value in predicting the in-vivo function of islets in this model. Thus, none of the biochemical indices (EC, IE nor SSI) are able to predict the in-vivo effectiveness or function of transplanted islets in the NOD-SCID mouse model.

Non-heart-beating donors (NHBDs) are generally not deemed suitable for whole organ pancreas donation but could provide a significant additional source of pancreata for islet transplantation if it was demonstrated that NHBD islets functioned no differently from

islets isolated from heart-beating donors (HBDs). The recovery of islets from NHBDs was comparable to that of control HBD. In-vitro assessment of NHBD islet function revealed function equivalent to those isolated from HBD, and NHBD islets transplanted to NOD-SCID mice efficiently reversed diabetes. A single donor transplant from a NHBD resulted in a state of stable insulin independence in a type 1 diabetic recipient. Thus, normally functioning islets can be isolated successfully from NHBD pancreata, suggesting that NHBDs may provide an untapped source of pancreatic tissue for preparation of isolated islets for clinical transplantation.

Rapamycin (sirolimus) is a macrolide fungicide with immunosuppressant properties that is used in human islet transplantation. Little is known about the effects of rapamycin on islets and MIN-6 cells (mouse clonal insulin-producing cells that are used experimentally as a β-cell model). Rapamycin had a dose-dependent, time-dependent, glucose-independent deleterious effect on MIN-6 cell viability. At day 1, using the MTT method (this mitochondrial succinate dehydrogenase activity assay is used as an indirect measure of cell viability), 0.01 nM rapamycin reduced cell viability to 83 ± 6% of control ($p<0.05$). Using the calcein AM method (a fluorescent marker of live cells), at day 2, 10 nM rapamycin caused a reduction in cell viability to 73 ± 5% of control ($p<0.001$). Furthermore, 10 and 100 nM rapamycin caused apoptosis in MIN-6 cells as assessed by the TUNEL assay (apoptotic nicked DNA is fluorescinated and detected at the single cell level by flow cytometry). Compared to control, there was a 3.1 ± 0.6 fold increase ($p<0.01$) in apoptosis in MIN-6 cells treated with 10 nM rapamycin. A supra-therapeutic rapamycin concentration of 100 nM significantly impaired glucose- and carbachol-stimulated insulin secretion of rat islets and had a deleterious effect on the viability of rat and human islets. Thus, currently there is no evidence that therapeutic concentrations of rapamycin have any in-vitro deleterious effect on islets.

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AIR_{ar}	acute insulin response to arginine
AIR_g	acute insulin response to glucose
ANOVA	analysis of variance
AP-1	activator protein-1
AST	arginine stimulation test
ATG	equine antithymocyte globulins
ATP/ADP	adenosine triphosphate/adenosine diphosphate ratio
AUC_{C-p}	area under the C-peptide curve
AUC_i	area under the insulin curve
BCA	bicinchoninic acid
BD	bladder drainage
BMI	body mass index
BSA	bovine serum albumin
CCH	carbachol
CII	cold ischaemic index
CIT	cold ischaemia time
CMV	cytomegalovirus
CNI	calcineurin inhibitors
DCCT	Diabetes Control and Complications Trial
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
4EBP1	eukaryotic initiation factor 4E binding protein-1
EC	exocrine contamination
ED	enteric drainage
eIF-4F	eukaryotic initiation factor-4 complex
ESRF	endstage renal failure
ethD-1	ethidium homodimer-1

FKBP12	FK506 binding-protein
G	glucose
GBM	glomerular basement membrane
GI	gastrointestinal
GRF	glomerular filtration rate
HBD	heart-beating donor
HDL	high-density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HITP	Human Islet Transplantation Program
HUP	Hospital of the University of Pennsylvania
IDDM	insulin-dependent diabetes mellitus
IE	insulin enrichment
IEQ	islet equivalent
IFG	impaired fasting glycaemia
IGT	impaired glucose tolerance
IL	interleukin
INS	insufficient
IPTR	International Pancreas Transplant Registry
IVGTT	intravenous glucose tolerance test
JDFI	Juvenile Diabetes Foundation (International)
K_G	glucose disposal
LDL	low-density lipoprotein
mAb	monoclonal antibody
MAP	mitogen-activated protein
MHC	Major Histocompatibility Complex

MMF	mycophenolate mofetil
6-MP	6-mercaptopurine
MPA	mycophenolic acid
mTOR	mammalian target of rapamycin
MTT	C,N-diphenyl-N'4-5-dimethyl thiazol-2-yl tetrazolium bromide
NF-AT	nuclear-factor-of-activated T cells
NF-κB	nuclear factor kappa B
NHBD	non-heart-beating donor
NICE	National Institute for Clinical Excellence
NIDDM	non-insulin-dependent diabetes mellitus
NIH	National Institutes of Health
NODAT	new-onset diabetes after transplantation
NOD-SCID	non-obese diabetic mouse with severe combined immunodeficiency disease
OGTT	oral glucose tolerance test
OKT3	muromonab-CD3
PAK	pancreas after kidney transplant
PBS	phosphate-buffered saline
PHAS-1	phosphorylated heat- and acid-stable protein
PI	propidium iodide
PSI	perifusion stimulation index
PTA	pancreas transplant alone
PV	portal venous drainage
RATG	rabbit antithymocyte globulins
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute tissue culture medium
SD	standard deviation
SEM	standard error of the mean

SPK	simultaneous pancreas and kidney transplant
SSI	static stimulation index
SV	systemic venous drainage
T	transplant number
TGN	thioguanine nucleotides
TNF	tumour necrosis factor
TPMT	thiopurine methyltransferase
TUNEL	transferase-mediated dUTP nick-end labeling
UK PDS	UK Prospective Diabetes Study
UNOS	US United Network of Organ Sharing
WHO	World Health Organisation
XO	xanthine oxidase

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Publications arising from this study

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Chapter 1

An Introduction to Diabetes Mellitus and Transplantation

Chapter 1 Introduction

1.1 Diabetes Mellitus

1.1.1 Introduction

Diabetes mellitus is defined as a metabolic disorder of multiple aetiology, characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism, resulting from defects of insulin secretion, insulin action or both (1). Prior to the discovery of insulin in the early 1920s, diabetes was invariably fatal within two years in young patients. Insulin therapy has revolutionised the treatment of diabetes and permitted young patients with diabetes to live a long and full life. It has however, introduced a new set of problems such as the risk of hypoglycaemia and the development of long-term macrovascular and microvascular complications.

The diagnosis of diabetes is based on blood or venous plasma glucose concentrations, taking into account the presence or absence of symptoms (1). Often, the initial test is a random plasma glucose. In the presence of symptoms of diabetes (thirst, polyuria and weight loss), a random plasma glucose concentration of greater than 11.1 mmol/L is sufficient to diagnose diabetes. In the absence of symptoms one further diagnostic glucose measurement is required for confirmation. A plasma glucose of less than 5.5 mmol/L indicates that the diagnosis of diabetes is extremely unlikely. Random plasma glucose concentrations between 5.5 mmol/L and 11.1 mmol/L are non-diagnostic and need to be repeated or a fasting plasma sample taken. A fasting plasma venous glucose concentration of greater than 7.0 mmol/L is diagnostic of diabetes mellitus (the previous diagnostic cut-off value was 7.8 mmol/L). A fasting plasma glucose concentration between 6.1 and 7.0 mmol/L places the patient in the category of impaired fasting glycaemia (IFG). Patients with a random plasma glucose in the non-diagnostic range and a fasting plasma glucose of less than 7 mmol/L should have an oral glucose tolerance test performed. In this test, the person is required to take an oral 75g-glucose load, and the plasma glucose concentration two hours later is measured. Two-hour plasma glucose concentrations of greater than 11.1 mmol/L indicate the presence of diabetes mellitus (irrespective of the fasting plasma glucose level), while concentrations between 7.8 and 11.1 mmol/L indicate impaired glucose tolerance (IGT). IGT represents an intermediate category between normoglycaemia and diabetes. Patients with IGT are more likely to develop diabetes and are at higher risk of cardiovascular

disease than normoglycaemic individuals (2). IFG is analogous to IGT, and is the preferred method of determining impaired glycaemic control according to the American Diabetes Association guidelines (3). The two methods of detecting impaired glycaemic control do not always detect the same patients. Whilst a fasting sample is easier to perform than an OGTT, it is not as reliable in detecting patients who are at risk of subsequent cardiovascular events (4).

In 1999 the World Health Organisation (WHO) updated its relevant classification system for diabetes in an attempt to make the classification more clinically relevant (Table 1). The categories are based on the aetiology of diabetes, rather than the treatment, as was the case in the previous classification (5). This has avoided the confusion caused by the terminology of insulin and non-insulin dependent diabetes.

1.1.2 Type 1 diabetes mellitus

Type 1 diabetes, previously referred to as insulin-dependent diabetes mellitus (IDDM), is characterised by pancreatic β-cell destruction and absolute insulin deficiency. β-cells are destroyed by an autoimmune process, and antibodies to glutamic acid decarboxylase, which is located in the cytoplasm of β-cells, are found in 70-80% of all patients with type 1 diabetes, but less than 1% of the general population (6). In common with most autoimmune diseases, there is a strong genetic predisposition to type 1 diabetes. Approximately 50% of the genetic predisposition can be attributed to the presence of a non-aspartic residue at position 57 of the beta chain of the human leukocyte antigen class II DQ molecule (7).

Type of diabetes	Definition
Type 1 Autoimmune or Idiopathic	Beta-cell destruction usually leading to absolute insulin deficiency
Type 2	May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance
Other specific types	Genetic defects of insulin function Genetic defects of insulin action Diseases of the endocrine pancreas Endocrinopathies Drug- or chemical-induced Infections Uncommon immune-mediated disease Other genetic syndromes
Gestational diabetes	Includes impaired glucose tolerance and diabetes in pregnancy

Table 1. The WHO classification of diabetes mellitus (1).

As β-cells are destroyed, insulin levels fall below that required to maintain normoglycaemia and patients often present subacutely with the classical triad of symptoms: thirst, polyuria and weight loss. If these warning symptoms are not heeded or if destruction of β-cells is particularly rapid, the person may develop absolute insulin deficiency and become ketoacidotic. The rate of β-cell destruction varies between individuals, but in general is relatively rapid; therefore type 1 diabetes has a short latent period prior to detection. As a state of absolute insulin deficiency exists, insulin therapy is required from the onset of diagnosis.

Type 1 diabetes tends to be diagnosed in younger people, usually before 35 years of age. The incidence rate of type 1 diabetes in the United Kingdom has been estimated at

13.5 per 100,000 in persons less than 15 years of age, and this incidence is increasing (8). Diabetes is the most common metabolic disease in the young. The Scottish Study Group for the Care of Young Diabetics demonstrated there is a rising incidence of diabetes in the under 16 years age-group, with an annual incidence of 25 per 100,000 population and a near tripling of new cases in the last 30 years (9).

1.1.3 Type 2 diabetes mellitus

Type 2 diabetes, previously known as non-insulin dependent diabetes mellitus (NIDDM), is associated either with insulin resistance or a defect in insulin secretion, with or without insulin resistance (10). There is no autoimmune β-cell destruction, thus the deficiency in insulin is relative rather than absolute, and indeed systemic insulin levels are often high. Type 2 diabetes, as currently defined, is likely to result from a number of heterogeneous pathological processes. Whilst the aetiology of the condition is uncertain, it is strongly associated with obesity (11). There is a stronger genetic predisposition to type 2 diabetes than type 1 diabetes, but the underlying susceptibility genes have not yet been elucidated (12).

As there is no absolute deficiency of insulin, patients rarely develop ketoacidosis, and often they do not require insulin therapy in the initial stages of the disease. The development of relative insulin deficiency is often insidious, thus impaired glucose tolerance or diabetes may be present for many years prior the diagnosis of diabetes (13). These individuals remain at increased risk of diabetic complications, and indeed up to 25% of patients with newly diagnosed type 2 diabetes already exhibit evidence of complications (14).

In America, the prevalence of diabetes in persons over 18 years of age increased from 4.9% to 7.3% between 1990 and 1998, an increase of almost 50%. This was primarily due to the increased prevalence of type 2 diabetes. In parallel, an almost 80% increase in the prevalence of obesity from 11.1% to 19.8% was observed, with a high degree of concordance between obesity and diabetes ($p<0.001$) (15,16). In 2000, it was estimated that around 1.4 million people in the UK have diabetes (17). At least a million more are thought to have diabetes but are undiagnosed (18,19,20). In the UK, it has been predicted that there will be 20% more people with type 2 diabetes alive in 2030 than in

2000 with a 20-30% increase in microvascular and macrovascular complications. Undoubtedly, this will have a significant economic impact on health care provision (21).

1.1.4 Complications of diabetes

The discovery of insulin by Banting and Best in 1922 revolutionised the treatment of diabetes, such that for the first time, a diagnosis of diabetes in a young person was compatible with relative longevity (22). However exogenous insulin delivery cannot replicate the endogenous mechanisms that control the secretion of insulin, consequently normoglycaemia cannot be continuously maintained. The chronic exposure to hyperglycaemia produces long-term complications, which are classified as microvascular or macrovascular.

Microvascular complications are specific to diabetes and include retinopathy, neuropathy and nephropathy. Over 90% of patients with type 1 diabetes diagnosed before 30 years of age will develop evidence of retinopathy after 15 years of diabetes (23). After 30 years of diabetes, approximately 60% will develop the more serious proliferative retinopathy, which potentially can lead to blindness (23,24). The most common forms of neuropathy are a peripheral symmetrical sensorimotor neuropathy and autonomic neuropathy, which can affect gastric motility, erectile function, bladder control and vascular tone. In the Pittsburgh Epidemiology of Diabetes Complications Study, 30% of patients who had type 1 diabetes for 20 years developed distal symmetrical polyneuropathy (25).

Macrovascular complications are due to accelerated atherosclerosis, and include ischaemic heart disease, peripheral vascular disease and cerebrovascular disease. The macrovascular complications are not specific to diabetes, but are more common in patients with diabetes (26,27,28). This is particularly true for type 2 diabetes, which is often accompanied by other vascular risk factors such as hypertension, dyslipidaemia and obesity (29).

1.1.5 Unstable diabetes

Unstable (or brittle) diabetes implies glycaemic instability in type 1 diabetics sufficient to cause life disruption. Glycaemic instability can manifest as recurrent admissions to hospital with diabetic ketoacidosis or hypoglycaemia. As there is no generally agreed

definition of unstable diabetes, estimates of prevalence vary from 0.3% to 'almost all diabetics', although it is likely that the prevalence lies somewhere between 0.1 to 0.5% (30). Of those type 1 diabetics with unstable diabetes, the vast majority (59%) have recurrent admissions solely with ketoacidosis, 17% with hypoglycaemia and 24% recurrent admissions with both. Most patients are females between the ages of 15 and 25 years. Diabetologists caring for those patients considered there to be an organic cause (autonomic neuropathy, subcutaneous insulin resistance, dementia, coeliac disease, chronic renal failure, hypopituitarism or steroid treatment) in less than 10% of cases. The rest were considered to be behavioural or psychosocial in origin, attributable to domestic problems, anorexia, denial, self-control or alcohol or drug addiction (30). Most patients with unstable glycaemia become more stable with time, however, not all patients live long enough to stabilise; there is a significant morbidity and mortality associated with unstable glycaemia and in addition a much poorer quality of life (31). Thus, although the prevalence of unstable glycaemia in the type 1 diabetic population is likely to be low, these patients have a poor quality of life and consume a disproportionate amount of health-care resources.

1.1.6 Discussion

Parenteral insulin therapy does not cure diabetes; it simply controls diabetes by lowering blood glucose levels, often sub-optimally. Parenteral insulin regimes attempt to replicate the physiological patterns of β-cell insulin release, however the finely-tuned release of the counter-regulatory hormones insulin, glucagon and others to maintain normal blood glucose levels are unachievable with current pharmacological technology. The chronic exposure to hyperglycaemia can lead to the long-term complications described previously. However, tightening glycaemic control (and thus reducing the long-term risk of complications) comes at a price. The Diabetes Control and Complications Trial Research Group showed that intensive insulin therapy to tighten diabetic control resulted in a three-fold increase in severe hypoglycaemia (32). Acute hypoglycaemia can cause drowsiness, confusion, disorientation, coma, cardiac arrhythmia and death. Recurrent, prolonged hypoglycaemic attacks are associated with permanent neurological deficits such as hemiparesis, memory impairment, diminished language skills, decreased abstract thinking capabilities and ataxia.

Thus type 1 diabetic patients are dependent on life-long parenteral insulin for survival and are in the unenviable position of setting (if they can) their glycaemic control somewhere on the spectrum between two extremes. They can chose tight glycaemic control (with reduced risks of long-term complications but increased risks of hypoglycaemia), poor glycaemic control (with increased risks of long-term complications but reduced risks of hypoglycaemia), or, as is usually the case, somewhere in between.

The concept of a cure for diabetes is the driving force behind innovations such as pancreatic and islet transplantation; the aim being to render diabetics normoglycaemic and independent of insulin, improve day-to-day quality of life and ameliorate secondary complications.

1.2 Diabetic nephropathy

1.2.1 Definition and diagnosis

Diabetic nephropathy is a chronic glomerular disease characterised by persistent proteinuria, increasing blood pressure and declining renal function in a patient who has diabetes. Clinically, diabetic nephropathy is diagnosed as the presence of proteinuria (>300mg albuminuria/24 hours) in a person with diabetes who has no other cause of renal disease and no concurrent urinary infection. It is a major cause of morbidity and mortality in persons with diabetes. The presence of proteinuria in a patient with type 1 diabetes confers an eight-fold increased risk of death from cardiovascular disease compared with diabetic patients with no proteinuria (33). This represents a relative mortality that is a 37 times greater than in the general population. In addition, diabetic nephropathy is now the commonest cause of end-stage renal disease in the Western world (34).

The detection of proteinuria is therefore key to diagnosing diabetic nephropathy. Overt nephropathy with proteinuria can be detected by urinary dipstick, a simple qualitative measurement. However, urinary dipsticks for proteinuria have a minimum detection limit of 100-200 mg/L, and thus are insufficiently sensitive to detect smaller concentrations of albumin (microalbuminuria). Thus the urinary dipstick is primarily used as a screening tool to detect overt nephropathy. Following a positive test, the

amount of proteinuria should be quantified. There are three methods of quantifying the degree of proteinuria (Table 2). Historically the standard method has been a 24 hour urine collection for albumin excretion, but this is problematic, as it requires the patient to collect their urine each time they micturate. This can be simplified by performing a timed overnight collection, and calculating the rate of albumin excretion. A more practical approach for screening purposes is to determine the albumin/creatinine ratio in a spot urine sample. By referencing the albumin concentration to the creatinine concentration, allowance is made for the state of hydration of the patient.

	24hr urinary albumin excretion (mg)	Timed overnight collection (µg/min)	Albumin/creatinine (mg/mmol)
Proteinuria	>300	>200	>30
Microalbuminuria	30-300	20-200	3-30
Normoalbuminuria	<30	<20	<2.5 (F) <3.5 (M)

Table 2. Methods of quantification of albuminuria.

1.2.2 Epidemiology

The incidence and prevalence of diabetic nephropathy have been steadily increasing over the last 20 years, and it has now become the single most common cause of end-stage renal failure in the Western world (34). In 2000, it accounted for over 43% of all new cases of end-stage renal failure in the USA. A large part of the 52% increase in the incidence of end-stage renal failure in the USA between 1991 and 2000 can be accounted for by the 86% increase in diabetic nephropathy during this period. The increased incidence of diabetic nephropathy was primarily due to a dramatic rise in the number of patients with type 2 diabetic nephropathy. This accounted for over 86% of all new cases of diabetic nephropathy in 2000. The median age for developing end-stage renal disease was 48 and 65 years for patients with type 1 and type 2 diabetic nephropathy respectively.

The incidence of diabetic nephropathy is not increasing as rapidly in the UK. In 2001 in England and Wales, diabetic nephropathy was the single most common cause of new cases of end-stage renal failure, but it accounted for only 18.6% of all cases (35). The median age for developing end-stage renal disease was 51 and 65 years for patients with

type 1 and type 2 diabetic nephropathy respectively. In contrast to the USA, patients with type 2 diabetic nephropathy comprised only 31% of all diabetes patients on renal replacement therapy.

Diabetic patients have increased morbidity and mortality on renal replacement therapy compared to non-diabetic patients. In 2001 in England and Wales, patients less than 65 years old had one-year survival rates on dialysis of 82% and 92% for those with and without diabetes respectively. The equivalent rates for those over 65 years old are 72% and 78%. Furthermore, patients with diabetes are less likely to receive a renal transplant, which is the only form of renal replacement therapy compatible with long-term survival in diabetic patients. In 2001 in England and Wales, the proportion of patients who had a renal transplant as the mode of renal replacement therapy was 37% and 13% for patients with type 1 and type 2 diabetes respectively. This compares unfavourably with the 51% observed for non-diabetic patients.

In summary, diabetic nephropathy is the single most common cause of end-stage renal failure in the Western world and its incidence continues to rise. This places a huge demand on already stretched healthcare resources. Individuals with diabetes and proteinuria are 37 times more likely to die of cardiovascular disease than age-matched non-diabetic individuals. Patients with end-stage renal failure due to diabetic nephropathy have one-year survival rates on dialysis of between 72% and 82%. This is worse than many forms of cancer. Hence any interventions that can reduce the incidence or progression of diabetic nephropathy could potentially have a significant impact on the individual with diabetes and healthcare finances.

1.2.3 Natural history

The natural history of diabetic nephropathy can be divided into a series of distinct stages that relate glomerular structural changes to clinical observations. The first evidence of diabetic nephropathy is glomerular hypertrophy, which is associated with hyperfiltration, and can be detected clinically as a raised glomerular filtration rate (GFR). The earliest histological abnormalities detected are thickening of the glomerular basement membrane (GBM) and diffuse mesangial expansion. These have both been detected within two years of the onset of diabetes (36). These changes are associated with an increase in blood pressure although it is still within the normal range (there may

be loss of the nocturnal dip). It is unknown whether the increase in blood pressure is primary or secondary, but it does predict the subsequent development of microalbuminuria (37). In the early stages of GBM thickening and mesangial expansion there is no increase in urinary protein excretion.

As the mesangial expansion increases, the first clinical signs of the development of kidney dysfunction becomes apparent. Microalbuminuria occurs and blood pressure rises by approximately 3mmHg per year. Mesangial expansion correlates closely with the presence of proteinuria (38). The presence of microalbuminuria is associated with a 24 times greater risk of subsequent overt nephropathy, and hence it functions as an excellent screening toll for identifying those at risk of nephropathy (39).

As diabetic nephropathy progresses, mesangial expansion is accompanied by early glomerulosclerosis. Around this time, proteinuria can be detected by dipstick testing; therefore this phase is known as overt proteinuria. As the mesangium accumulates, it occupies a greater proportion of the glomerulus, resulting in a smaller surface area available for filtration (38). In conjunction with increasing glomerulosclerosis, there is a progressive decline in GFR, until endstage renal disease is reached, usually within seven years of the onset of proteinuria (40).

1.2.4 Role of hyperglycaemia in the pathogenesis of diabetic nephropathy

It is well established that poor glycaemic control is associated with increased risk of subsequently developing nephropathy (40,41,42). The Diabetes Control and Complications Trial (DCCT) showed that aggressive glycaemic control in patients with type 1 diabetes could reduce the risk of developing nephropathy and the progression of early nephropathy (32). In this study intensive glycaemic control was compared to standard therapy in the primary and secondary prevention of nephropathy. Intensive control reduced the risk of developing microalbuminuria by 34% ($p=0.04$) and 43% ($p=0.001$) in the primary and secondary prevention groups respectively. It also reduced the risk of progression to proteinuria by 56% in the secondary prevention group ($p=0.01$).

The effect of improved glycaemic control remained after four years of follow-up despite the fact that the difference in glycaemic control between intensive and conventional

groups narrowed significantly (43). It is primarily based on the evidence from this study that the UK National Service Framework for diabetes recommends a target HbA1c of less than 7% for all patients with diabetes (44).

The evidence for a beneficial effect of intensive glycaemic control in retarding progression in patients who already have established nephropathy is less convincing. One study has shown a reversal of the histological lesions of diabetic nephropathy in patients at ten years following a successful pancreatic transplant (45). It does appear, however, that in advanced diabetic nephropathy the relative importance of systemic and intraglomerular hypertension may mask the effect of glycaemic control. This is in keeping with a study which showed that in patients with diabetic proteinuria who had good blood pressure control, the fall in glomerular filtration rate correlated significantly with glycaemic control (46).

1.2.5 Role of hypertension in the progression of diabetic nephropathy

While hyperglycaemia is essential for the pathogenesis of diabetic nephropathy, it alone is insufficient. This is vividly demonstrated by two cases in which patients with long-standing diabetes had co-existing unilateral renal artery stenosis (47,48). In the adequately perfused kidney, there was histological evidence of diabetic glomerulosclerosis, while in the contralateral stenosed kidney, no diabetic lesions were observed despite the existence of an identical metabolic environment. This illustrates the importance of haemodynamic factors in the pathogenesis of nephropathy.

The strongest evidence for the beneficial effect of lowering blood pressure on the progression of diabetic nephropathy comes from studies in type 2 diabetics (49). In this study, patients were assigned to either tight or less-tight blood pressure control, and followed up prospectively for a mean period of 8.4 years. The mean blood pressure in the tight control group was significantly lower than in the less-tight group (144/82 versus 154/87, $p<0.0001$). At six years of follow-up there was a 29% reduction in the progression to microalbuminuria (defined as urinary albumin concentration >50 mg/L, $p=0.009$), and a non-significant reduction in the progression to proteinuria (defined as urinary albumin concentration >300 mg/L, $p=0.06$). Based primarily on this data, the current National Institute for Clinical Excellence (NICE) guidelines recommend a target

blood pressure of 140/80 for patients with diabetes who have no evidence of nephropathy and 135/75 for diabetics with either microalbuminuria or proteinuria (50).

1.3 Pancreatic Transplantation

1.3.1 Historical overview

The aim of the surgeons who first attempted pancreatic transplantation was a belief that those diabetic patients with end-stage renal failure required normalisation of their glycaemic control by pancreatic transplant to ensure the sustained viability of the transplanted kidney. This proved to be not entirely correct. However, the relative success (at that time) and perceived benefits of pancreatic transplant (with or without kidney) provided continued impetus to the development of pancreatic transplantation programs.

The first human pancreas transplant took place in December 1966 at the University of Minnesota and was reported in 1967 (51). The recipient was a diabetic patient with chronic renal failure, who in addition to a simultaneous kidney transplant (SPK), also received a segmental duct-ligated pancreas graft transplanted into the extraperitoneal position in the left iliac fossa. Vascular reconstruction was performed to the recipient external iliac vessels. This resulted in immediate insulin independence. Graft function persisted for two months when the patient died of sepsis, related to surgical complications. Between 1966 and 1973, the University of Minnesota group went on to perform 13 whole pancreas transplants (52). Of these 13 transplants, seven were performed with internal pancreatic duct exocrine drainage via a Roux-en-Y duodenojejunostomy. Thus the transplanted exocrine pancreas drained into the donor duodenum and then into the recipient's jejunum. Cutaneous graft duodenostomy was performed on another five patients. In these cases the exocrine pancreatic secretions drained into the donor duodenum and then externally via a graft duodenostomy. One patient had the donor papilla of Vater anastomosed to the recipient's bowel. The first 11 patients were uraemic diabetics (ten had SPK transplants and one had a pancreas transplant alone, in a patient who remained on dialysis) and three were non-uraemic diabetic patients (pancreas transplant alone). It was thought at this stage in the evolution of kidney transplantation that for kidney transplants to be successful correction of the diabetic state was required, although this was later challenged (53, 54). Indeed, none of

the pancreas allografts in the ten uraemic SPK patients rejected, all losses resulting from technical complications (thrombosis, infection, anastomotic leak) or death with a functioning graft. One uraemic SPK patient remained insulin-independent for over a year and died with a functioning pancreatic graft after the reinstitution of dialysis as a consequence of renal artery stenosis (52). This was the longest documented functioning graft until a series of SPK segmental transplants draining into the ureter were performed in the early 1970s in New York, which produced a recipient who remained insulin-independent for five years (55). This series of pancreatic transplants at the University of Minnesota ended in 1973 with the expectation being that islet transplantation would imminently surpass pancreatic transplantation (56). This turned out to be overly optimistic and in 1978 a clinical pancreatic transplant program was reinstituted at the University of Minnesota (57).

Surgeons in Sweden (1976-1982) also attempted enteric drainage of pancreatic secretions by performing a Roux-en Y pancreaticojejunostomy, however, as in previous cases, pancreatic and enterocutaneous fistulae occurred in all patients (58,59,60).

Many attributed the early failures of pancreatic transplant to complications related to the graft duodenum and hence in the late 70s and early 80s, several groups tried segmental pancreatic grafts (body or tail of the pancreas) which did not involve transplanting the donor duodenum. However the management of exocrine secretions continued to be a major problem and thus various methods were devised to control the exocrine drainage.

In 1978, surgeons in France attempted segmental pancreas transplantation with obliteration of the exocrine pancreas by inducing pancreatic duct occlusion by injecting the duct with Neoprene (polychloroprene), a synthetic polymer (61,62). Although exocrine pancreatic function was eliminated, foreign body inflammation, pancreatitis and graft damage ensued and hence, overall this technique was not deemed successful.

Other groups including the University of Minnesota attempted segmental pancreatic transplants with free intraperitoneal drainage of the exocrine secretions (63,64). Although the first recipient of an open-duct graft (PAK) achieved insulin-independence for 17 years, the open-duct graft procedure was successful in less than half of cases (65). Free peritoneal drainage of pancreatic secretions led to recurrent chemical

peritonitis and ascites often requiring graft irradiation and occasionally graft loss (63,66).

Thus alternative measures to manage exocrine pancreatic secretions were required. Initial attempts at anastomosing the pancreatic duct to the recipient ureter were unsuccessful as the procedure was technically challenging and necessitated an ipsilateral nephrectomy. In addition, anastomotic leaks were common and to avoid them permanent silicone stents had to be inserted (67).

In the early 80s the University of Wisconsin modified the urinary drainage technique and drained the pancreatic duct into the recipient bladder (68). This reduced the acute complication rate experienced by Gliedman using the ureter (69). Nghiem and Corry made further modifications at the University of Iowa in 1987, by transplanting the whole pancreas and donor duodenum and anastomosing the donor duodenum to the recipient bladder (70). This technique was quickly adapted by most centres and remained the dominant surgical technique for management of pancreatic graft exocrine secretions into the 90s (71).

The advantage of bladder drainage is that although the complication rate is not necessarily lower than enteric drainage, the acute morbidity and severity of the complications are less (71). Also, in patients with a solitary pancreas transplant (PTA), a reduction in urinary amylase is a useful marker of organ rejection. Indeed, in pancreatic rejection, a reduction in urinary amylase can precede hyperglycaemia by several days. Although a decline in urinary amylase is sometimes preceded or accompanied by a rise in serum pancreatic enzyme levels, there have been several reports of rejection episodes occurring when only the urinary amylase level declined (72). With a SPK transplant, bladder drainage is not so crucial, as a rising serum creatinine indicates renal rejection (and concomitant pancreatic rejection if from the same donor). Indeed in SPK transplants serum creatinine rises before pancreatic exocrine and endocrine dysfunction occurs.

The chronic complications of bladder drainage include recurrent bladder infections, haematuria, acidosis and dehydration. If the complications are sufficiently severe, enteric conversion can be performed. The first conversion was reported by the

University of Cincinnati in 1987 (73). In one series of bladder drainage transplants, the enteric conversion rate was 10% (71).

Segmental pancreas transplantation did not completely disappear as this technique did facilitate the use of living donors, which was first attempted in 1979 and since then the University of Wisconsin has performed over 100 living donor transplants (74). Initially, recipients of living-donor pancreata were advantaged, as the incidence of rejection was less than that for cadaveric pancreata. However as immunosuppression and the results of cadaveric pancreatic transplants improved, the incentive to perform segmental living donor transplants receded (75).

The consensus site for pancreatic transplant has varied little; the vast majority are situated in the recipient's pelvis, with arterial anastomosis of a donor Y-iliac extension graft to join the superior mesenteric and splenic arteries on the pancreas to the recipient right common iliac or external iliac artery. A variety of techniques have been used for venous drainage. Several patients had drainage of segmental pancreas transplant venous effluent into the recipient's portal system, either via the recipient's splenic vein, superior mesenteric vein or inferior mesenteric vein. In the 1980s portal drainage of pancreas graft venous effluent was performed in a few patients in several centres (76,77,78,79). However, it wasn't until a series of cases of routine use of portal venous drainage in SPK transplants was reported in 1992 that others adopted the technique (80,81). Other groups used the recipient's iliac vein to drain the transplanted pancreatic venous effluent. It makes more physiological sense for the transplanted graft venous effluent to drain via the recipient's portal system and avoid the relative hyperinsulinaemia associated with systemic drainage (82).

In summary, whole organ pancreatic transplant started in the late 1960s and continued until the early 1970s. Thereafter segmental transplantation, which facilitated living donor transplantation, was popular from the late 1970s to the early 1980s, but then was largely replaced with a return to whole-organ transplant. Enteric drainage, pancreatic duct injection and subsequently bladder drainage have been successively dominant from the early 1970s to the present date.

1.3.2 Simultaneous pancreas and kidney transplantation (SPK)

This is the preferred procedure in type 1 diabetics with endstage or near endstage renal disease (ESRF). Patients with type 1 diabetes with impending ERSD who have minimal or limited secondary complications of diabetes and are between the ages of 20 and 40 years are considered optimal candidates for SPK. One exception to this is the young diabetic patient for whom a suitable living related renal transplant is available. A living related renal transplant offers excellent long-term results with less immunosuppression than is required for SPK transplantation. However, not all type 1 diabetics with renal failure are acceptable candidates. It has been reported that only 64 per cent of diabetic patients screened are actually accepted for SPK. Severe cardiovascular illness has been identified as the main criteria for limiting patient selection. Patients who have undergone cardiac angioplasty or coronary artery bypass can be accepted but only if they have adequate left ventricular function without demonstrable ischaemia. Blindness, history of major amputation, or history of cardiac disease are considered to be relative contraindications to SPK. Although these diabetes-related problems are not reversible, there are a number of patients who are well adjusted to these complications and hence potentially can lead productive lives after dual organ transplantation.

Another factor to be considered is the timing of the transplantation. Pre-emptive transplantation offers the additional advantage of halting the diabetic complications before uraemia develops. Pre-emptive transplantation refers to the use of transplantation for primary renal replacement before dialysis commences. The aim is to take advantage of the possible benefits of transplantation over dialysis. These include improved survival, reduced costs, and reduced morbidity. Furthermore, if the increased waiting times, the variable progressive nature of diabetic complications along with the diminished survival that type 1 diabetics have on dialysis are taken into account, it can be argued that SPK transplantation should be carefully thought of as a potential treatment for diabetic patients before dialysis (83).

1.3.3 Pancreas transplantation alone (PTA)

Ideally, solitary PTA should be performed before the development of diabetic complications such as the need for a renal transplant. However, at present no reliable markers exist to predict, before the appearance of early lesions, which diabetic patients will develop complications. In some USA centres, PTA has been reserved for those

patients with very unstable diabetes or hypoglycaemic unawareness that is life-threatening.

Indications for PTA include two or more diabetic complications including evidence of early diabetic nephropathy such as microalbuminuria, proteinuria or early histological changes but with relatively preserved renal function (creatinine clearance greater than 70 mL/minute). Other indications may include glucose hyperlability, defined as frequent episodes of hypoglycaemia without frank symptoms which lead to a significantly poor quality of life and increased risk of trauma or sudden death. Nevertheless, when non-uraemic diabetics are concerned, the morbidity and mortality associated with the long-term immunosuppression and the surgical procedure itself must be weighed against the benefits of reversing or halting the progression of secondary end-organ diseases, reducing the risk of hypoglycaemic events, and improving quality of life. In summary, PTA is only appropriate in non-uraemic patients where the problems of diabetes are perceived to be more serious than the potential problems of immunosuppression. In the diabetic patients whose metabolic control is so fragile that their life is chaotic, PTA may be their only hope for a better lifestyle (83).

1.3.4 Pancreas after kidney transplantation (PAK)

In type 1 diabetic patients with a well functioning kidney transplant, sequential PAK transplant has been advocated because these patients are already receiving chronic immunosuppression. The benefits of a subsequent PAK transplant are improved quality of life and the fact that a functioning pancreatic allograft will likely prevent or reverse early diabetic changes in the existing kidney transplant. However, only patients with stable and adequate renal transplant function (creatinine clearance greater than 50 mL/minute) should be considered for a PAK transplants. Patients with marginal function of their transplanted kidneys should instead be considered for a SPK (or no pancreas) because intensified calcineurin inhibitor therapy used postoperatively may have a detrimental effect on renal function (83).

1.3.5 Current practice

The various current techniques for pancreatic transplantation can be broadly classified according to the type of exocrine drainage performed. Bladder drainage (BD) is currently the most common method of duct management because of the ability of this

procedure to allow monitoring of urinary amylase as a marker of pancreatic rejection, especially when PTA and PAK are performed. Due to recent improvements in immunosuppression and antimicrobial prophylaxis, enteric drainage (ED) is increasingly being utilised as a method for managing the exocrine secretions. ED is associated with a significant reduction in urological and metabolic complications with no increase in septic complications (84). Indeed of all SPK transplants performed in 1998, ED was performed in over 50% of cases, whereas in 1994, ED was performed in only 6% (85). BD is reserved for PTA cases, to allow monitoring of the urinary amylase. In contrast to BD, with ED the length of the donor duodenum is not as critical as ED pancreatic secretions are reabsorbed in the distal bowel segment.

Currently, there are two types of venous drainage practised and the preferred method remains controversial. Systemic venous drainage (SV) is used in over 90% of transplant centres and is associated with a favourable outcome (86). It involves anastomosis of the donor portal vein to the recipient's common or external iliac vein or vena cava. However, a theoretical disadvantage of SV drainage is the high levels of insulin in the systemic peripheral circulation. Hyperinsulinaemia has been shown in some experimental systems to be associated with insulin resistance and dyslipidaemia (87).

Portal venous drainage (PV) is the preferred technique in some centres and is claimed to be more physiological than SV drainage. Although follow-up is limited, it is claimed that this technique results in excellent graft survival rates and a reduced number of surgical complications. (88). The current technique of PV drainage is based on the technique described by Shokouh-Amiri et al (89) and involves anastomosis of the donor portal vein to the recipient's superior mesenteric vein, which subsequently drains into the recipients portal vein. In addition to the potential physiological advantage, PV drainage is technically easier to perform than SV drainage and anastomosis to a mesenteric vein does not increase the risk of thrombosis (90).

In the majority of cases, a donor Y- (right common or external) iliac artery extension graft is used to join the superior mesenteric and splenic arteries on the pancreas.

Segmental pancreas transplantation is used as a therapeutic option in the few US centres offering live donor transplantation and in two European centres, which continue to

perform segmental transplants from cadaveric donors (86). Segmental pancreas donation involves dividing the graft pancreas at the neck to give two pancreas segments – the head, which is supplied by the superior mesenteric artery and portal vein and the body and tail, which is supplied by the splenic artery and vein. Transplantation of both these segments has been described, although currently the tail is the preferred segment for transplantation (in live donation). As with the majority of whole pancreatic transplants, the recipient iliac vessels are used to reconstruct the vascular supply of the donated pancreatic segment. Hence segmental pancreas transplantation has systemic venous drainage and the theoretical risks attached therein. Methods of duct management with segmental pancreas grafts include BD and duct injection. As there is no graft duodenum to anastomose to the bladder, the segmental pancreatic duct is anastomosed to the bladder mucosa. As the exocrine pancreatic secretions drain into the bladder the urinary amylase can be monitored for evidence of segmental pancreas rejection. Another alternative for controlling exocrine secretions, described previously, is to inject the duct with silicone or neoprene. The disadvantage of this approach is that monitoring of rejection by urinary amylase is not possible and complications may occur.

1.3.6 Outcomes

1.3.6.1 Introduction

The International Pancreas Transplant Registry (IPTR) is located at the University of Minnesota, Minneapolis and, in cooperation with over 200 centres, maintains a database of all reported pancreas transplants and the pre-transplant and post-transplant courses worldwide. Biostatistical analyses are performed regularly and the results are published or presented at international and national scientific meetings or on-line (91).

1.3.6.2 Type of transplant

As of June 2003 19,600 patients have received pancreas transplants. Over 14,300 pancreas transplants were reported from USA sites and over 5,300 from non-USA sites. In the USA, from 1998 to 2002, the majority of all transplant cases per year were SPK (about 79%), with PAK increasing significantly over time to about 14% per year and PTA increasing to about 6%. Pancreas transplants outside the USA, reported to the IPTR over a similar period, reveals that as in the USA, the vast majority of cases are SPK (92%), but compared to US totals the proportion of PAK (5%) and PTA (3%) transplants was lower.

1.3.6.3 Success of pancreatic transplants

From 1997 to May 2003, the patient and graft survival rates for primary cadaveric pancreas transplants at 1 year are described in Table 3.

	Patient 1 year survival rates (%)		Pancreas graft 1 year survival rates (%)	
Transplant type	US (n=4818)	Non-US (n=1649)	US (n=4805)	Non-US (n=1726)
SPK	95	96	85	85
PAK	98	INS	79	INS
PTA	95	INS	79	INS

Table 3. Patient and graft 1 year survival rates for primary pancreatic transplants from 1997 to 2003 for each type of transplant in the US and non-US countries (91). SPK, simultaneous pancreas-kidney; PAK, pancreas after kidney; PTA, pancreas transplant alone; INS, insufficient numbers to calculate.

The US pancreas graft survival rates from 1998 to 2003 compare favourably with the rates from 1994 to 1998 (SPK 83%, PAK 71% and PTA 64%) and from 1987 to 1993 (SPK 76%, PAK 47% and PTA 48%). In the US SPK recipients the simultaneous kidney graft 1 year survival rate was 92%. There were insufficient non-US solitary pancreas transplants to calculate meaningful survival rates (91).

1.3.7 Risks and benefits

Although initially beset with dismal survival rates, advances in surgical technique, immunosuppression, anti-viral prophylaxis and post-transplant monitoring have had a significant positive impact in lowering the morbidity and mortality associated with the transplant technique (92).

As described previously, the results of PTA in carefully selected individuals are impressive with 1-year graft survival of over 80% and patient survival as high as 98% (92,93). However, the procedure is still associated with significant risk of morbidity. Although technical complications have diminished (with improvements in surgical technique and immunosuppression), there are still problems with rejection, graft pancreatitis, anastomotic leak or thrombosis which can lead to an extended hospital stay and on occasions death. However these risks have to be weighed against the potential benefits of a successful whole pancreas transplant. These include freedom from insulin

injections, blood glucose monitoring and dietary restrictions all of which would significantly improve the quality of life, especially in those diabetics with glycaemic instability and hypoglycaemic unawareness (94).

There is no doubt that pancreatic transplantation has a beneficial effect on diabetic complications (despite the transplant often occurring late in the course of the disease), although it takes some time for these positive effects to become significant. It can take a decade of excellent glycaemic control in non-uraemic recipients, following pancreas transplantation, for there to be any reversal of diabetic lesions in the native kidney (95). More importantly, type 1 diabetic recipients of SPK transplants survive significantly longer than diabetic recipients receiving kidney-alone grafts (96,97).

In conclusion, despite the improvements in patient and graft survival rates and the considerable progress made, whole organ pancreas transplantation is a complex surgical procedure that requires life-long immunosuppression. Consequently, whole organ pancreas transplantation is usually restricted to those type 1 diabetic patients with advanced chronic disease, as the risks of the procedure may outweigh the risks of the disease.

1.4. Immunosuppression

1.4.1 Introduction

The immunosuppressive regimens used in pancreas allograft recipients are the same as those for other organs. From the 1960s to the early 1980s the only available maintenance immunosuppressants were azathioprine and steroids. In 1980, the 1-year pancreas graft survival rate overall was 20 per cent (98). Following the introduction of cyclosporine for general use in the mid-1980s, the 1-year graft survival rates reached approximately 75 per cent for SPK transplants and approximately 50 per cent for PAK and PTA cases (99). Tacrolimus and mycophenolate mofetil came into use in the mid-1990s (100,101) and current 1-year pancreas graft survival rates are over 80 per cent in all categories (71).

1.4.2 Induction therapy

1.4.2.1 Introduction

Polyclonal and monoclonal antibody (mAb) preparations are used primarily as induction agents, that is, drugs that are prophylactically administered in the peri-transplant period in an attempt to prevent acute rejection. Prophylaxis against early acute rejection is especially beneficial in managing the recipient with delayed graft function. The agents provide effective immunological cover during a period when calcineurin inhibitors are either delayed or administered in sub-therapeutic doses until graft function improves. Although equine antithymocyte globulins (ATG), rabbit antithymocyte globulins (rATG) and muromonab-CD3 (OKT3) have been used extensively in induction therapy, the original indication for all 3 agents was not prophylaxis, but rather the treatment of acute rejection.

1.4.2.2 Polyclonal antibodies

Polyclonal antibody preparations have been used for immunosuppressive therapy in transplantation for more than two decades. These preparations are purified immunoglobulin preparations derived from animals after immunisation with human thymocytes that contain antibodies with multiple, distinct antigen-combining sites or epitopes.

1.4.2.3 Monoclonal antibodies

A mAb is an antibody derived from a single clone that is active against a single target antigen. mAbs are manufactured using the hybridization technique to produce immortal hybridomas; murine myeloma cells are fused with antibody-producing B cells from the spleens of mice immunized against a particular antigen. The resulting hybridoma yields an infinite supply of purified, antigen-specific antibody against that particular antigen. Because each molecule of antibody is produced by descendants of a single B cell and will react with only one specific antigen, this is a monoclonal antibody.

1.4.2.4 Mechanisms of action

The first mAb to be approved for clinical use in humans was OKT3. OKT3 is a murine monoclonal IgG2a antibody that specifically reacts with the T-cell receptor-CD3 complex on the surface of circulating human T cells. OKT3 binds to a glycoprotein (the 20-kd epsilon chain) on the CD3 complex to activate circulating T cells, resulting in a

transient activation of T cells, release of cytokines, and blocking of T-cell proliferation and differentiation. Nearly all functional T cells are transiently eliminated from the peripheral circulation. Although T cells reappear in the circulation during the course of treatment, these cells are CD3-negative and are not capable of T-cell activation. T-cell function usually returns to normal within approximately 48 hours of discontinuation of therapy.

OKT3 acts in 2 phases (102). During the first phase, which begins immediately after injection, circulating T cells are depleted, primarily as a result of opsonization in the liver and cytolysis. The second phase of OKT3 action involves antigenic modulation. The CD-3 complex on the cell surface is removed, producing immuno-incompetent T cells, without further depletion of the T-cell population (103).

The evaluation of OKT3 as specific therapy for rejection in human renal transplant recipients began in 1980 (104). This study provided strong evidence that OKT3 produced significant results in the treatment of renal graft rejection. Subsequent prospective randomized trials yielded similar results (105). OKT3 was subsequently shown to be of benefit in the treatment of corticosteroid-resistant rejection in renal transplant patients who have received prophylactic cyclosporin (106). OKT3 has also been used to treat acute rejection in liver and heart transplant recipients (107,108,109). OKT3 initially was reserved for rescue therapy for acute rejection, but later was also used prophylactically to prevent acute rejection in the early postoperative period (109,110,111),

OKT3 is associated with a wide spectrum of side effects, most of which are relatively minor. Most occur almost immediately after administration of the first dose, but some are delayed for days or weeks. All patients experience a self-limiting first-dose response, usually limited to fever, chills, and mild pulmonary and GI symptoms. Approximately 5% experience more serious reactions such as cardiopulmonary distress, seizures, encephalopathy, meningitis, renal insufficiency, and graft thrombosis. Hypersensitivity reactions are rare.

Recent research has focused on the role of the interleukin (IL)-2 receptor in acute allograft rejection. T-cell proliferation, a central event leading to graft rejection, is triggered by the interaction of IL-2 with its receptor on activated T cells. Anti-CD25 mAbs selectively block IL-2 receptors (IL-2 receptor antagonists) on activated T-helper cells (112).

IL-2 is a major growth factor for activated T lymphocytes, and antibodies reacting with the Tac-chain component of the IL-2 receptor can prevent allograft rejection in animals. Because Tac chains are expressed only on a small fraction of activated lymphocytes, mAbs against the IL-2 receptor may offer a more specific means of immunosuppression than polyclonal antilymphocyte globulin in prophylaxis against graft rejection. Modification of mAbs through genetic engineering has obviated some of the problems historically associated with mAbs. By replacing most of the murine portion of the mAb with human amino acid sequences (to form a chimeric mAb or humanized mAb), problems with antigenicity and short serum half-life are eliminated. There are 2 types of anti-CD25 mAbs: chimeric (approximately 75% human and 25% murine protein) and humanized (approximately 90% human and 10% murine). Basiliximab is the chimeric mAb and daclizumab is the humanized mAb that binds specifically to the alpha or Tac subunit of the human high-affinity IL-2 receptor that is expressed on the surface of activated lymphocytes. All chimeric antibodies contain "xi" (for example basiliximab) and all humanised antibodies contain "zu" (for example daclizumab) within their names.

Daclizumab and basiliximab are comparable in terms of their clinical effectiveness. Both have been shown in large, prospective, randomized, controlled, blinded trials to decrease the incidence of acute kidney allograft rejection when combined with a cyclosporin-based triple therapy regimen. Both agents are well tolerated without side effects or evidence of toxicity (112).

1.4.2.5 Use of induction in pancreatic transplantation

Induction therapy is usually included in immunosuppressive protocols for recipients of whole-pancreas transplants. Indeed induction therapy is used with greater frequency for pancreas transplant recipients than other solid organ recipients. This is because SPK, PAK and PTA recipients all exhibit a higher risk of rejection than recipients of other solid organ transplants. There are few published formal multicentre, randomised,

prospective trials assessing the use of induction therapy, rather its use has been guided by practical experience. Nonetheless, in the USA in 2001 78% of solitary pancreas transplant recipients (PTA and PAK) and 75% of SPK transplants received induction therapy, compared to 59% of kidney transplant recipients (113).

The type of induction therapy has also changed over the years. In the USA, 100% of SPK transplants between 1994 and 1997 used muromonab-CD3 or ATG. Since 1998, the use of daclizumab, basiliximab and rATG has supplanted these. The use of rATG for all kinds of pancreas transplants increased in the USA from 0.7% in 1998 to 54% in 2001 and from 1992 to 1997, almost all cases of induction therapy involved the use of either muromonab-CD3 or ATG. Between 1998 and 2001, basiliximab use rose in the USA from 7% to 32%, daclizumab from 15% to 21% and rabbit antithymocyte globulin rose from 0.4% to 29% (113).

Whole pancreas induction therapy in the US currently involves a T-cell-depleting agent (57% in 2001) and/or an interleukin-2 receptor (IL-2R) antagonist (48% in 2001). For comparison, amongst recipients of other organ transplants who received induction therapy in 2001, 21% of kidney transplants, 4% of liver transplants, 28% of heart transplants and 15% of lung transplants received T-cell-depleting agent (113).

In 2001 many solitary pancreas transplant recipients received more than one induction agent, typically rATG and daclizumab. This dual strategy is unusual in that it is not replicated in other whole-organ transplants. Indeed in the US in 2001, of those receiving induction therapy prior to SPK transplant, 53% utilised an IL-2 receptor antagonist (basiliximab or daclizumab) and 36% received a T-cell depleting agent (rATG, OKT3, or ATG) (113).

1.4.3 Mechanism of action of immunosuppressants

Maintenance immunosuppressive agents used for pancreas and other whole-organ transplantation fall into the following categories

- Corticosteroids
- Calcineurin inhibitors (such as cyclosporine and tacrolimus)
- Antimetabolites (such as azathioprine and mycophenolate mofetil)

- Others (such as rapamycin and cyclophosphamide)

To understand the mechanisms of action of these immunosuppressants it is necessary to describe the pathophysiological mechanisms that underpin the T cell response to the donor graft antigen. In order to activate the recipient T cell, T cell receptors must engage

- Foreign Major Histocompatibility Complex (MHC) molecules and
- Donor antigens.

In addition there must be costimulation of T cells by molecules such as CD80 and CD40. The presentation of donor alloantigen to recipient T cell can occur by two mechanisms-

The direct pathway

Donor dendritic cells express high levels of MHC antigen and present the donor antigen directly to the recipient T cell, which is activated in the presence of costimulation molecules (114,115,116).

The indirect pathway

Recipient dendritic cells invade the graft and uptake, process and present alloantigens to recipient T cells (117).

The relative contribution of these two pathways is poorly understood. Nonetheless, the consequence of antigen presentation is activation and proliferation of T cells which orchestrate the recruitment of numerous effector mechanisms such as B cells, natural killer cells, macrophages, neutrophils and eosinophils, resulting in graft rejection. Thus the role of T cells in graft rejection is critical. The immunosuppressants used in transplantation can be shown to have their main effect on the T cell intracellular signalling pathway or cytokine receptor, either in the process of activation of the T cell or in the subsequent proliferation of T cells (118).

Upon alloantigenic binding to the plasma membrane T cell receptor, increased cytoplasmic calcium activates calcineurin (a serine threonine phosphatase), which activates cytoplasmic transcription factors nuclear-factor-of-activated T cells (NF-AT), nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (Figure 1). These factors

migrate to the nucleus and induce the expression of genes that encode for interleukin-2 (IL-2), -4, -7, -9 and –15, and interferon-γ. These cytokines, when released from the activated T cells are involved in rejection of the transplant. In addition IL-2 binds to IL-2 receptors on T cells, which activates downstream phosphorylation of the mammalian target of rapamycin pathway (mTOR) and map kinase pathways. Both pathways converge to allow cell cycle progression in T cells stimulated by IL-2 and hence T cell proliferation.

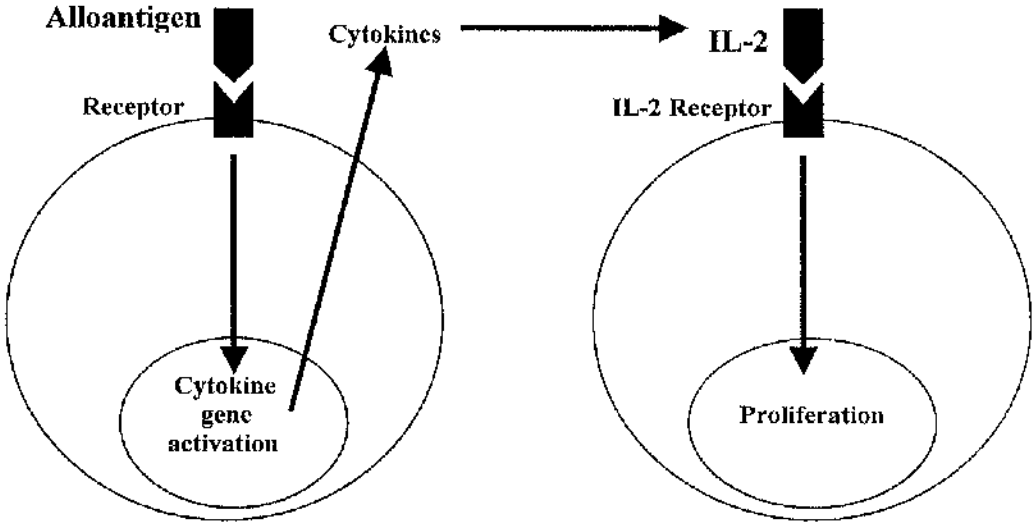


Figure 1. The role of interleukin-2 and the mechanism of stimulation, activation and proliferation of T cells following presentation of alloantigen.

1.4.4 Corticosteroids

Corticosteroids have been used as immunosuppressants since the inception of allo-transplantation. They exert their immunosuppressive effect by preventing activation of macrophages, inhibiting antigen presentation and reducing their function as effector cells. In addition, they inhibit T cell cytokine production and thus subsequent T cell proliferation (119). Unfortunately there are numerous side-effects from steroids including hypertension, dyslipidaemia, accelerated atherosclerosis, osteopaenia, avascular necrosis of the joints, cataracts, weight gain and steroid-induced diabetes. Thus their use in the context of transplantation in diabetics is far from ideal.

1.4.5 Calcineurin Inhibitors (CNIs)

1.4.5.1 Mechanism of action

In transplantation, the commonly used CNIs are cyclosporin and tacrolimus. The CNIs have their effect by binding with their respective binding protein to form a complex, which inhibits the phosphatase activity of calcineurin and thus prevents downstream translocation of cytoplasmic transcription factors to the nucleus and subsequent inhibition of cytokine formation (Figure 2) (120).

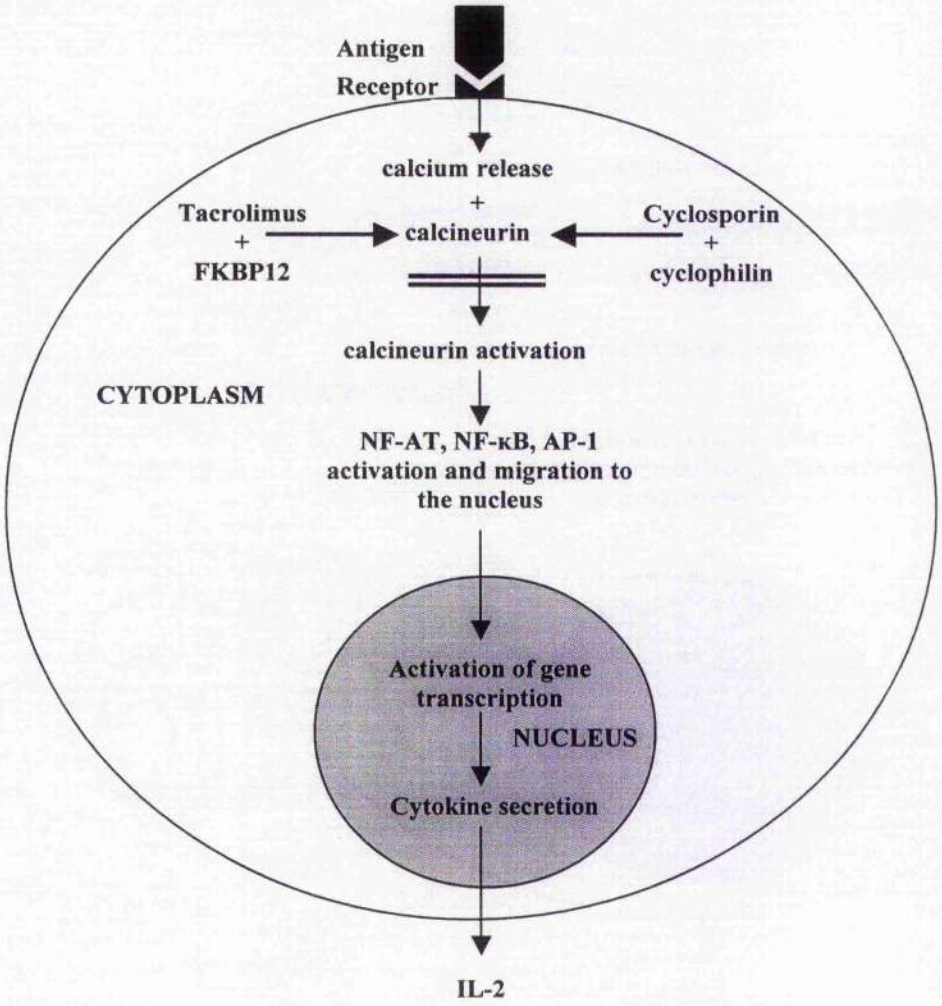


Figure 2. **The intracellular signalling pathway and nuclear gene transcription involved in T cell cytokine synthesis and the inhibitory effect of CNI on the pathway (121,122).**

1.4.5.2 Clinical use

Cyclosporin is a lipophilic cyclic peptide that has been used in organ transplantation since the early 1980s and is historically the most important immunosuppressant employed in renal transplantation. Indeed its use has led to a substantial improvement in the success of organ transplants (123,124,125). It has also been used in patients with autoimmune diseases such as rheumatoid arthritis, lupus erythematosis and type 1 diabetes mellitus.

Tacrolimus is a macrocyclic triene, which has similar immunosuppressant properties to cyclosporin. It is highly effective in preventing allograft rejection (121) and in the management of autoimmune disorders and skin disorders (126,127).

In large, prospective, randomised, multicentre trials in adults and children receiving solid organ transplants, tacrolimus was as least as effective or provided better efficacy than cyclosporin in terms of patient and graft survival, treatment failure rates and the incidence of biopsy-proven acute and corticosteroid rejection episodes (121,128).

1.4.5.3 Adverse effects

One of the major adverse effects of CNIs is nephrotoxicity, both acute and chronic (129,130). Acute nephrotoxicity is dose-dependent and reversible, whereas chronic nephrotoxicity is often associated with irreversible interstitial fibrosis of the renal tissue and afferent arteriolopathy (131). Following renal transplantation it is often difficult to differentiate between CNI-associated nephrotoxicity and chronic rejection (132). This nephrotoxic effect also limits the use of CNIs in other clinical settings such autoimmune disease or non-renal transplantation (133). Several studies demonstrated no difference in renal function between cyclosporin-treated and tacrolimus-treated renal transplant patients (128,134).

Infectious complications following transplantation would appear to be common to both cyclosporin and tacrolimus, ranging in severity from urinary tract infection to cytomegalovirus infection. Indeed the frequency and type of infection is similar in both cyclosporin- and tacrolimus-treated renal transplant patients (N128,135).

As the main cause of death in renal transplant recipients is cardiovascular or cerebrovascular disease it is important that the immunosuppressants used do not have an adverse effect on the recipient's lipid profile nor systemic blood pressure, both risk factors for the development of vascular disease. In renal transplant patients, those on a cyclosporin regime have significantly higher total cholesterol, LDL cholesterol, triglyceride and systemic blood pressure and significantly lower HDL cholesterol than those on a tacrolimus regime (128,134,135,136). However, although tacrolimus has a healthier cardiovascular risk-factor profile than cyclosporin, it is still to be established whether this results in a clinically significant reduction in vascular outcomes.

Tacrolimus is associated with an early increase in new-onset diabetes after transplantation (NODAT); however, the long-term incidence of NODAT is possibly similar to cyclosporin regimens. In the long-term, tacrolimus may have a less detrimental effect on glucose metabolism because the steroid requirement is markedly less when compared to cyclosporin-based regimes and because some studies have used lower dosage and target trough tacrolimus concentrations (138,139).

Malignancy is a well-recognised adverse effect of immunosuppressants. Studies have shown there to be no difference in the incidence of malignancy between renal transplant patients treated with tacrolimus and cyclosporin. Indeed the reported incidence of malignancy in one study was less than 1% (128,135).

Other adverse effects of CNIs include tremor, which is more frequent in tacrolimus-treated patients than cyclosporin-treated patients. However, hirsutism, gingivitis and gum hyperplasia are significantly more frequent in cyclosporin-treated renal transplant patients than those treated with tacrolimus (128, 134,135).

1.4.6 Antimetabolites

1.4.6.1 Azathioprine

Azathioprine is the prodrug of 6-mercaptopurine, an analogue of adenine and hypoxanthine. Azathioprine is initially converted to 6-mercaptopurine (6-MP) in the liver, possibly in a non-enzymatic manner. 6-MP is catabolised by one of two competing mechanisms. Xanthine oxidase (XO), an intracellular enzyme present in liver and the gastrointestinal tract (but not in haematopoietic tissue), metabolises 6-MP to the

inactive 6-thiouric acid. Alternatively, 6-MP can be catabolised by transmethylation via the thiopurine methyltransferase enzyme (TPMT) to the inactive methylmercaptopurine. A third enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) converts 6-MP to active and cytotoxic thioguanine nucleotides (TGNs), which accumulate in tissues and are either catabolised or incorporated into RNA or DNA. This results in inhibition of cell proliferation and in the context of transplantation, azathioprine acts as an immunosuppressant by preventing cytokine-driven proliferation of T cells. Both XO and TPMT can further metabolise active TGNs to inactive products. Thus low enzymatic activity of TPMT can lead to the accumulation of toxic metabolites, with consequent tissue toxicity. As TPMT is the principal inactivation pathway for cytotoxic TGNs in haematopoietic tissue (and XO is absent), haematopoietic toxicity of azathioprine is largely TPMT-dependent (139,140).

TPMT is a cytosolic enzyme that exhibits genetic polymorphism. There are nine variant alleles associated with TPMT enzymatic activity and TPMT genotypes correlate well with in-vivo red blood cell TPMT activity (141). Approximately 90% of individuals have high TPMT activity phenotype, which corresponds to the homozygous wild-type genotype. The intermediate-activity phenotype is associated with the presence of one mutant allele (heterozygotes) at the TPMT gene locus and affects approximately 10% of the population. Homozygotes have deficient TPMT activity and account for less than 0.3% of the population (142,143). TPMT genetic polymorphism is clinically relevant in that those individuals with sub-optimal TPMT activity are more intolerant of azathioprine and more susceptible to haematopoietic toxicity than the homozygous wild-type genotypes (144,145). The TPMT phenotype of an individual can be measured by a radiochemical assay that measures the methylation of mercaptopurine using [^{14}C-methyl]-S-adenosylmethionine as the methyl donor, using the patient's erythrocytes as the enzyme source (146). Alternatively, TPMT genotyping can be performed by means of polymerase chain reaction analysis that can detect mutations on the TPMT genomic DNA (147,148). Knowledge of a patient's TPMT phenotype or genotype prior to the commencement of azathioprine allows dosage adjustments to be made to avoid myelosuppression in the intermediate-activity patients and therapeutic failure in the high-activity patients.

In the UK, azathioprine is licensed for use in organ transplantation, rheumatoid arthritis, maintenance of remission of inflammatory bowel disease and myasthenia gravis. In many cases, administration of azathioprine allows a reduction in the dose of corticosteroids, thus reducing the risk of developing steroid-related complications.

Dose-related bone marrow suppression, including neutropenia, anaemia and thrombocytopaenia, is the commonest adverse effect of azathioprine and is usually associated with TPMT activity. Hepatic toxicity, including cholestatic jaundice, nausea, vomiting, abdominal pain, diarrhoea, hypersensitivity reactions (with fever, headache, arthralgia, rhabdomyolysis and cardiovascular, renal, pulmonary and hepatic involvement), pancreatitis, eosinophilia, neurotoxicity and photosensitive eruptions have all been reported and are thought to occur independently of TPMT activity (149). Some studies have shown that the level of methylmercaptopurine, the inactive metabolite of mercaptopurine, may be related to hepatotoxicity (150,151).

1.4.5.2 Mycophenolate mofetil (MMF)

Mycophenolate mofetil is also a prodrug, which is metabolised to mycophenolic acid (MPA). MPA reversibly and uncompetitively inhibits inosine monophosphate dehydrogenase, which is an essential enzyme required to sustain the guanine nucleotide pool and de novo DNA synthesis (152,153). Both B and T lymphocytes (unlike neutrophils) are dependent on this pathway for DNA and RNA synthesis (they cannot utilise the scavenger pathway) and hence inhibition of this pathway prevents proliferation of activated T cells (154). In lymphocyte cell-lines, MPA suppresses new DNA synthesis, expression of cell surface T-cell activation markers and cytokine production (155, 156).

In addition MMF disrupts the glycosylation of adhesion molecules involved in the attachment and infiltration of lymphocytes. Also, in experimental in-vitro models, MMF inhibits human arterial smooth muscle proliferation, which, if applicable clinically, may have a beneficial effect on arterial disease which has a critical role in chronic graft rejection (157).

Pooling of data from several studies has demonstrated that MMF can reduce acute rejection and graft loss due to rejection following renal transplant by approximately

50% compared to placebo or azathioprine in patients also taking corticosteroids and cyclosporin at one year (158,159). At 3 years, studies showed a trend of decrease in graft loss from all causes and graft loss due to rejection in the MMF group, but not to a level of statistical significance (160). At present the 3 year data have not been pooled. There is also accumulating evidence that MMF can be used as rescue therapy for refractory acute rejection (despite treatment with OKT3 or ALG) following renal transplantation and is more effective than azathioprine and intravenous corticosteroids (all patients received cyclosporin) up to 12 months (161).

Another potential benefit of MMF is that in maintenance therapy, its use may allow the reduction in dose or cessation of other immunosuppressants such as corticosteroids and cyclosporin, thus reducing the exposure of transplant recipients to the toxic effects of these drugs. Thus, there is emerging evidence that MMF may have a role in the maintenance phase of the management of renal transplantation.

The main adverse effects of MMF are gastrointestinal (diarrhoea and vomiting), haematological (neutropenia and thrombocytopaenia) and infective. In one study, there was an increase in each of these adverse effects compared to placebo and those effects were more prevalent in the higher dose MMF group (3 mg/day) than the lower dose (2 mg/day) group. Indeed the marginal benefits on the incidence of rejection of using the higher dose of MMF over the lower dose were outweighed by increased adverse effects (162). Other studies showed no difference in the rate of opportunistic infection between MMF and control (azathioprine) groups. However, one study showed an increase in cytomegalovirus (CMV) infection in the MMF group (163), but another showed no difference between rates of CMV infection (158). In both these studies, neutropenia, lymphoma and gastrointestinal disorders were more frequent in the MMF groups.

1.4.7 Rapamycin

Refer to Chapter 4, page 108.

1.5 New-onset diabetes after transplantation

1.5.1 Introduction

The life expectancy of type 2 diabetics is reduced by 8-10 years and atheromatous vascular disease (coronary artery and cerebrovascular disease) is the cause of 70% of such early deaths (164). Vascular disease is 2-3 times more common in type 2 diabetics than non-diabetics and those that present in their 40s and 50s have a twofold increased total mortality (165,166). The UK PDS demonstrated that in patients with type 2 diabetes, coronary artery disease was significantly associated with baseline increased concentrations of low density lipoprotein cholesterol (LDL), decreased concentrations of high density lipoprotein and increased triglyceride concentration, haemoglobin A_{1C}, systolic blood pressure, fasting plasma glucose concentration and a history of smoking, all modifiable risk factors. Indeed, there was an increased risk of coronary artery disease with haemoglobin A_{1C} of >6.2%, the upper limit of normal, and an increased risk of 11% for each incremental rise of 1% in haemoglobin A_{1C} (167). It has to be noted however, that in terms of reducing the risk of coronary heart disease in individuals with type 2 diabetes, reduction in blood pressure has a greater impact than reduction of haemoglobin A_{1C}.

However, the increased cardiovascular morbidity and mortality associated with diabetes is not restricted to the non-transplant population. It has been shown that diabetic kidney transplant recipients have a significantly higher mortality from ischaemic heart disease than diabetics in the normal population (168) and that diabetes is the most important risk factor for developing both cerebrovascular and peripheral vascular disease in kidney transplant recipients (169). Furthermore, traditional risk factors associated with cardiovascular disease (for example, diabetes, hypertension and dyslipidaemia) are also risk factors for chronic graft rejection (170).

New-onset diabetes after transplantation (NODAT) is recognised as a significant adverse effect of many immunosuppressants and as recipients of organ transplants survive longer, the secondary microvascular and macrovascular complications of diabetes mellitus have assumed ever-greater importance (171,172). Thus any additional iatrogenic vascular risk factors must be considered significant. Furthermore, cyclosporin, steroids and tacrolimus are not only diabetogenic but can also cause

dyslipidaemia and hypertension, all additional cardiovascular risk factors. It is for this reason that sirolimus and mycophenolate mofetil are considered to be beneficial as at present they are thought not to be diabetogenic, which in the context of pancreas and islet transplantation is desirable.

1.5.2. Definition, incidence and prevalence

Estimating the incidence and prevalence of NODAT is problematic as many investigators have used different diagnostic criteria. The following criteria have been published;

- WHO guidelines, whereby two fasting venous plasma glucose concentrations above 7 mmol/L are required (173)
- Fasting venous plasma glucose of more than 8.4 mmol/L on three separate occasions or an abnormal oral glucose tolerance test (174)
- Two fasting venous plasma glucose concentrations of more than 7.8 mmol/L and an abnormal oral glucose tolerance test (175)
- Three fasting venous plasma glucose concentrations of more than 7.8 mmol/L but no confirmatory oral glucose tolerance test (OGTT) (176)
- Two random venous plasma glucose concentrations above 11.1 mmol/L (177)
- A random venous plasma glucose above 22.2 mmol/L at any point or over 11.1 mmol/L for two weeks or any patient requiring insulin for over two weeks (178).

In addition, many researchers attempting to estimate the incidence and prevalence of NODAT have observed transplant patients for less than 12 months. This is an insufficient period of follow-up post-transplant as NODAT may develop years after transplant. As a consequence of different diagnostic criteria and variable, insufficient duration of follow-up, the published reported incidence of NODAT has varied significantly from 2% to 53% (179). In one study, the cumulative incidence of NODAT, analysed retrospectively in 11,659 transplant patients was 9.1% at 3 months, 16% at 12 months and 24% at 3 years (N180).

In order to standardise the diagnosis and management of NODAT, experts devised the International Consensus Guidelines under the auspices of the International Diabetes

Foundation (181). The guidelines suggest that the criteria for diagnosing NODAT should be the same as those for diagnosing diabetes in the non-transplant population (1).

1.5.3 Natural history, pathogenesis and risk factors

Following transplantation, the risk of developing NODAT is greatest during the first six months. However, there is a progressive linear increase in the diagnosis of NODAT thereafter (182). In some cases the development of NODAT is similar in many ways to the natural history of type 2 diabetes, with an insidious onset initially associated with impaired glucose tolerance and lack of symptoms, ultimately resulting in overt glucose intolerance with symptoms of hyperglycaemia (183). It differs from type 2 diabetes in that on occasions, overt NODAT may resolve, leaving the patient with impaired glucose tolerance (as defined by OGTT) for many years after remission of overt diabetes (184). NODAT after pancreatic transplantation can occur as a consequence of many factors including graft ischaemia, graft rejection, steroids, and drug-induced or autoimmune damage to islets. A single mechanism of NODAT has not been clearly established and a unified hypothesis has not emerged. Calcineurin inhibitors (CNI) such as cyclosporin and tacrolimus may have their diabetogenic effect by binding to calmodulin, which is physiologically involved in β-cell insulin secretion. Indeed it has been demonstrated that calmodulin inhibitors can restore insulin secretion in cyclosporin-treated rat islets (185). In addition the selective localisation of FKBP12 and calcineurin in islets compared to acinar tissue, might explain why the toxic pancreatic effects of tacrolimus are exclusive to the endocrine pancreas and have no effect on the exocrine pancreas (186). Studies in rat insulinoma cell-lines demonstrated that tacrolimus inhibited insulin mRNA transcription and hence insulin synthesis. Withdrawal of tacrolimus allowed insulin mRNA transcription and insulin synthesis to return to normal. Furthermore, glucose uptake into rat striated muscle cell-lines and the number of insulin receptors was not affected by tacrolimus (187). This suggests that the diabetogenic effect of tacrolimus is not caused by the induction of peripheral insulin resistance, but by a reduction in β-cell synthesis and secretion of insulin.

It has been proposed that corticosteroids cause NODAT by a variety of mechanisms such as decreased insulin receptor number and affinity, impaired peripheral glucose uptake in muscle, impaired suppression of endogenous insulin production or release of

free fatty acids. Indeed there may also be a genetic predisposition. It is likely however, that the predominant defect is peripheral insulin resistance (188).

Although tacrolimus and cyclosporin are considered to be steroid-sparing agents (by facilitating a reduction in dose of concomitant steroid), they are both potent inhibitors of the cytochrome p-450, the pathway responsible for steroid metabolism. Thus even patients on tacrolimus or cyclosporin and low doses of steroids may develop Cushingoid features including impaired glucose tolerance.

Apart from the immunosuppressants used, there are other recipient risk factors which are positively associated with NODAT (181);

- pre-existing impaired glucose tolerance
- age over 40 years
- metabolic syndrome (with normal glucose tolerance)
- a family history of type 2 diabetes
- African or Hispanic ethnicity
- obesity
- pre-transplant hepatitis C infection.

1.5.4 Consequences of developing NODAT

It has been demonstrated consistently that the development of NODAT is associated with a significant decrease in graft function and survival compared with controls in kidney transplant recipients up to 12 years post-transplant (176,189). Also, liver transplant recipients with NODAT are more likely to experience acute rejection than controls (190). In addition to the graft effects of NODAT, many studies have demonstrated reduced patient survival compared to controls (175,183). Supporting the theory that glucose intolerance has a deleterious effect on post-transplant outcome is the observation that simultaneous pancreas-kidney transplants in diabetic patients have improved long-term survival compared to cadaveric kidney transplantation alone (191).

1.5.5 Consensus Guidelines

The International Expert Panel Meeting convened by the International Diabetes Foundation made a series of recommendations regarding the screening, diagnosis,

treatment and management of NODAT in 2003 (181). The guidelines aim to reduce the incidence and impact of new-onset diabetes after transplantation by providing appropriate management strategies for transplant recipients. These management strategies are extremely detailed and involve;

- standardisation of diagnostic criteria of NODAT which are consistent with the criteria for diagnosing diabetes in the non-transplant population
- detailed clinical decision pathways for the pre-and post-transplant assessment of glycaemic status with recommendations based on the results
- recommendations on individualised immunosuppressive therapy depending on glycaemic status
- assessment of other vascular risk factors and the management thereof
- specific recommendations detailing the step-wise therapeutic approach to the management of NODAT
- target levels for LDL cholesterol, HbA_{1c} and blood pressure

1.6 Islet Transplantation

1.6.1 Historical overview

The concept of transplanting just islets and not the whole pancreas is enticing as it potentially avoids the risks associated with transplantation of the non-endocrine pancreas. However, until recently, it has been extremely difficult to achieve this goal successfully. Over 30 years ago, islet transplantation successfully reversed diabetes in rodents, however, early attempts at replicating this success in humans failed as a consequence of ineffective immunosuppression and poor quality, low-yield islet preparations.

Two important developments in the evolution of human islet transplantation occurred in the late 1960s. Separation of pancreatic islets from the non-endocrine components was technically challenging. However, distending the pancreatic duct with a salt solution and injecting collagenase into the duct, allowed enzymatic digestion of the pancreas. Furthermore, it was discovered that islets could be separated from the digested acinar tissue based on their differential density in sucrose gradients (192).

These improved techniques allowed investigators to ameliorate chemically-induced diabetes in rats by injecting allografted islets into the peritoneum (193,194). Thereafter intraportal injection of islets became the preferred site in rat experiments (195). Rat models allowed further improvements and refinements to be made to the process of islet transplantation, thus leading to a series of clinical islet allograft transplants in human type 1 diabetics immunosuppressed with azathioprine and steroids. Seven patients received dispersed pancreatic tissue into the peritoneal cavity or portal vein. However, although insulin-requirements were reduced and the procedure proved to be safe, no patient achieved insulin-independence (194). Insulin-independence following islet transplantation did not occur until 1978, when a group in Zurich, Switzerland embolised digested non-purified islet tissue into the recipient's spleen (with a simultaneous kidney transplant) (196).

In 1986 the Ricordi chamber was introduced; a semi-automated mechanical and enzymatic procedure which separated islets from pancreatic acinar tissue. This facilitated considerable improvements in the quality and yield of islets and is still considered a major advance of its time (197).

In 1989, nine patients undergoing abdominal multivisceral (including pancreas) resection for malignancy received intraportal single-donor human islets. 50% of patients achieved and maintained insulin-independence until death from recurrence of malignancy (198). Subsequent successes using steroid-free immunosuppression (high dose tacrolimus) were reported, representing the first experience with less diabetogenic immunosuppression (199).

In the 90s further discoveries and improvements were made in the islet isolation procedure. Liberase enzymes replaced traditional collagenase, which is a crude and variable fermentation by-product of Clostridium histolyticum. This resulted in improved islet yield, viability and functionality. In addition, lot-to-lot consistency is assured, with every manufactured lot adhering to the same enzyme activity specifications. Also, each lot is tested for endotoxin to ensure consistently low levels (200). Other improvements included the use of a cooled COBE-cell apheresis system (201) and the use of less toxic osmotic gradients for islet purification (202).

Despite the improvements in process and the technical advances made, only 10% of type 1 diabetics transplanted with human islets and reported to the Islet Transplant Registry between 1974 and 2000 were able to discontinue insulin therapy for more than one year (203). However, 28% did have sustained C-peptide secretion (204). Further peri-transplant improvements included intensive insulin and anti-oxidant therapy, vitamin D, anti-T cell induction and alternative immunosuppressants (for example mycophenolate mofetil and cyclosporin). However, despite reporting improved rates of insulin-independence at some centres, overall the results remained poor (204,205).

1.6.2 The Edmonton protocol (206)

It became apparent that the following factors were responsible for the poor success rate of human islet transplantation-

- inadequate islet potency
- inadequate islet transplant mass
- inadequate prophylaxis against allograft rejection or autoimmunity
- use of diabetogenic immunosuppressants

These issues led to the establishment and implementation of a new protocol, which addressed each of these limitations.

Inadequate islet potency

Islet function was optimised by using a low-endotoxin collagenase enzyme, transplanting the islets immediately (to limit cold ischaemia) and avoiding exposure to xenoproteins (fetal calf serum).

Inadequate islet transplant mass

Each transplant recipient received on average 11,000 islet equivalents/ kg body weight, using islets from two donors.

Sub-optimal immunosuppression

Potent, less diabetogenic, steroid-free immunosuppressants were used. Thus corticosteroids were replaced with daclizumab (a CD25 monoclonal antibody) in addition to low-dose tacrolimus and sirolimus (206).

Recent follow-up in over 50 patients treated with the Edmonton protocol reveals a 1-year insulin-independence rate of 80%, with a 3-year islet graft function (persisting C-peptide secretion) rate of 90%, a considerable improvement on previous results (207). In addition, initial results from the nine-centre Immune Tolerance Network trial have shown that this protocol can be replicated elsewhere (208). Innovative refinements continue to be made to the protocol. The most significant recent advances include

- the development of single-donor transplants (previously recipients required two and sometimes three donors) (209)
- refining islet culture to improve purity and allow transport between centres (210)
- confirmation that steroid-free, low-dose tacrolimus and sirolimus is effective for patients receiving islets after kidney transplants (211)
- introduction of anti-inflammatory and calcineurin-inhibitor-sparing regimes (212)

1.6.3 Limitations

Despite the recent and continuing improvements and success with human islet transplantation, there are still associated problems and adverse sequelae. As the long-term risks of human islet transplantation and immunosuppression are unknown, the procedure is only being offered to those type 1 diabetic patients who have unstable diabetes to the extent that they have a significant morbidity and mortality without transplant. Thus the majority of type 1 diabetics are at present considered to be unsuitable for islet transplantation. If there was to be a relaxation in the inclusion criteria for transplantation, and more type 1 diabetics became eligible for transplantation, there would be insufficient pancreata available to meet demand. Currently most centres require two (and on occasions three) pancreata per recipient.

Although islet transplantation avoids surgery there are still risks associated with the procedure. As islets are infused into the portal vein via a percutaneous approach, there is a risk of bleeding after the procedure (10%) and portal vein thrombosis (less than 0.5%). This may increase with repeated infusions (213). In addition, although the incidence of life-threatening sepsis, post-transplant lymphoma and malignancy are extremely low to date, the long-term effects of the current immunosuppressive regimes are unknown and unquantified. Drug-related side effects include mouth ulceration, hypertension,

dyslipidaemia, anaemia, accelerated nephropathy and diabetogenicity (207). Thus, although considerable improvements have been made in the field, further refinements are required to enhance the success of islet transplantation.

1.6.4 Current technical innovations

1.6.4.1 Islet culture

It is not yet clear whether islets should be cultured prior to transplantation. Several transplant centres do maintain islets in culture prior to transplantation, using culture media specifically designed for islets (214). The aim of this approach is twofold. Islet culture may improve the purity of islets, but also culturing islets prior to transplantation allows time for the patient to travel to the centre for transplantation, instead of relocating many months prior to the transplant. In addition, in the future, if any tolerogenic intervention or immunological preconditioning is required, then this could take place in the window between removal of the pancreas and transplantation. At present many centres are investigating whether it is possible to increase the islet yield in-vitro, during culture. Factors that may potentially increase islet mass include islet growth factors such as hepatocyte growth factor and islet neogenesis-associated peptide (215,216).

1.6.4.2 Islet engraftment

Once transplanted into the portal vein, many islets die (207). This may be as a consequence of pre-existing central necrosis in the larger islets; however, there is also some emerging evidence that the high levels of tissue factor present in human islets may provoke significant platelet binding and islet injury. Specific blocking antibodies have been used to disrupt this pathway in-vitro with the aim, ultimately, of protecting transplanted human islets in-vivo (217). In addition, in rodents, attempts have been made to promote islet neovascularisation by inserting the vascular endothelial growth factor gene into the islet genome (218,219). Other strategies for improving islet engraftment include the use of anti-inflammatory antibodies such as etanercept (a soluble TNF-receptor antibody) (220), anti-macrophage therapy, antioxidant therapies, vitamin D and the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors such as pravastatin (221). Indeed all these approaches have been shown to be of some benefit in preclinical studies.

1.7 Tolerance Induction

1.7.1 Introduction

The aim of tolerance induction is to manipulate the recipient's immune response such that the transplanted allograft can survive and function without the use of immunosuppressants. Although this has been achieved successfully in non-human models it has been extremely difficult to achieve in humans. Outwith the sphere of human islet transplantation, a limited degree of success has been achieved in other fields. Selected patients have been weaned off immunosuppressants following kidney and liver transplants, initially by reducing the dose and frequency of administration and in some cases, stopping immunosuppressants completely. Although a significant number of patients rejected their transplant and had to recommence immunosuppressants, a small proportion remained stable with preserved graft function without any therapeutic immunosuppression (222,223,224). In these cases it was considered that microchimerism was responsible for the graft tolerance (225). In another study, thymoglobulin induction followed by tacrolimus monotherapy in renal, hepatic and pancreatic transplant recipients allowed more than 50% of patients to be reduced to tacrolimus dosing once weekly (226). In addition numerous living-donor liver transplant children have been weaned off all immunosuppression, with continued graft function (227,228). Why some patients are able to tolerate allografts without any immunosuppression is not clear. Furthermore it is unclear how long graft tolerance will last and whether there is accelerated chronic rejection (229,230).

At present, there are several avenues being explored with regard to tolerance and immune protection of isles. These include;

- costimulation blockade
- use of immuno-privileged sites for islet implantation
- intrathymic administration of donor antigens
- haematopoietic chimerism

Prevention of rejection via manipulation of the recipient immune response has been attempted with various combinations of conventional, generalised immunosuppressive drugs, polyclonal or monoclonal antibodies, and chimeric molecules that target key

components of the immune system, by donor antigen administration, and by transplantation of haematopoietic cells to induce chimerism. Using a variety of approaches, it is now possible to prevent rejection of allogeniec islets in rodents, dogs, monkeys, and humans, but reports of donor-specific tolerance in larger animals and humans have remained more sporadic (231)

Experimental approaches to the prevention of rejection have been designed to block one or more of the myriad of interactions that occur between T cells and antigen-presenting cells. Blockade of this afferent arm of the immune response can prevent the generation of efferent responses, such as the development of cytotoxic T cells and antibodies, thus enhancing graft survival. Approaches that solely target molecules involved in the effector immune response have generally not been as effective at prevention of rejection or induction of tolerance. Such agents can be effective when used in combination with other drugs or biological agents that act earlier in the immune cascade by targeting cells that escape blockade at earlier time points (231).

A variety of strategies have been used to block receptor-ligand interactions, thus suppressing or altering T cell signalling and activation and leading to T cell clonal deletion, anergy, or regulation. Polyclonal and monoclonal antibodies, recombinant molecules, generalised immunosuppressive drugs and strategies incorporating administration of donor antigen, have been utilised to achieve these effects, with prolongation of graft survival a desired effect and induction of tolerance the ultimate goal.

Several monoclonal antibodies specific for T cells and T cell subsets have been tested in islet transplant models. With regards to pan-T-cell specific reagents, both anti-CD2 and anti-CD3 specific monoclonal antibodies have been tested in animal models. Peri-transplant administration of anti-CD2 has been reported to prolong the survival of murine pancreatic islet allografts and of rat islets in murine recipients (232,233). Administration of anti-CD3 led to prolonged graft survival in murine islet allograft recipients, with some experiencing permanent engraftment, although tolerance was not achieved, and injection of donor strain leucocytes resulted in rejection in recipients with long-term surviving grafts (234). Also, peri-transplant treatment of non-human primates with an anti-CD3-immunotoxin conjugate, plus a short course of cyclosporin and

methylprednisolone, resulted in long-term islet survival (235). Taken together with data that demonstrates permanent remission of diabetes in anti-CD3 treated NOD mice with recent disease onset, anti-CD3 treatment appears to have significant potential as therapy for patients with type 1 diabetes who undergo islet transplantation (236).

With regards to T-cell subsets, a single course of a depleting anti-CD4 monoclonal antibody was shown to result in indefinite islet allograft survival in mice (237,238). In addition, allografted mouse islets were permanently accepted in anti-CD4 or anti-CD4 plus CD8 treated mice, but not in recipients treated with anti-CD8 alone or with the pan-T-cell reagent, anti-Thy 1,2 (239).

1.7.2 Costimulation blockade

A great deal of research has focused on antirejection approaches that block T cell signal 2 activation (costimulation), whilst leaving signal 1 antigen activation unaffected. At the time of transplantation, blockade of co-stimulation leads to incomplete activation and may result in the induction of antigen-specific unresponsiveness and operational tolerance. Several pathways of co-stimulation have been elucidated, and their manipulation explored as a potentially therapeutic means of tolerance induction. LFA-1, CD154 and CD28 have received considerable attention because of their fundamental roles in T-cell activation (240).

Mice receiving an allogeniec islet graft with a short course of anti-LFA-1 antibody resulted in long-term graft acceptance and donor-specific tolerance (240,241).

Similarly, islet transplantation performed across a fully mismatched histocompatibility barrier was accepted permanently after transient treatment aimed at blockade of the CD28-CD80/86 interaction alone (242,243) or in combination with blockade of CD40-CD154 interaction (244). Blockade was obtained by the administration of a soluble chimeric molecule (cytotoxic T-lymphocyte antigen 4 immunoglobulin, CTLA-4-Ig) that prevents CD28 engagement and of an anti-CD154 monoclonal antibody.

CD154 blockade has also been explored as a part of a strategy comprising the administration of donor-specific spleen cell subpopulations. Convincing data have been presented that transient blockade of co-stimulation together with the administration of

donor-specific cell subsets, can lead to permanent acceptance of allogeneic islet grafts (245).

1.8 Aims

The aims of this study were to

- develop in-vitro biochemical tests to assess the quality of human islet preparations
- perform these biochemical tests systematically on all procured human pancreata and islet preparations
- assess whether any donor or islet isolation factors correlate with the results of the in-vitro biochemical tests
- assess whether human islets can restore normoglycaemia when transplanted into the NOD-SCID mouse
- investigate the relationship between the in-vitro biochemical tests and in-vivo islet function in the NOD-SCID mouse model
- assess whether donor characteristics have any effect on the function of transplanted islets in the NOD-SCID mouse
- investigate the effect of warm ischaemia on in-vivo tests of islet function
- assess the effect the immunosuppressant rapamycin has on MIN-6 cells, rat islets and human islets.

Chapter 2
In-Vitro Islet Quality Tests

Chapter 2 In-Vitro Islet Quality Tests

2.1 Introduction

During the last two decades, advances in islet biology have resulted in a greater understanding of the mechanisms involved in regulated insulin secretion by islets of Langerhans. The main events can be summarised as follows. Glucose oxidation by the β-cell is essential for insulin secretion. In particular, glucokinase, the first step in glycolysis, has been convincingly shown to be the β-cell glucose sensor (246). β-cell metabolism of glucose results in an increase in the ATP/ADP ratio leading to closure of the K_{ATP} channel, depolarisation of the β-cell, and influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels. The subsequent increase in intracellular Ca^{2+} then activates insulin exocytosis. The possibility of other signalling pathways involved in glucose-induced insulin secretion has also been suggested (247-252). These crucial biochemical pathways are indispensable to the correct functioning of a β-cell, and by extension, to transplanted islets. However, few islet transplant centres have addressed these issues in a systematic fashion.

In the Edmonton study, seven patients with unstable type 1 diabetes were successfully transplanted with human islets and in many cases became insulin-independent (206). They characterised the islet allografts by;

1. Cold ischaemia time (CIT)

CIT is defined as the duration from cross clamping of the donor aorta to implantation of the islets. The mean ± SD duration of CIT in 16 islet preparations was 13.9 ± 9 hours.

2. Immunohistochemical analysis

Immunohistochemical staining allowed the composition of each islet preparation to be calculated. In 16 islet preparations the mean ± SD percentage of β-cells present was 24 ± 12 %, α-cells was 10 ± 5 % and amylase-containing cells was 30 ± 15 %.

3. Total number of islets per isolation

One islet equivalent (IEQ) is the standard unit used to report the volume of islets. One IEQ has a diameter of 150 microns. The mean ± SD total number of islets per isolation was 357,336 ± 109,042, with a range from 125,317 to 591,278 IEQ.

4. Total number of islets infused

Of the seven patients who received islets, one patient required four separate islet transplants and the rest received two separate islet preparations. The mean ± SD number of IEQ infused per patient was 11,547 ± 1604 per Kg of recipient's body weight (the actual mean ± SD number of islets transfused per patient was 816,767 ± 56,047). The mean ± SD number of islets transfused in the first transplant was 389,016 ± 73,769, 374,926 ± 107,962 in the second transplant, 125,317 in the third transplant (only one patient) and 244,453 in the fourth transplant (only one patient).

5. Mean static stimulation index (SSI)

The in-vitro SSI was calculated by dividing the insulin secreted by islets exposed to 20 mM glucose by the insulin secreted by islets exposed to 2.8 mM glucose. The mean ± SD SSI was 6.5 ± 5, with a range from 3.0 to 23.5.

As part of the follow-up studies, an attempt was made to relate the quality and quantity of the transplanted islets (as defined by some of the above variables) to the clinical outcome following transplantation in 17 patients (207). As this series contained more patients than the first paper (206), the mean averages differed slightly. For example, the mean ± SEM number of IEQs transplanted at the first transplant was 374,283 ± 20,247 and 391,647 ± 32,921 IEQs at the second transplant. Each patient received a mean ± SEM of 850,035 ± 37,911 IEQs (or 12,330 ± 581 IEQs/Kg recipient body weight).

In order to compare the quality and quantity of islets with the subsequent success or otherwise of the islet transplant, several post-transplant measures of glycaemic control were calculated by performing the following tests;

1. Oral glucose tolerance test (OGTT)

OGTTs were performed two weeks after the first transplant and then at two weeks, three and six months off insulin and every 6 months thereafter. The test was performed in the fasting state using 75 g of oral glucose, with blood samples drawn at baseline and 30, 60, 90 and 120 minutes.

2. Intravenous glucose tolerance test (IVGTT)

IVGTTs were performed between the first and second transplants, 1, 3 and 6 months after becoming insulin independent after the second transplant, and every 6 months thereafter. The test was performed in the fasting state using 50% dextrose, 300 mg/Kg body weight, given over one minute after two baseline samples (-10 and 0 minutes) for glucose, insulin and C-peptide were drawn. Sampling was then at 3, 4, 5, 7, 10, 15, 20, 25 and 30 minutes, with time 0 being the start of the infusion.

3. Arginine stimulation test (AST)

ASTs were performed between the first and second transplants, 1, 3 and 6 months after becoming insulin independent after the second transplant and every 6 months thereafter. 5 g arginine HCl was infused intravenously over 30 seconds into the patient in the fasting state, and insulin levels were checked at the following time periods: -10, 0, 2, 3, 4, 5, 7 and 10 minutes.

The IVGTT allowed the calculation of the acute insulin response to glucose (AIR_g) based on the mean of the insulin level at 3, 4 and 5 minutes after the infusion less the mean basal insulin level. Glucose disposal (K_G) was calculated as the slope of the natural log of the glucose values from 10 to 30 minutes. The areas under the curve for insulin and C-peptide (AUC_i and $AUC_{C\text{-}p}$) were calculated as the area under the curve above baseline over 30 minutes post-infusion. Acute insulin response to arginine (AIR_{arg}) was calculated by taking the mean of the three highest values at 2, 3, 4 and 5 minutes post-infusion less the mean basal value (6). These values were compared with various measures of islet quality and quantity;

1. Total number of islets transplanted and cold ischaemia index

The total number of IEQ transplanted correlated with all measures of insulin reserve and glucose disposal at mid-transplant and 3 months after transplant (Table 4). This

correlation was stronger at 3 months than at 1 month (207). The Edmonton group devised the cold ischaemic index (CII), which is calculated by multiplying the total number of IEQs infused by 10^{-3} and dividing by the cold ischaemia time (hours). The CII for each infusate was summed as a total for each patient. The correlation between all measures of insulin reserve and glucose disposal and CII was stronger than the correlation with number of IEQs infused (207).

	number	r	p value
Islet equivalents versus			
AIR_g	26	0.463	0.017
AIR_{arg}	9	0.789	0.011
AUC_I	26	0.501	0.009
$AUC_{C\text{-}p}$	26	0.522	0.006
K_G	26	0.49	0.011
CII versus			
AIR_g	26	0.589	0.002
AIR_{arg}	9	0.827	0.006
AUC_I	26	0.684	<0.001
$AUC_{C\text{-}p}$	26	0.728	<0.001
K_G	26	0.684	<0.001

Table 4. Relationship of both IEQ transplanted and the cold ischaemic index with measures of insulin secretion and glucose disposal at 3 months post-transplantation (207).

2. In-vitro SSI

The authors describe the absence of a correlation between the SSI and AIR_g, fasting glucose and meal tolerance test-stimulated glucose levels. They make no mention of any correlation between in-vitro SSI and AUC_i, $AUC_{C\text{-}p}$, AUC_g and K_g (253).

3. Islet purity

Again, the authors describe the absence of a correlation between the islet purity and AIR_g, fasting glucose and meal tolerance test-stimulated glucose levels and make no mention of any correlation between islet purity and AUC_i, $AUC_{C\text{-}p}$, AUC_g and K_g (253).

In summary, it can be concluded that;

- the greater the number of islet equivalents transplanted, the greater the insulin reserve and glucose disposal in the transplant recipient.

- the greater the cold ischaemic index (or the lower the cold ischaemia time) the greater the insulin reserve and glucose disposal in the transplant recipient (206,207,253).

Despite the improvement in insulin reserve and glucose disposal that occurs post-transplant, these patients still have a substantially reduced insulin reserve compared to controls. The reason for this is not clear and remains to be elucidated.

The first sets of parameters are aimed at assessing β-cell function and responsiveness to glucose by measuring the dynamics of insulin release, both in a static and perifusion system. To assess the degree of exocrine contamination, the amylase content of both the whole pancreas and islet preparation are measured. Measuring the insulin content of both the whole pancreas and islet preparation allows us to quantify the increase in insulin concentration as a consequence of the islet isolation procedure. By staining the islet preparations with dithizone (which stains insulin-containing cells red) it is possible to calculate the purity of the islet preparation.

2.2 Materials and Methods

2.2.1 Pancreas procurement procedures

Pancreata were harvested by a number of Surgeons in the Pennsylvania, New Jersey and Delaware States, USA and the procedures were approved by the Institutional Review Board of the University of Pennsylvania, USA.

HBD pancreata were harvested using standard multi-organ recovery techniques. University of Wisconsin Solution was used for preservation of both HBD and NHBD pancreata. For NHBDs, life-support was withdrawn and death declared following cardiac asystole. A five-minute interval between pronouncement of death and initiation of organ recovery was observed. In all cases, the time to aortic cannulation and initiation of organ perfusion was less than 7 minutes. The period from withdrawal of life support to aortic cross-clamp was defined as the warm ischaemia time.

2.2.2 Donor data

Donor data was collected by Paige Oliver, Secretary, Hospital of the University of Pennsylvania (HUP), Philadelphia, USA.

The US United Network of Organ Sharing (UNOS) and local Gift of Life organisations procured pancreata from selected and appropriate donors and organised distribution and transport to the Hospital of the University of Pennsylvania (HUP) Human Islet Transplantation Program (HITP). Each donated pancreas had a unique identifying code (UNOS number), which tracked with the pancreas and subsequent islet preparation. On arrival at the HUP, each pancreas was given a local identifying T (transplant) number, which also remained with and identified the specimens derived from it throughout their distribution. Each pancreas arrived with a form, which described the following donor details-

- Age and gender
- Height, weight and body mass index (BMI)
- Referring institution
- Whether the donor had type 2 diabetes or not
- Whether the donor was a HBD or NHBD

2.2.3 Islet isolation

Performed by Dr Shaoping Deng, Research Assistant Professor, Department of Surgery, University of Pennsylvania, Philadelphia, PA, USA.

Pancreas preparation and phase I digestion (re-circulation phase)

The pancreas was trimmed and a small sample removed for measurement of insulin content, amylase activity and protein content. The whole pancreas was then perfused and distended with collagenase (Liberase, 0.5g in 350mL of Hanks solution), cut into several small pieces and placed into the digestion chamber (Figure 3). A stainless steel filter was placed over the upper half of the chamber and the chamber closed. The whole digestion circuit was filled with Hanks solution and the chamber secured in a mechanical shaker.

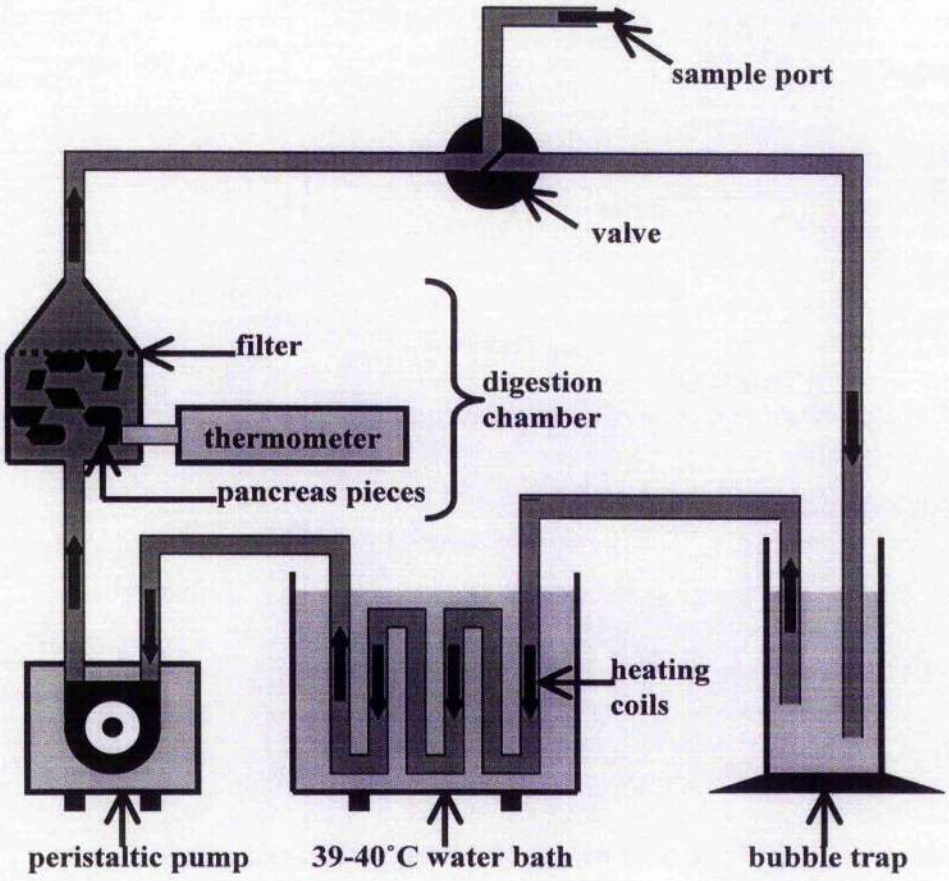


Figure 3. Islet isolation procedure.

As the digestion proceeded there was an increase in the amount of dispersed digested pancreatic tissue. The digestion was considered complete when an increase in the amount of tissue liberated from the chamber was observed, most or all of the islets were free of the surrounding acinar tissue, intact islets were observed, and the acinar tissue became finer (smaller cell clusters). The amount and size of acinar tissue, number of islets, percent free and score were recorded. In order to stop the collagenase digestion process, room temperature tissue-culture medium (RPMI tissue-culture medium) was added to the system and the heat-exchange coils removed from the water-bath. A

peristaltic pump maintained a constant flow rate round the circuit. When tissue started appearing in the stream (around 10 minutes), small samples were removed from the sample port, stained with dithizone (which stains insulin, and hence islets, red) and observed under the microscope.

Collection / Digestion phase II

Cold (2 to 8°C) human serum albumin was added to twenty 50 mL conical flasks. Digest from the system was sequentially added to fill each of the conical flasks, until no more islets were observed (typically between conicals 12 and 20). At this stage the system was refilled with tissue-culture medium and the whole process repeated until there were no islets left in the circuit.

Washing and centrifugation

The conical flasks were centrifuged and all, but 3 to 5 mL, of the supernatant was removed. The remaining supernatant and pellet were aspirated and transferred to a clean conical flask containing tissue-culture medium with human serum albumin. Any residual tissue remaining in the flasks was washed with additional tissue-culture medium (CMRL 1066) and added to the flask. The digest was then washed three times by resuspending the tissue, centrifuging the flask, aspirating and discarding the supernatant, then adding tissue-culture medium with human serum albumin to the pellet.

Cobe cell separation (Figure 4)

Islets were then purified on a Cobe 2991 cell separator using a continuous gradient. An equal volume of low- and high-density material was transferred to the two chambers of the gradient-maker, allowing for the creation of a continuous gradient. The digest was pumped into the Cobe bag and spun for three minutes. This process dispersed the digest along a density gradient and allowed low-density tissue (for example islets) to be separated from high-density tissue (for example acinar tissue). Thereafter, the Cobe bag was compressed, expelling the contents and the different-density fractions (from low to high) were collected in conical flasks containing tissue-culture medium. A small sample was taken from each flask to assess islet count, purity and quality score. The purified islets were then washed three times and then incubated at 22°C in tissue-culture medium (CMRL 1066) plus human serum albumin.

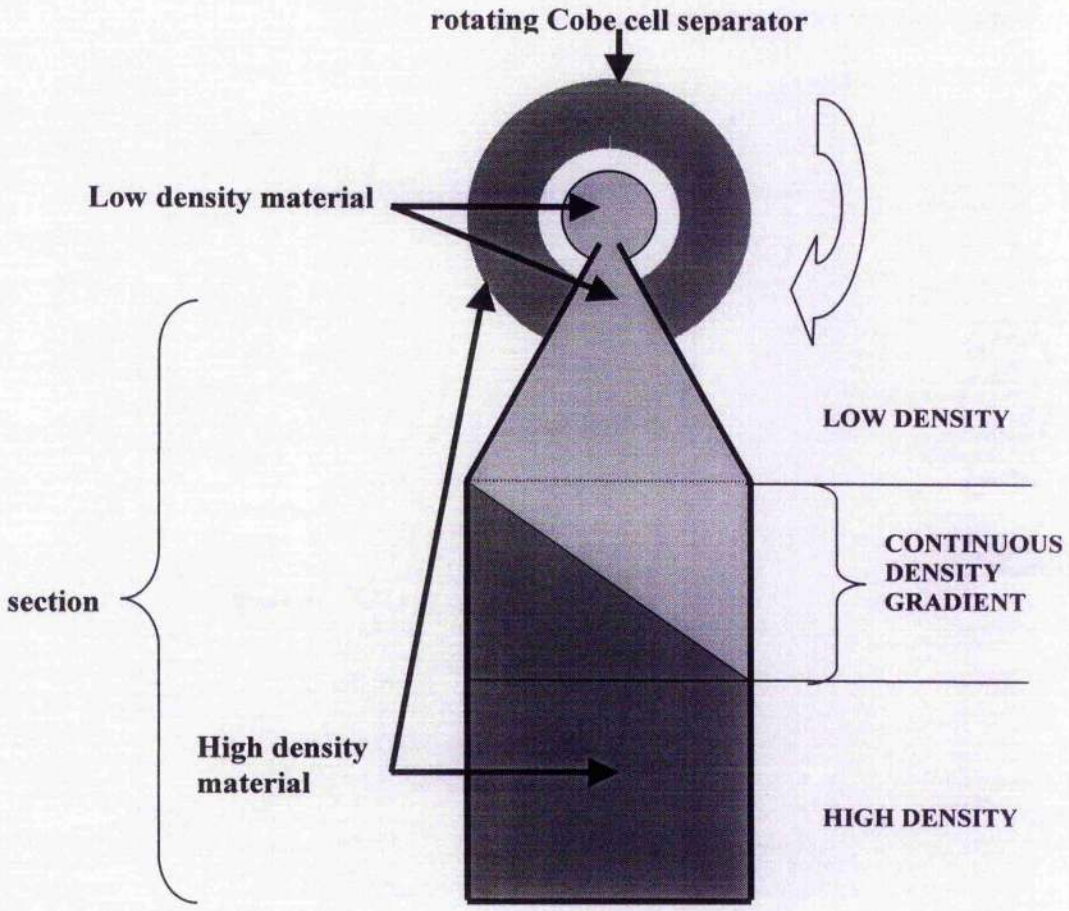


Figure 4. Cobe cell separator.

2.2.4 Static insulin secretion test

1.2 ml of 2.8 mM and 25 mM glucose tissue-culture medium (RPMI), supplemented with 1 M HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin was added in triplicate to a 24-well non-tissue culture treated plate. The plate was then placed in a 37°C incubator under conditions of 95% air and 5% CO_2 for 1 hour. The human islet preparation was supplied by the human islet isolation and culture laboratory in supplemented CMRL medium in a 250 mL tissue culture flask. By removing 0.2 mL of this islet suspension and counting the number of islet equivalents present it was possible

to calculate the total volume of suspension required to yield 300 islet equivalents without requiring to hand-pick islets. This volume of islet suspension was pipetted into a 10 mL polypropylene conical tube and the islets allowed to gravitate to the bottom of the tube. The supernatant was removed and discarded and the islets washed once with the 2.8 mM glucose RPMI solution. The islet pellet was then resuspended in 2.6 mL of the 2.8 mM glucose RPMI solution. 800 μL of the homogeneous suspension was removed (for sonication for measurement of protein and insulin content and amylase activity) and pipetted into a 10 mL polypropylene conical tube. The remainder of the suspension was aliquoted (300 μL each) into the low-glucose (3 of 2.8 mM) and high-glucose wells (3 of 25 mM, resulting in a glucose concentration of 20 mM). The plate was then incubated at 37°C for 2 hours under conditions of 95% air and 5% CO_2. After incubation 700 μL of the supernatant was removed (without any islets) and sent for RIA insulin analysis.

2.2.5 Sonication of islets

The sample was centrifuged for 2 minutes at 200 RPM and the supernatant removed. The islet pellet was resuspended in 1 mL of TH buffer (50 mM HEPES and 1% Triton X-100 (ethylene glycol octyl phenyl ether)) and sonicated for 3 minutes on ice. The sample was then centrifuged and the supernatant removed for measurement of protein and insulin content and amylase activity.

2.2.6 Sonication of whole pancreas specimen

A frozen piece of human pancreas was supplied by the human islet isolation and culture laboratory in a 50 mL polypropylene conical tube and allowed to thaw on ice. A 3 x 3 x 3mm cube of pancreas was dissected and cut into as many small pieces as possible. The minced pancreas was added to 1 mL of TH buffer (50 mM HEPES and 1% Triton X-100) in a 10 mL polypropylene conical tube and sonicated for 5 minutes on ice. The sample was then centrifuged and the supernatant removed for protein and insulin content and amylase activity.

2.2.7 Protein assay

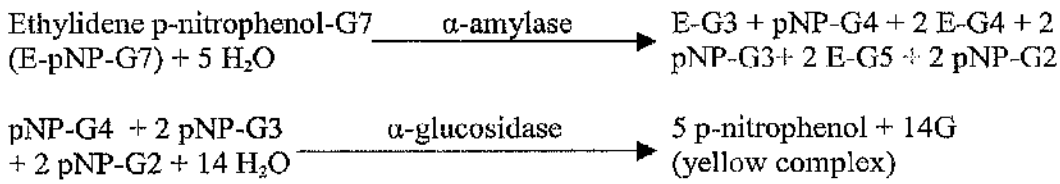
The Pierce Micro BCA Protein Assay kit was used to measure protein levels (254). Protein reduces Cu^{2+} to Cu^{1+} in an alkaline medium, which is chelated with

bicinchoninic acid (BCA), to give a purple-coloured complex which exhibits a strong absorbance at 562 nm, that is linear with increasing protein concentrations.

100 µL of sample or standard was added in duplicate to a 96-well microplate. To each well, 100 µL of reagent was added manually and incubated at 37°C for 1 hour. The absorbance of each well was measured by a Wallac Victor microplate reader and the sample protein concentration calculated according to the standard curve, which is linear from 1 to 200 µg/mL.

2.2.8 Amylase assay

The Sigma Infinity Amylase Reagent kit was used to measure amylase activity (255). This is a colorimetric kinetic assay, which measures the rate of formation of yellow p-nitrophenol at a wavelength of 405 nm.



Where G = glucose.

50 µL of sample was added in duplicate to a 96-well microplate. The Wallac Victor dispensed 50 µL of reagent (heated to 37°C) to a well then measured the absorbance over a two minute period, before moving on to the next well. The software calculated the mean change in absorbance per second (ΔOD/sec). To calculate the amylase activity in U/mL, the following formula was used –

$$\text{Amylase activity (U/mL)} = \frac{\Delta OD/sec \times 60 \times TV}{SV \times E \times P}$$

Where TV = total reaction volume, in this case 0.1 mL

SV = sample volume, in this case 0.05 mL

E = millimolar extinction coefficient of p-nitrophenol, in this case 10.13

P = pathlength, in this case 0.354 cm

2.2.9 Calculation of quality indices

Standardisation

The amount of amylase and insulin in the islet preparation and whole piece of pancreas depends on the mass of tissue present in the sample. In order to standardise the amylase and insulin concentrations, these values were divided by the protein concentration of the sample. Thus amylase activity is expressed as Units/mg of protein and insulin concentration as ng/mg of protein.

Static Stimulation Index

The Static Stimulation Index (SSI) quantifies the ability of each islet preparation to secrete insulin in response to glucose. It is calculated as follows-

$$SSI = \frac{\text{mean insulin secretion (ng/mL) at 20 mM glucose}}{\text{mean insulin secretion (ng/mL) at 2.8 mM glucose}}$$

Exocrine contamination

The Exocrine Contamination (EC) quantifies the degree of pancreatic exocrine contamination of the final islet preparation and is calculated as follows-

$$EC\ (\%) = \frac{\text{sonicated islet amylase activity (U/mg of protein) x 100}}{\text{sonicated pancreas amylase activity (U/mg of protein)}}$$

Insulin enrichment

The Insulin Enrichment (IE) quantifies the degree of enrichment of the islet preparation in comparison to the whole piece of pancreas and is calculated as follows-

$$IE\ (\text{fold increase}) = \frac{\text{sonicated islet insulin conc. (ng/mg of protein)}}{\text{sonicated pancreas insulin conc. (ng/mg of protein)}}$$

2.2.10 Perifusion stimulation index

Performed by Dr Marko Vatamaniuk, Post-doc Fellow, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA, USA.

100 representative human islets were handpicked and counted and placed in a plastic perifusion chamber. The perifusion apparatus consisted of computer-controlled fast-performance HPLC system, which allowed programmable rates of flow and glucose concentration in the perifusate, a 37°C water bath and a fraction collector. The perifusate was Krebs-HEPES buffer (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1

mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4 and 0.25% bovine serum albumin) and equilibrated with 95% O_2 and 5% CO_2.

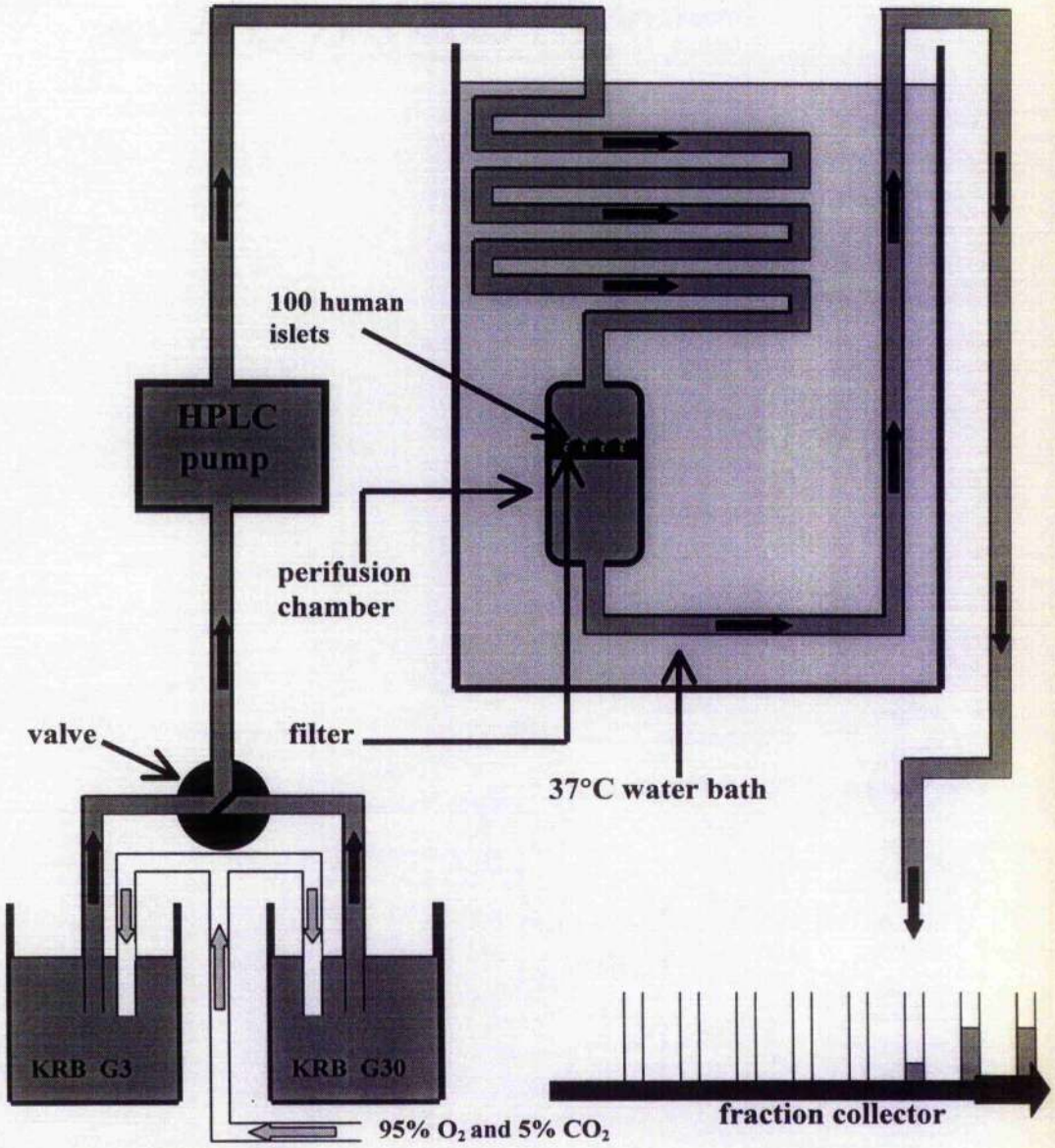


Figure 5. Perifusion system.

The islets were initially exposed to 3 mM glucose in Krebs-HEPES buffer and the insulin secreted measured in the samples collected by the fraction collector. Thereafter the glucose concentration was increased to 30 mM and the insulin secreted by the islets measured. Knowing the flow rate allowed the insulin secretion to be expressed as ng/mL, thus allowing the perifusion stimulation index (PSI) to be calculated.

$$PSI = \frac{\text{insulin secretion (ng/mL) at 30 mM glucose}}{\text{insulin secretion (ng/mL) at 3 mM glucose}}$$

2.2.11 Statistics

Statistical analyses were performed using GraphPad Prism software version 3.00 and Minitab Release 13 for Windows. Data are presented as mean ± S.E.M. Statistical significance of differences between groups was analysed by the unpaired t test and between multiple groups, one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison tests. Correlation between variables was calculated by normalising the non-Gaussian data by logarithmic transformation, then calculating the Pearson correlation. A p value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 In-vitro tests

The protein content of each islet preparation and pancreas was measured to quantify the amount of tissue present (Figures 6 and 7). This allowed different islet preparations and pancreata to be compared and also allowed standardisation of the insulin content and amylase activity of each islet preparation and pancreas.

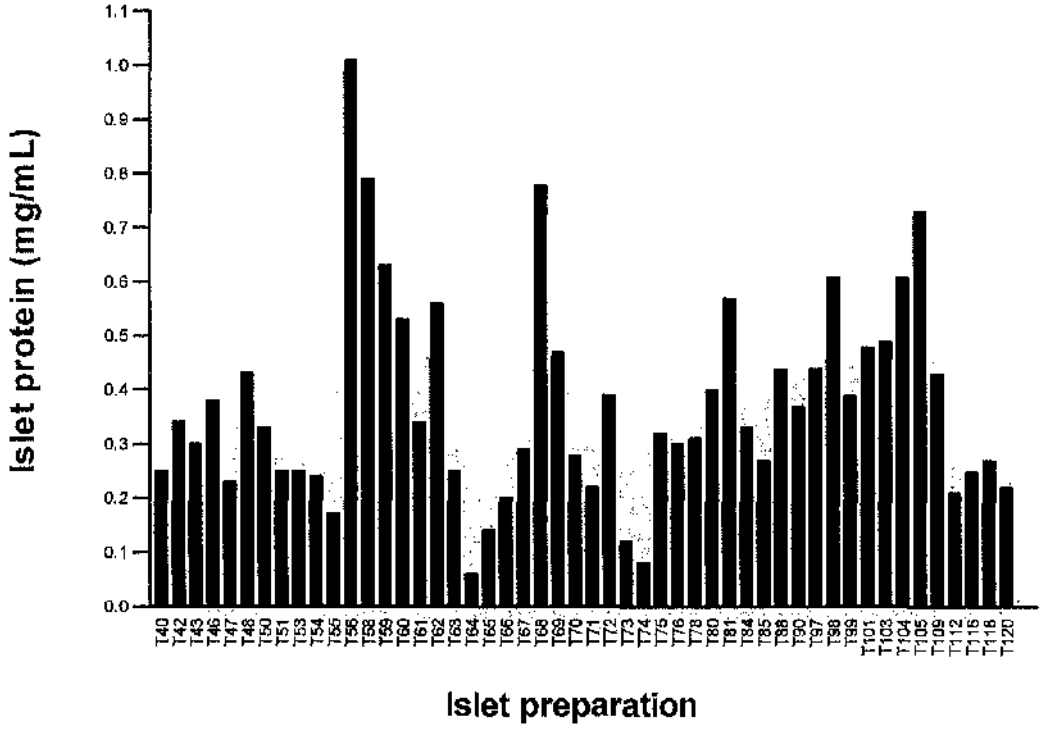


Figure 6. **Protein concentration in each sonicated human islet preparation (T).**

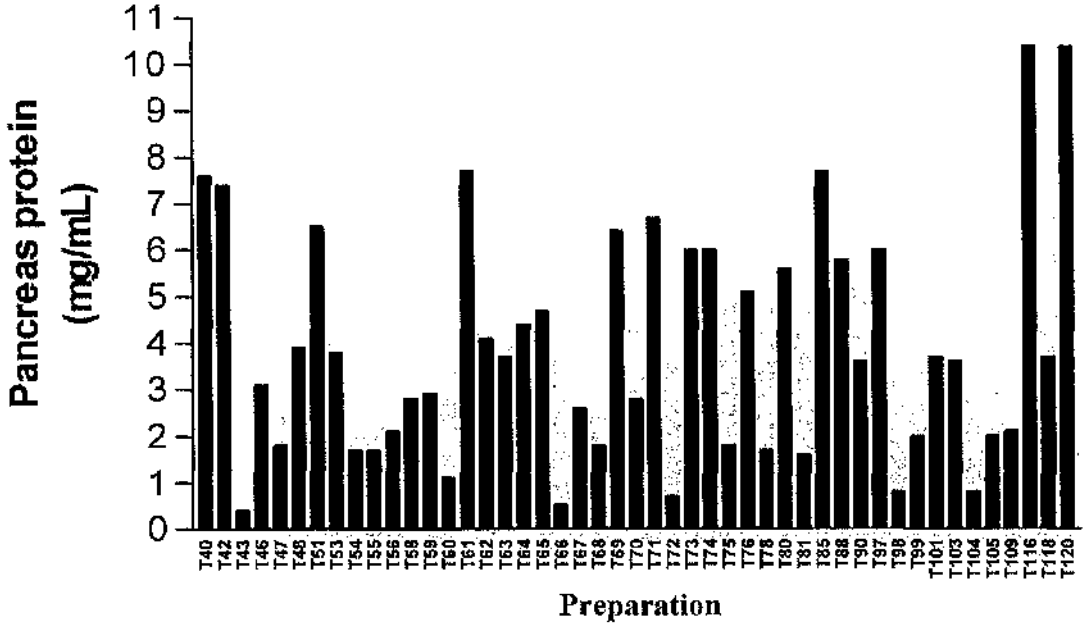


Figure 7. **Protein concentration in each sonicated human pancreas specimen (T).**

The mean protein content of 50 islet preparations was 0.38 ± 0.03 mg/mL, whereas the mean protein content of 47 pancreata was 3.9 ± 0.4 mg/mL.

The amylase activity of each islet preparation and pancreas was measured to quantify the degree of success at removing the exocrine component from the pancreas during the islet isolation procedure. To allow comparisons between preparations the amylase activity was divided by the protein concentration (Figures 8 and 9). Thus the mean amylase activity of 50 islet preparations was 0.059 ± 0.014 U/mg protein, whereas the mean amylase activity of 47 pancreata was 3.4 ± 0.3 U/mg protein.

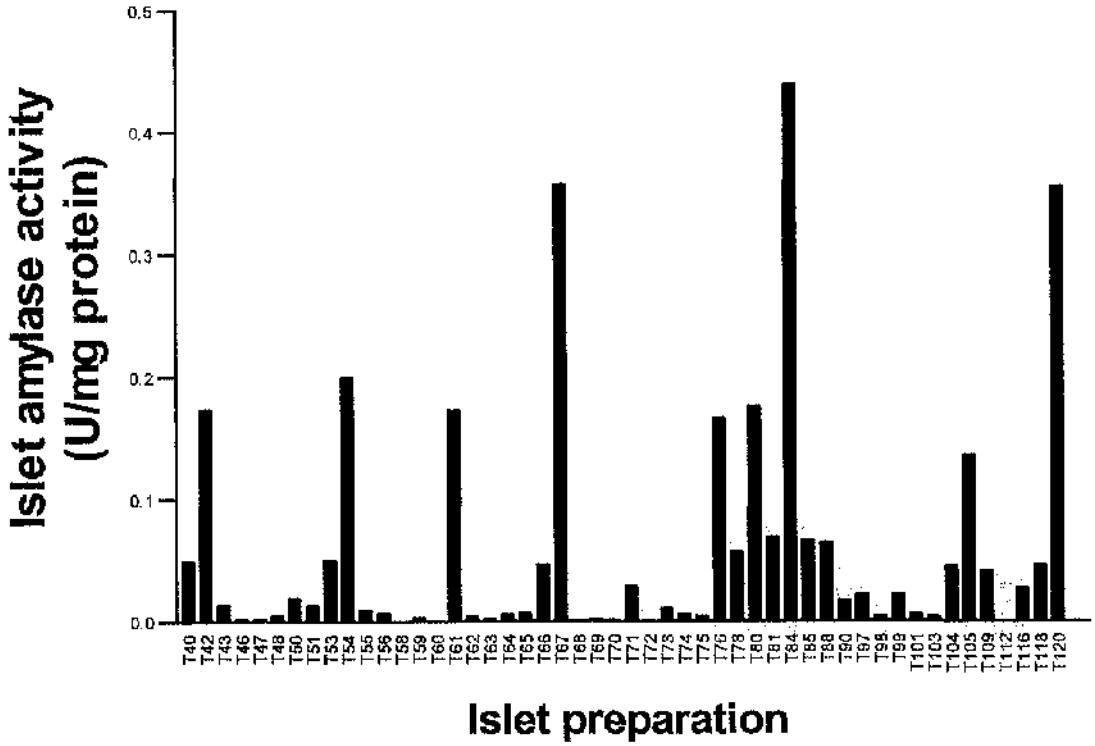


Figure 8. **Amylase activity in sonicated human islet preparation standardised to protein concentration.**

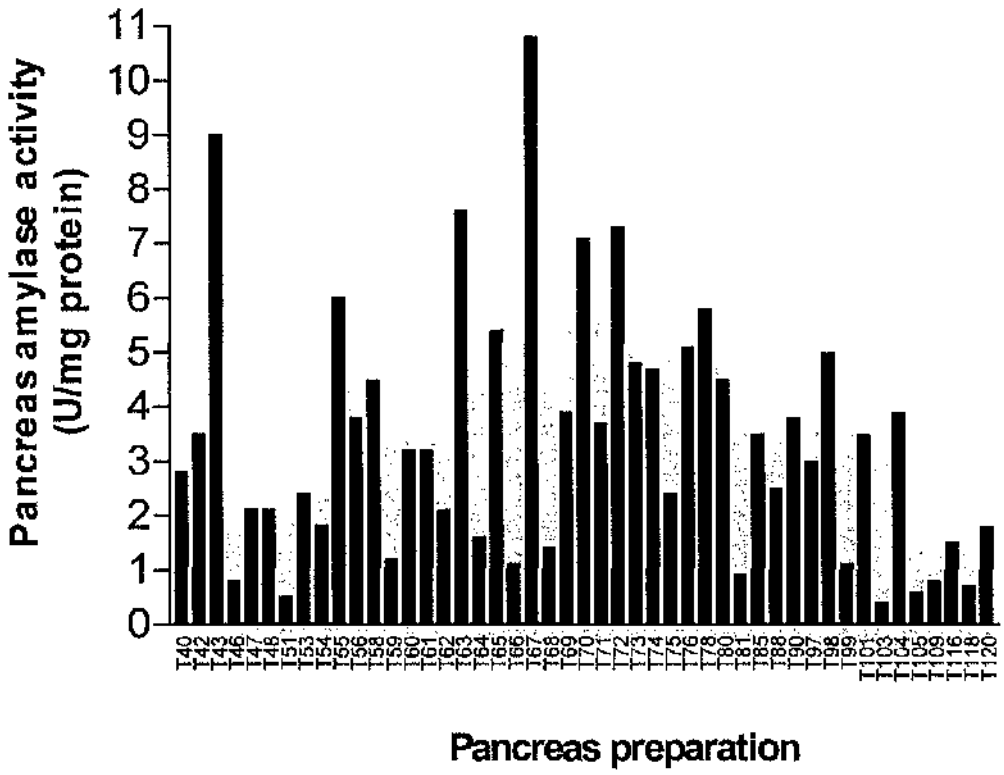


Figure 9. Amylase activity in sonicated human pancreas specimen standardised to protein concentration.

Exocrine contamination (EC) was calculated by dividing the standardised islet amylase activity by the corresponding pancreatic amylase activity and expressing the number as a percentage. This allowed us to quantify and then compare each islet isolation procedure (Figure 10). The mean exocrine contamination in 47 islet preparations was 2.5 ± 0.7%.

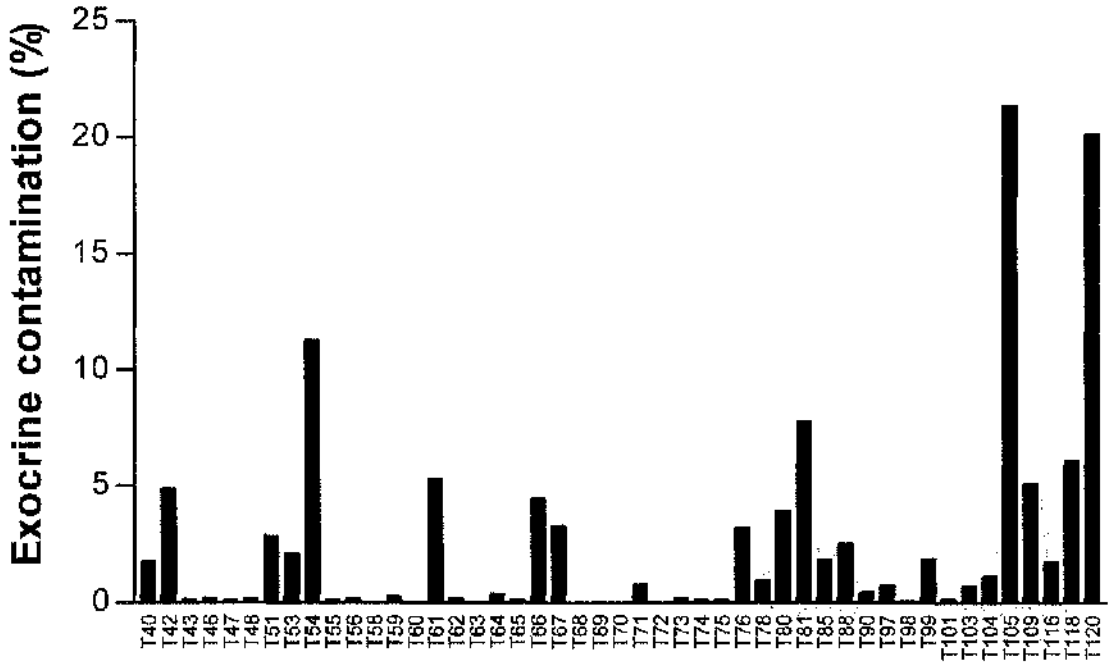


Figure 10. Exocrine contamination of each human islet preparation.

The insulin content of each islet preparation and pancreas was measured to quantify the degree of success at removing all the insulin-containing tissue from the pancreas. Each preparation was compared by dividing the measured insulin concentration by the protein concentration (Figures 11 and 12). Thus the mean insulin content of 50 islet preparations was 4.1 ± 0.6 μg/mg protein, whereas the mean insulin content of 47 pancreata was 0.093 ± 0.02 μg/mg protein.

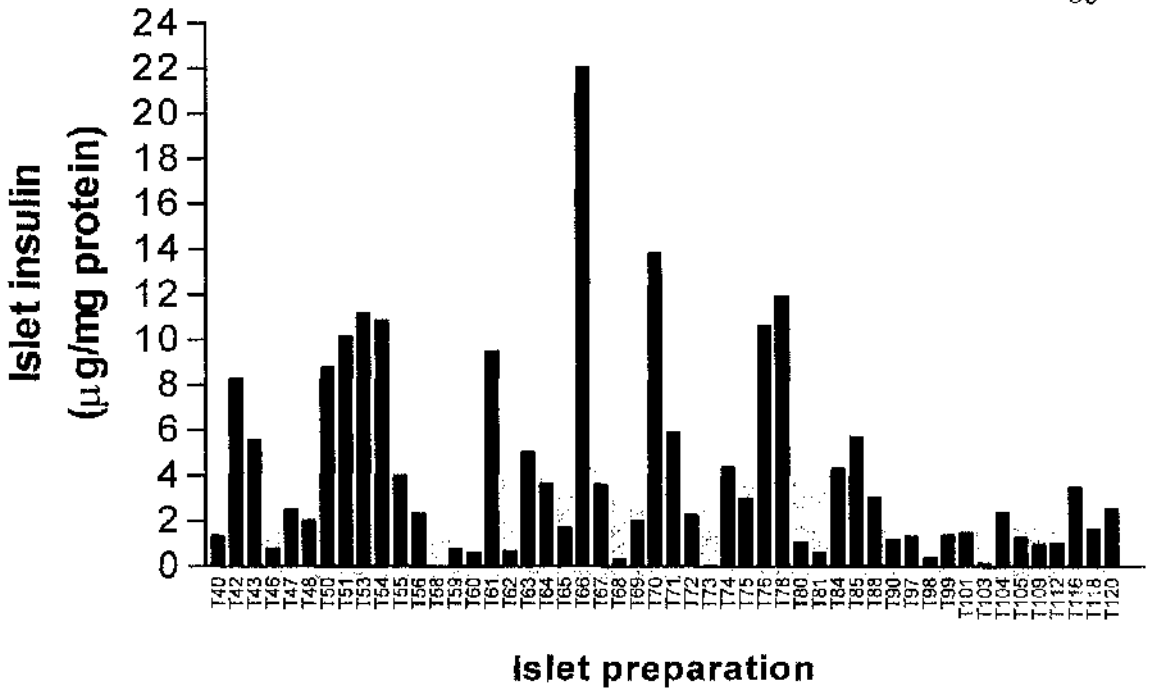


Figure 11. **Insulin concentration in sonicated human islet preparation standardised to protein concentration.**

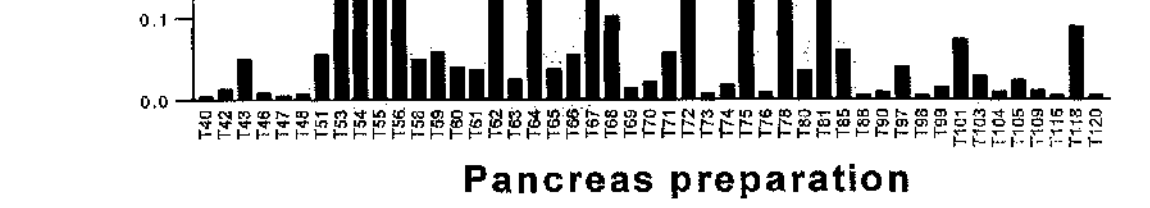


Figure 12. **Insulin concentration in sonicated human pancreas specimen standardised to protein concentration.**

Insulin enrichment was calculated by dividing the standardised islet insulin content by the corresponding pancreas insulin content and expressing the number as a 'fold

increase'. This allowed us to quantify and then compare each islet isolation procedure (Figure 13). The mean insulin enrichment (fold increase) in 47 cases was 180 ± 37.

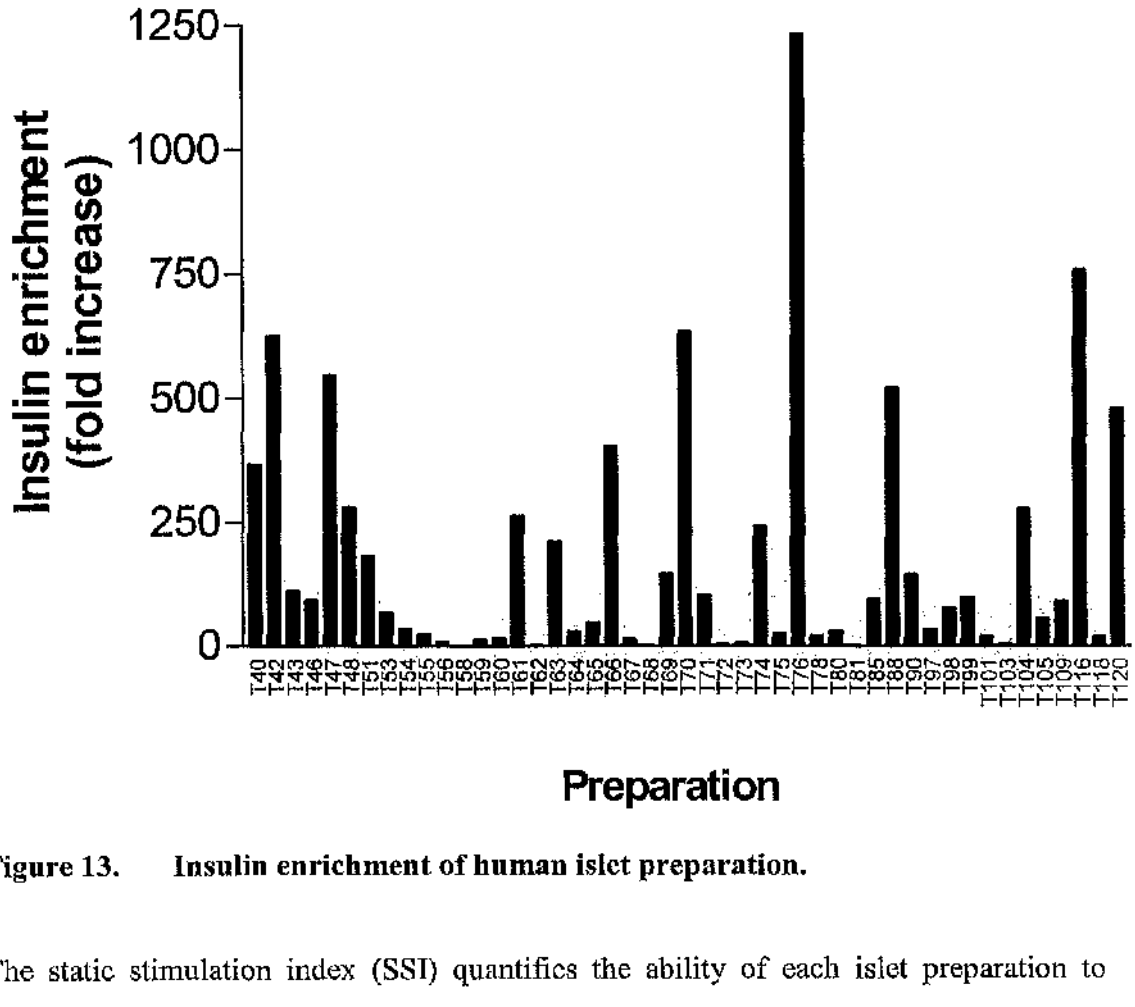


Figure 13. Insulin enrichment of human islet preparation.

The static stimulation index (SSI) quantifies the ability of each islet preparation to secrete insulin in response to glucose. It was calculated by dividing the mean insulin secretion at 20 mM glucose by the mean insulin secretion at 2.8 mM glucose (Figure 14). The mean SSI of 50 islet preparations was 1.47 ± 0.08.

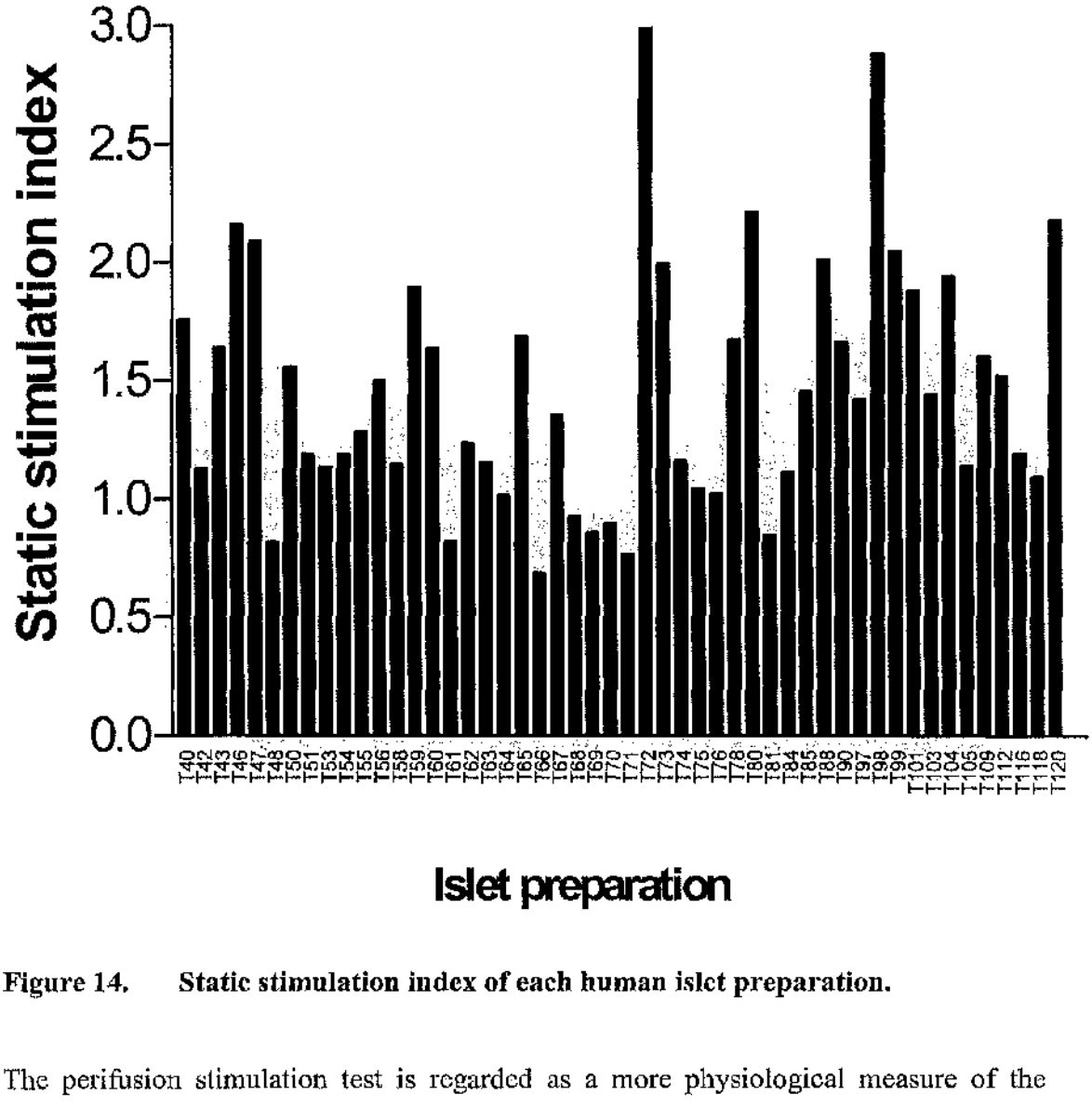


Figure 14. Static stimulation index of each human islet preparation.

The perifusion stimulation test is regarded as a more physiological measure of the responsiveness of islets to glucose. The perifusion stimulation index (PSI) was calculated by dividing the insulin secretion at 30 mM glucose by the insulin secretion at 3 mM (Figure 15). The mean PSI of 37 islet preparations was 9.8 ± 1.1.

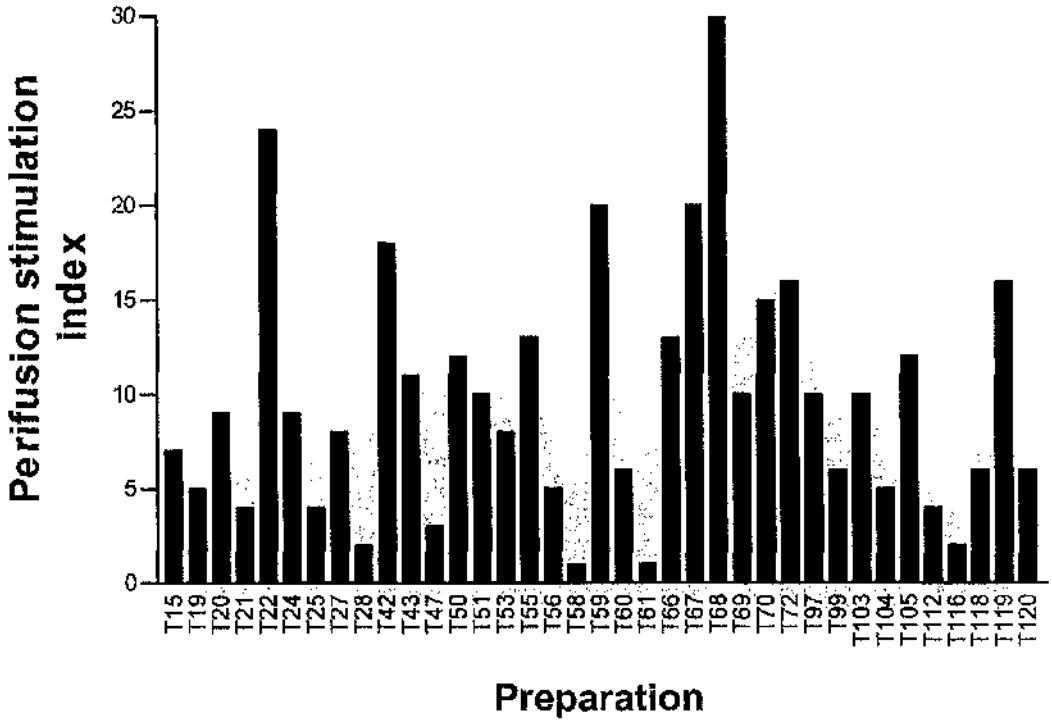


Figure 15. Perifusion stimulation index of each human islet preparation.

2.3.2 Correlation between donor, pancreatic, isolation and islet characteristics and test results

Figures 10, 13, 14 and 15 demonstrated considerable variability between different pancreas/islet preparations. To assess whether there were any donor or islet isolation factors that were associated with the results of the biochemical tests, the Pearson correlation and level of significance were calculated. Non-Gaussian data was normalised by logarithmic transformation (Table 5).

	PSI	SSI	log IE	log EC	Purity	log IEQ/g	Total IEQ	Panc wt	log CIT	log BMI
Age	-0.13	-0.09	-0.08	0.11	-0.05	-0.03	0.00	0.17	-0.02	0.03
log BMI	0.12	0.24	0.09	0.16	-0.06	-0.10	0.14	**0.30****	-0.02	
log CIT	0.26	0.04	0.14	-0.04	-0.04	-0.07	0.08	-0.10		
Panc wt	**0.35***	-0.06	0.09	-0.01	-0.06	-0.02	0.14			
Total IEQ	-0.23	0.10	0.09	**0.34***	-0.07	0.16				
log IEQ/g	-0.25	0.11	-0.03	-0.26	0.12					
Purity (%)	-0.15	0.15	0.06	0.13						
log EC	-0.32	0.15	0.30							
log IE	-0.41	-0.09								
SSI	-0.19									

Table 5. **Pearson correlation between donor, pancreatic, isolation and islet characteristics and test results.** BMI, body mass index (Kg/m^2); CIT, cold ischaemia time (hours); Total IEQ, total number of IEQs obtained from the pancreas; IEQ/g, number of IEQ isolated per weight of whole pre-digested pancreas; EC, % exocrine contamination; IE, insulin enrichment (fold increase); SSI, static incubation index; PSI, perifusion stimulation index; * $P<0.05$, ** $P<0.01$.

The Pearson correlation between the perifusion stimulation index and the total pre-digestion pancreatic weight was 0.35 ($p<0.05$). The only other significant correlations were between the total IEQs obtained and the log of the exocrine contamination ($p<0.05$) and the total pre-digestion pancreatic weight and the log of the donors' BMI.

2.4 Discussion

It is notable that the results of each of the in-vitro tests display remarkable heterogeneity between islet preparations. This may indeed reflect accurately the actual qualitative differences between islet preparations, however, this is only an assumption. In the clinical arena, new tests or assays would be compared against a gold standard reference method using reference materials. Furthermore, new method evaluation involves assessing accuracy, precision, sensitivity and specificity of a test. Once in use, accuracy of a test would be continually assessed by participation in external quality assurance schemes and precision by repeatedly measuring the same internal quality controls to ensure that the test is performing in a reliable and consistent manner. Clearly in this research setting, with these kinds of tests, rigorous method evaluation is not possible. Thus when making any conclusion based on the data produced from these tests it has to be borne in mind that the only guarantor of consistency is the operator and this is far from objective.

The biochemical indices (exocrine contamination, insulin enrichment and static stimulation index) were developed to quantify the quality of the islet isolation procedure and the subsequent in-vitro function of the islet preparation. Indeed, with a mean exocrine contamination of 2.5 ± 0.7%, mean insulin enrichment (fold increase) of 180 ± 37 and mean static stimulation index of 1.47 ± 0.08, it could be concluded that on the whole, the islet preparations-

- have low levels of exocrine contamination
- have significant insulin enrichment
- release insulin in response to glucose.

This suggests that the HUP islet isolation procedure may be reasonably successful at separating the endocrine from the exocrine pancreas. In addition, the in-vitro glucose-stimulated increase in insulin secretion demonstrates that the islet preparation is capable, to some extent, of responding in-vitro in a physiological manner appropriate to the hyperglycaemic environment found in diabetes. The static incubation test used in this project is similar to that used by the Edmonton group (206), which achieved a considerably higher mean SSI of 6.5 ± 5, compared to 1.47 ± 0.08 in our study. As both

the islet isolation procedures and static incubation tests are similar, broadly similar results might be expected, however this was not the case. The reasons for this have not yet been established. The logical possibilities include the likelihood that either the HUP SSI test performs poorly or the HUP isolated islets don't function well. The only way to solve this issue would be to perform the HUP SSI test on the same islet preparation as another centre, however, this is impractical. In addition, as there is a paucity of published data on exocrine contamination and insulin enrichment of islet preparations it is impossible to compare these results with other centres. Whether our absolute level of exocrine contamination or insulin enrichment is good or bad is difficult to say.

Regarding the donor characteristics, perhaps it is not surprising that the greater the body mass index of the donor the greater the total pancreas weight, however this does not correspond to increased numbers of total islets nor IEQ per weight of pancreas. This might suggest that the increase in pancreatic weight associated with increased body mass index is related to an increase in weight of the non-endocrine component of the pancreas, possibly the fat component. There is no plausible scientific reason why there should be a relationship between perifusion stimulation index and total pre-digestion pancreatic weight, assuming that the increase in pancreatic weight is not associated with increased numbers of larger islets. Also, there is no convincing explanation as to why there is an association between total IEQs obtained and the log of exocrine contamination.

Chapter 3
In-Vivo Tests of Human Islets

Chapter 3 In-Vivo Tests of Human Islets

3.1 Introduction

Having collected the donor characteristics (age, sex, body mass index and diabetic status), developed the in-vitro tests of human islet function and establishing methods for estimating the exocrine contamination and insulin enrichment of the islet preparation, the next stage was to assess whether any donor characteristics or the in-vitro test indices could predict the curative efficacy of islet transplantation in the NOD-SCID mouse model. The curative potential of this islet material was determined by transplanting islet samples into NOD-SCID mice then measuring the glycaemic response as the biological endpoint in a standardised system. If this strategy was able to demonstrate that one particular index or donor variable was able to predict the curative efficacy of the islets when transplanted into NOD-SCID mouse then the next step would be to assess whether these findings are replicated when transplanting islets into human diabetic recipients. The ability to quantify the quality of each individual islet preparation prospectively, based on in-vitro testing and donor characteristics would offer several theoretical benefits. A quality score for each islet preparation would allow selection of the preparation most likely to be successful in restoring normoglycaemia in the diabetic recipient and thus the need for implantation of several islet preparations from several donors for each diabetic recipient might be avoided. For this approach to be successful there might be logistical problems, in that, each of the in-vitro tests developed cannot be measured by an automated analyser. Each is manually intensive and technically challenging and may take several hours to perform. For any of the in-vitro tests to be clinically worthwhile would require on-call staff to be always available, for an islet isolation that might take place irregularly and infrequently. Also, as the in-vitro tests are time-consuming, the islets to be used for transplantation would have to be cultured whilst awaiting the results of the quality tests. However, in terms of future research, the development of validated in-vitro quality tests would have numerous worthwhile applications. There is significant on-going research into changes in islet isolation procedures and islet culture conditions which might have a positive effect on islet efficacy. Instead of having to use the biological endpoint of transplanting islets into NOD-SCID mice, which is expensive and extremely time-consuming, initial

modifications in procedures and conditions could be assessed by performing the validated quality in-vitro tests.

Furthermore, it might be possible to predict islet efficacy based on certain donor characteristics, such as the age, gender, body mass index (BMI) or diabetic status of the donor. Again, this might allow a more refined selection of a suitable donor, to optimise the clinical benefits to the diabetic recipient.

More realistically, where donors are outnumbered by waiting potential recipients, the ability to quantify the quality of an islet preparation would not lead to islets being discarded because of their inferior quality, nor potential donors being rejected. It might be that those recipients, who receive an islet preparation with an unfavourable profile, would have to be scrutinised more closely post-transplant, or possibly subjected to an alternative post-transplant regime.

Insulin independence is often only achieved after infusion of two or three separate islet preparations procured from different donors. In light of the shortage of cadaveric donors and the fact that each recipient requires islets from multiple donors, isolated islet transplantation is unlikely to wholly replace whole organ pancreas transplantation. A potential source of donor pancreata that has not been thoroughly evaluated for islet transplantation is non heart-beating donors (NHBDs). NHBDs have life-support withdrawn and subsequently, cardiac arrest occurs at a variable interval prior to initiation of organ recovery (256), whereas, in traditional brain dead or heart-beating donors (HBDs) cardiac arrest occurs at the time of organ recovery. Thus warm ischaemia is defined as the time between cardiac arrest and organ cooling by cold perfusion. NHBD pancreata are not considered appropriate for whole organ transplantation by most transplant centres because of the concern of ischaemic injury leading to graft pancreatitis after reperfusion. However, whether pancreata procured from NHBDs can provide an adequate number of functional islets for successful isolated islet transplantation in humans is unknown.

There have been reports of successful recovery of islets from NHBD pancreata, however the function of the islet preparation has not been thoroughly studied and

attempts at human transplantation with NHBD islets have not been described (257,258). In this study, the ability to recover functional islets from NHBDs was evaluated (259).

3.2 Materials and Methods

3.2.1 Pancreas procurement procedures

The procurement procedures are described on page 72 (2.2.1).

3.2.2 Donor data

The collection of donor data is described on page 73 (2.2.2).

3.2.3 Islet isolation

The islet isolation procedure is described on page 73 (2.2.3).

3.2.4 Mouse transplantation

Performed by Dr Shaoping Deng, Research Assistant Professor, Department of Surgery, University of Pennsylvania, Philadelphia, PA, USA.

To evaluate the in-vivo function of islets, isolated human islets were transplanted into NOD-SCID mice. The mice were rendered diabetic by intraperitoneal injection of a β-cell toxin (two doses of streptozotocin, 150 mg/Kg on day 0 and day 2). NOD-SCID mice were transplanted with 1000 islets under the left kidney capsule. Islet graft function was monitored by blood glucose measurement on day 1, 3, 5, 7, 14, 28, 60 and 100. At the end of the experiment (100 days post-transplantation), the kidney bearing the transplanted islet graft was removed and blood glucose levels monitored in the animal for the next week to confirm that a functioning islet graft was responsible for the maintenance of normoglycaemia.

3.2.5 Statistics

Statistical analyses were performed using GraphPad Prism software version 3.00 and Minitab Release 13 for Windows. Data are presented as mean ± S.E.M. Statistical significance of differences between groups was analysed by the unpaired t test and between multiple groups, one-way analysis of variance (ANOVA) and Newman-Keuls

multiple comparison tests. Correlation between variables was calculated by normalising the non-Gaussian data by logarithmic transformation, then calculating the Pearson correlation. A p value of <0.05 was considered statistically significant.

3.3. Results

3.3.1 Mice transplantation

To evaluate the in-vivo function of islets, human islets were transplanted into streptozotocin-induced NOD-SCID mice and the blood glucose measured at intervals thereafter (Figure 16). The mean blood glucose concentration in 72 mice (20 experiments), immediately prior to transplantation, was 20.3 ± 0.4 mmol/L. 1 day after transplantation, the mean blood glucose concentration in 55 mice (20 experiments) fell to 11.6 ± 0.7 mmol/L. 3 days after transplantation, the blood glucose concentration in 50 mice (20 experiments) rose to 12.5 ± 0.8 mmol/L and subsequently fell again, such that by day 60 normoglycaemia had been achieved, with a mean blood glucose concentration in 48 mice (20 experiments) of 5.4 ± 0.4 mmol/L. The initial fall in blood glucose at day 1 may be attributed to necrosis of transplant β-cells and the subsequent release of insulin. The blood glucose then rises again by day 3 and thereafter starts to fall gradually as the transplanted islets release insulin appropriately in response to hyperglycaemia, such that from day 60 to day 100, normoglycaemia is maintained.

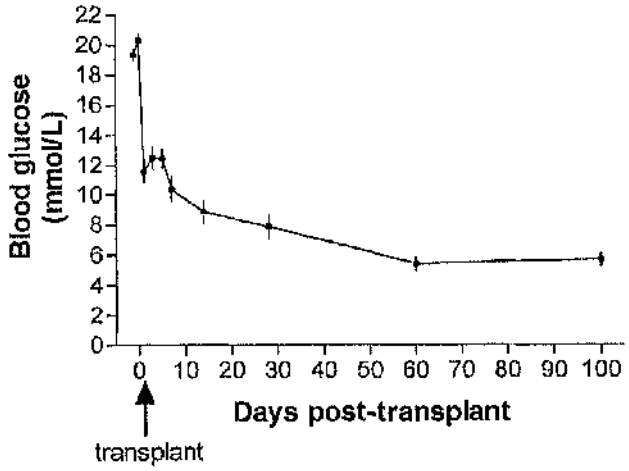


Figure 16. Mice blood glucose levels before and after transplanting human islets under the renal capsule of NOD-SCID mice (mean ± SEM).

3.3.2 In-vitro tests and their relationship to in-vivo tests of islet function

To assess whether the results of the in-vitro tests had any value in predicting the effectiveness of transplanted islets in the NOD-SCID mouse model, the mice were divided retrospectively into groups, for each in-vitro test, for the purposes of analysis. Grouping the mice into quartiles yielded too few mice in each group, so the mice were divided into three groups each containing equal numbers of experiments, that is, islet preparations from a single donor. However, for each islet preparation (from a single donor), there was often a different number of mice transplanted depending on the availability of mice at that time. The mean blood glucose concentration and SEM at the various time-points were calculated in each of the three groups and compared to assess whether there was a statistically significant difference or not.

With respect to EC (Figure 17), all groups of NOD-SCID mice were rendered normoglycaemic by the transplantation of human islets. The group with the lowest EC had lower mean glucose levels than the middle group. Both the low and middle groups had lower glucose levels than the high group. However, these differences were only significant at days 7 and 14 post-transplant. At day 7, there were significant differences between the low and middle groups ($p<0.01$) and between the low and high groups ($p<0.01$). At day 14, there were significant differences between the low and middle groups ($p<0.05$) and between the low and high groups ($p<0.05$).

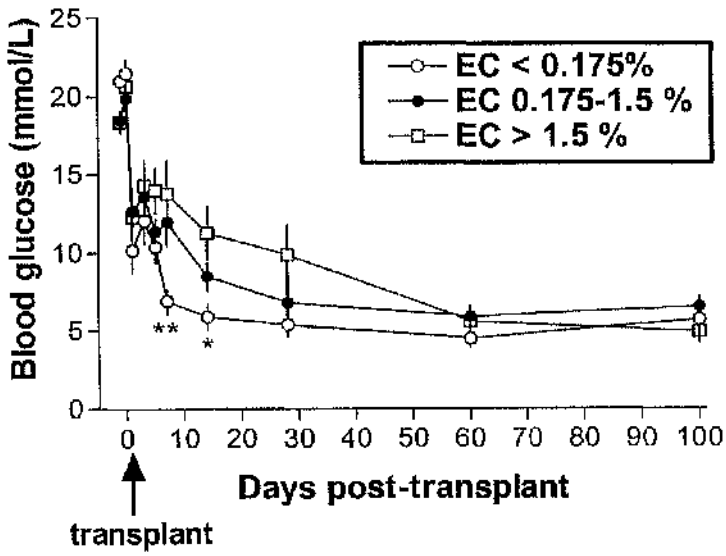


Figure 17. Effect of exocrine contamination (EC) of the human islet preparation on glycaemia, following human islet transplantation into NOD-SCID mice. * $P<0.05$, ** $P<0.01$.

Thus, in the first two weeks following transplant, the degree of exocrine contamination of the islet preparation may have some influence on the ability of the transplant to lower glucose levels, but ultimately exocrine contamination has no influence on whether the transplanted islets can restore normoglycaemia in the NOD-SCID mouse model.

Regarding IE (Figure 18), all groups of NOD-SCID mice were rendered normoglycaemic by the transplantation of human islets. There were no significant differences between each group at any time-point post-transplant. Thus, the degree of insulin enrichment of the islet preparation has little influence on the ability of the transplant to restore normoglycaemia in the NOD-SCID mouse model.

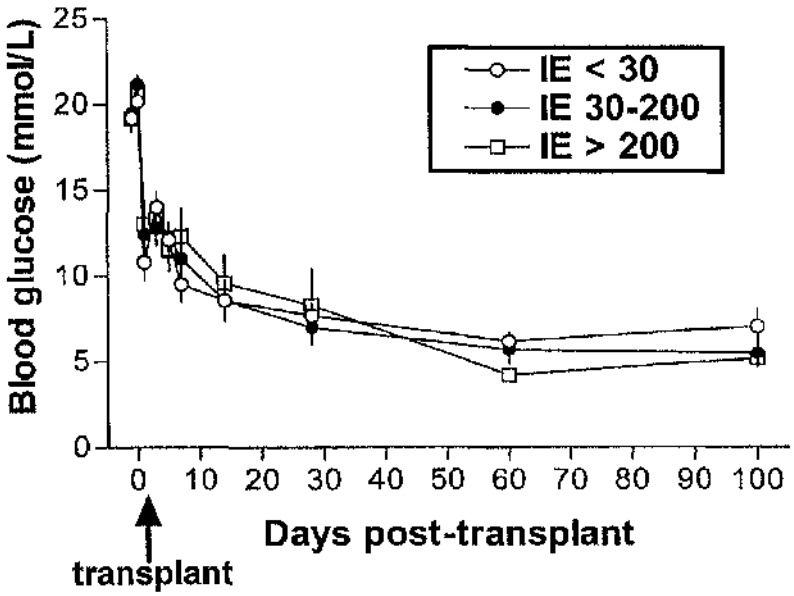


Figure 18. Effect of insulin enrichment (IE) of the human islet preparation on glycaemia, following human islet transplantation into NOD-SCID mice.

The SSI was used as an indicator of the in-vitro insulin-secretory response of isolated human islets to glucose. Ultimately, both the low and high SSI groups restored normoglycaemia (Figure 19). However, the middle group (with SS indices between 1.2 and 1.6) only ever achieved borderline glycaemic control. The middle SSI group had

significantly different mean glucose levels from the low and high groups at days 14, 28, 60 and 100 ($p<0.01$). Thus, human islets with low and high SSI can restore normoglycaemia in the NOD-SCID mouse model.

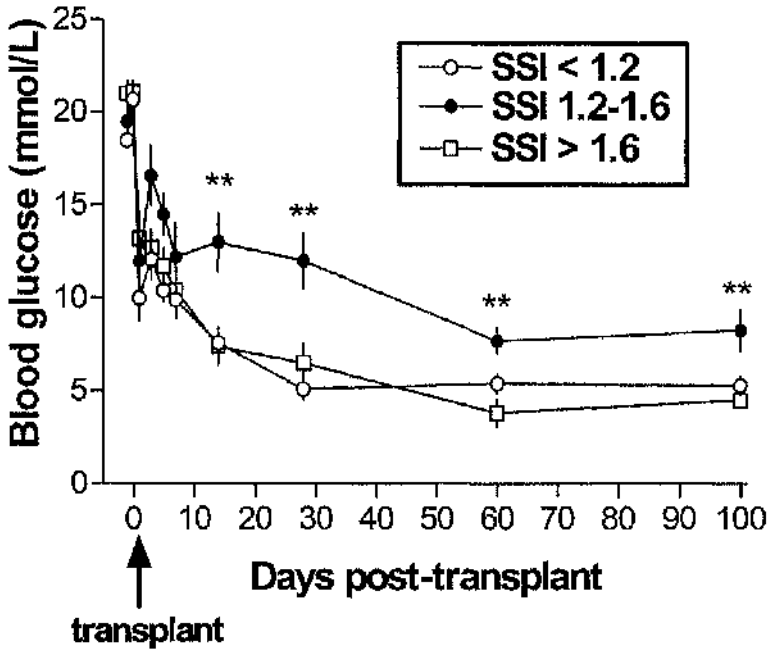


Figure 19. Effect of static stimulation index (SSI) of the human islet preparation on glycaemia, following human islet transplantation into NOD-SCID mice. ** $P<0.01$.

3.3.3 Donor characteristics and their influence on in-vivo tests of islet function

To investigate whether a donor's age, BMI, gender or diabetic status have any influence on the in-vivo function of transplanted islets, again the mice were divided into several groups retrospectively.

Those mice receiving islets from donors less than 40 years old, initially had a slower reduction in mean blood glucose concentration than those receiving islets from donors over 40 years old (Figure 20A). At day 1 and day 7 post-transplantation there was a significant difference in mean blood glucose. At day 1 the younger age group had a

mean glucose concentration of 13.1 ± 1.1 mmol/L (n=28, in 10 experiments) whereas the older age group had a mean glucose concentration of 10.1 ± 0.9 mmol/L (n=27, in 10 experiments), $p<0.05$. Also, at day 7 the younger age group had a mean glucose concentration of 11.8 ± 1.2 mmol/L (n=36, in 10 experiments) whereas the older age group had a mean glucose concentration of 8.2 ± 0.8 mmol/L (n=22, in 8 experiments), $p<0.05$. However, ultimately, both groups achieved normoglycaemia, which was maintained. Thus, the age of the donor only appears to have any influence on in-vivo islet function immediately following transplantation.

Although islets from female donors resulted in lower mean glucose levels than islets from male donors, both groups ultimately restored and maintained normoglycaemia in the diabetic mouse model and there were no significant differences between sexes (Figure 20B). Results are expressed as mean blood glucose concentration (mmol/L) ± SEM of 7 experiments (n=6 to 27) in the male group and 13 experiments (n=22-45) in the female group.

Islets transplanted from donors with a BMI <25 (normal) and >30 (obese) both restored normoglycaemia, with no significant differences between these groups (Figure 20C). However, those islets from donors with a BMI between 25 and 30 took longer to restore normoglycaemia, with significant differences between this middle group and the low group (BMI<25) at days 3 ($p<0.01$), 7 ($p<0.001$) and 14 ($p<0.05$) post-transplant. Results are expressed as mean blood glucose concentration (mmol/L) ± SEM of 6 experiments (n=10 to 21) in the BMI<25 group, 8 experiments (n=9 to 29) in the BMI 25 to 30 group and 6 experiments (n=9 to 22) in the BMI>30 group. Thus, ultimately, regardless of a donor's BMI, islets can restore and maintain normoglycaemia in this NOD-SCID mouse model.

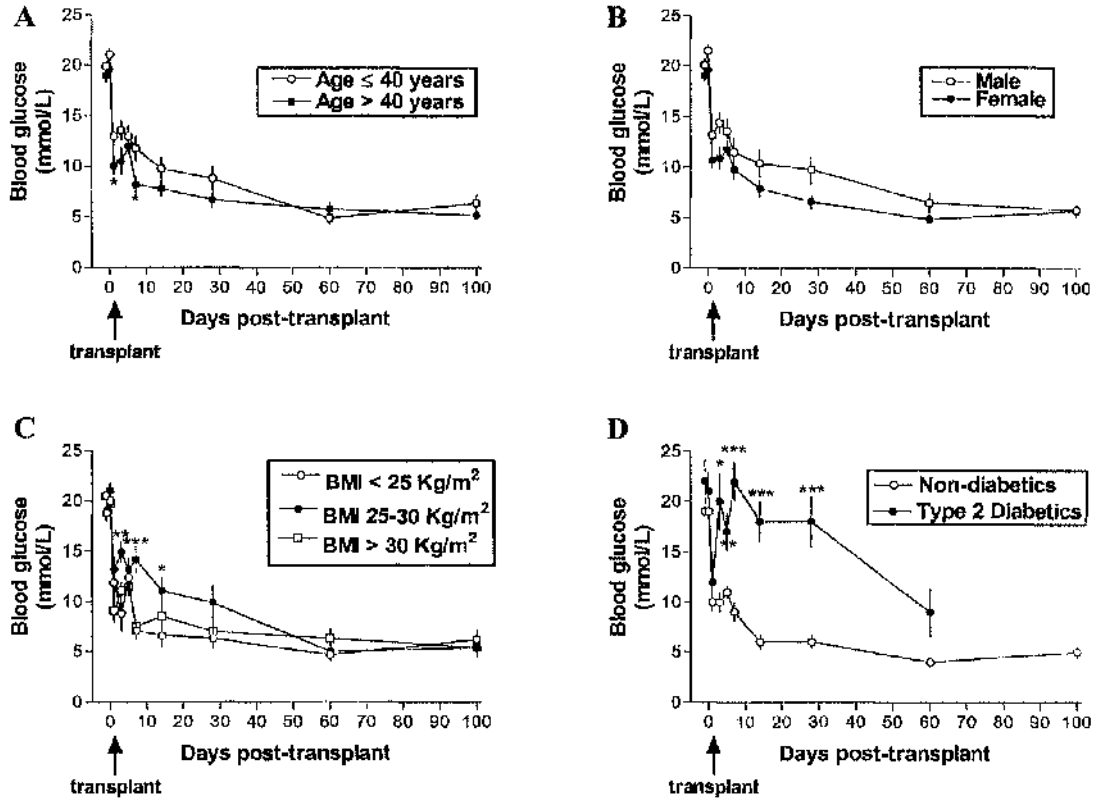


Figure 20. Effect of different donor age (A), sex (B), body mass index (C) and diabetic status (D) on glycaemia, following human islet transplantation into NOD-SCID mice. * P<0.05, ** P<0.01, *** P<0.001.

Islets from donors with type 2 diabetes had higher mean glucose levels than those from non-diabetic donors (Figure 20D). Results are expressed as mean blood glucose concentration (mmol/L) ± SEM of 14 experiments (n=20 to 50) in the non-diabetic group and of 2 experiments (n=4 to 8) in the diabetic group. Mean blood glucose levels in the type 2 diabetic group were significantly higher at days 3 ($p<0.05$), 5 ($p<0.01$), 7 ($p<0.001$), 14 ($p<0.001$) and 28 ($p<0.001$), compared to islets from donors without diabetes. Thus transplanted islets from diabetic donors do not function as well as those from non-diabetic donors in the NOD-SCID mouse model

3.3.4 Effect of the presence of warm ischaemia on in-vivo tests of islet function

HBD had no warm organ ischaemia as cardiac arrest occurs at the same time as organ recovery, whereas NHBD have life support withdrawn and organ recovery initiated at least five minutes after pronouncement of death (after cardiac arrest). Results are expressed as mean blood glucose concentration (mmol/L) ± SEM of 4 experiments (n=14) in the NHBD group and of 16 experiments (n=20 to 58) in the HBD group. Mice, transplanted human islets from either NHBD or HBD, had similar mean glucose levels with restoration and maintenance of normoglycaemia and no significant differences between groups (Figure 21). In addition, there were no other significant differences between the two groups (Table 6). Thus in this in-vivo model, NHBD can provide islets that function as well as islets from HBD.

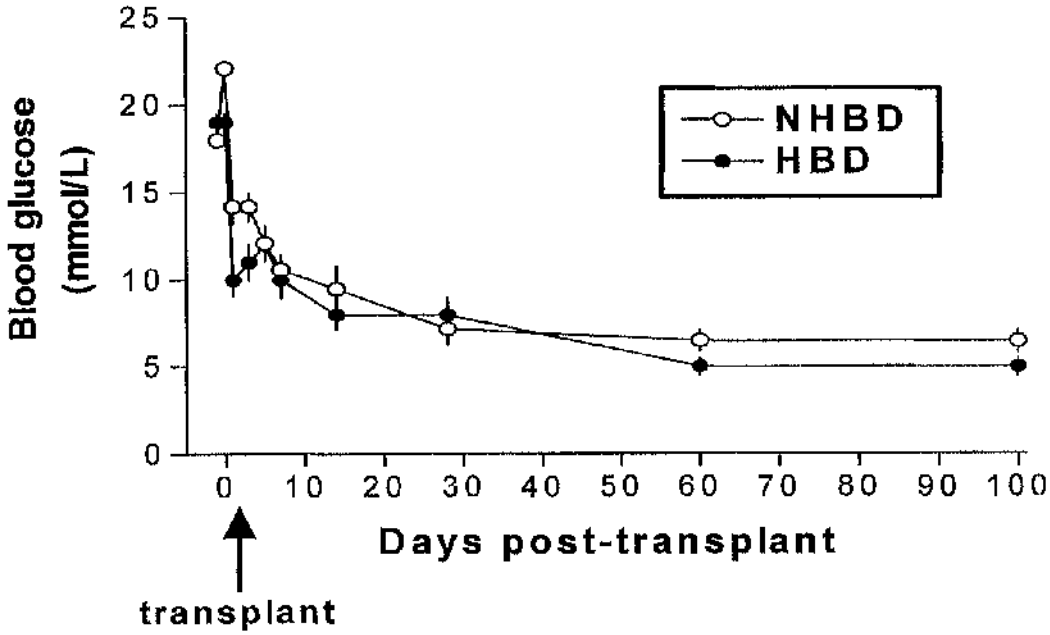


Figure 21. **NOD-SCID mice blood glucose levels following transplantation of human islets from HBD (no warm ischaemia) and NHBD (warm ischaemia present).** HBD, heart-beating donor; NHBD, non-heart-beating donor.

VARIABLE	NHBD mean ± SEM (n)	HBD mean ± SEM (n)	p value
Age (years)	41.2 ± 3.8 (9)	46.9 ± 1.6 (83)	0.189 (NS)
BMI (kg/m^2)	29.1 ± 2.6 (9)	27.8 ± 0.8 (107)	0.635 (NS)
CIT (hours)	5.7 ± 1.1 (9)	7.3 ± 0.4 (96)	0.190 (NS)
Pancreatic weight (g)	96.1 ± 14.0 (9)	89.8 ± 3.9 (104)	0.674 (NS)
Digestion time (min.)	60.0 ± 4.4 (9)	68.2 ± 2.1 (104)	0.113 (NS)
IEQ/g of pancreas	7315 ± 1894 (8)	7100 ± 1706 (71)	0.937 (NS)
Purity (%)	72.5 ± 10.3 (4)	63.1 ± 2.6 (82)	0.442 (NS)
EC (%)	3.8 ± 2.8 (7)	2.3 ± 0.6 (39)	0.610 (NS)
IE (fold increase)	139 ± 67 (7)	185 ± 43 (39)	0.578 (NS)
SSI	1.6 ± 0.2 (7)	1.5 ± 0.1 (41)	0.496 (NS)

Table 6. Donor, pancreatic, isolation and pre-transplant islet variables in NHB donors, compared to HB donors.
BMI, body mass index (kg/m^2); CIT, cold ischaemia time (hours); IEQ/g, number of IEQ isolated per weight of whole pre-digested pancreas; EC, % exocrine contamination; IE, insulin enrichment (fold increase); SSI, static incubation index; NS, not significant.

3.4 Discussion

In this study, transplanting human islets into diabetic mice effectively controls diabetes and restores and maintains normoglycaemia. At the end of the experiment (100 days post-transplantation), the kidney bearing the transplanted islet graft was removed by surgical nephrectomy. Thereafter the blood glucose concentrations rose to diabetic levels confirming that a functioning islet graft was responsible for the maintenance of normoglycaemia. The initial fall in blood glucose following transplantation is attributed to the death of insulin-containing cells, which release insulin in a pathological, uncontrolled manner. The blood glucose then rises again and then starts to fall gradually as the transplanted islets release insulin appropriately, in response to hyperglycaemia.

To assess whether the results of the in-vitro tests had any value in predicting the effectiveness of transplanted islets in the NOD-SCID mouse model, the mice were divided retrospectively into groups as described in Methods and Results. In the first two weeks following transplant, the degree of EC of the islet preparation may have some role in predicting the in-vivo function of islets, with those preparations of highest EC taking longer to restore normoglycaemia than the other groups. However, beyond 14 days post-transplant, EC is of no value in predicting the in-vivo function of islets, in this

model. Similarly the degree of IE and the SSI (with one exception) has no value in predicting the in-vivo function of islets in this model. The one exception is the middle SSI group (between 1.2 and 1.6). This middle group struggles to achieve the degree of normoglycaemia seen in the low and high SSI groups. Why islets with less glucose-stimulated insulin secretion (lower SSI) can perform better than islets with higher SSI (the middle SSI group) is difficult to explain.

Thus, none of the biochemical indices (EC, IE nor SSI) are able to predict the in-vivo effectiveness or function of transplanted islets in the NOD-SCID mouse model. This could be due to a variety of reasons. There could be problems with the validity of the tests. As this project is at the leading edge of this development, there is no previous similar experience of human islets to fall back on. That is, there are no available established human reference materials, so it is not possible to comment on the accuracy of the tests. In addition, in view of the finite availability of samples it is difficult to assess precision adequately. There is also the issue of the relevance of in-vitro tests. It may be that the short static incubation test in no way reflects the ability of the transplanted islets to respond to hyperglycaemia in-vivo. Indeed, in a static incubation test there is accumulation in the well of other hormones such as somatostatin, which inhibit insulin secretion (260). In addition, as this is an in-vitro test, the sympathetic and parasympathetic control of insulin secretion is absent. Also, it is possible that as there is so little exocrine contamination and such substantial insulin enrichment in most samples there is not a sufficient spread of values to permit adequate prediction of the in-vivo function of islets. However, most importantly, is the issue of IEQ dose-insulin response. It is possible that this model of in-vivo testing of islet function (with particular reference to the number of islets transplanted) results in a test with poor sensitivity. In other words, to discriminate between low and high quality islet preparations, perhaps fewer islets should have been transplanted. However, this problem will likely be addressed by the next phase of the HITP, which has already begun. Several brittle type 1 diabetic patients have now been transplanted with human islets and are subjected to intensive and thorough post-transplantation monitoring of glycaemic control and insulin secretion along similar lines to the Edmonton group (207,253). The in-vitro biochemical tests continue to be performed and at a later date comparisons will be made between these in-vitro results and the post-transplant data.

To investigate whether a donor's age, BMI or gender has any value in predicting the in-vivo function of transplanted islets, the mice were divided into several groups retrospectively. There are some significant differences between groups within the first 14 days post-transplant, but otherwise, islets from all groups were able to restore normoglycaemia in this model, no matter the donor's age, BMI or gender. It is clear that islets from donors with type 2 diabetes do not respond as well as those from donors without diabetes, as even by day 60 normoglycaemia is not restored in the type 2 diabetes group. This would be expected, as it is known that the pathological process in type 2 diabetes involves not only peripheral insulin resistance, but also β-cell dysfunction. Thus, if the NOD-SCID mouse model is a model that does indeed accurately reflect the transplanted in-vivo function of islets and allows for extrapolation to humans, it would seem reasonable that scrupulous attempts are made to ensure that donors do not have diabetes (which is often difficult to determine in critically-ill, stressed patients).

As NHBD islets performed as well as HBD islets in the in-vitro and in-vivo assays of islet function, this prompted the HUP HITP group to attempt a clinical transplant with islets procured from a NHBD. The transplanted NHBD preparation successfully reversed diabetes with infusion of islets from a single donor, a feat that has not been reported previously (259). It is believed that the duration of warm ischaemia may have a deleterious effect on islet viability (261,262). In this study, most of the pancreata procured had a warm ischaemia time of less than 30 minutes. Thus, this suggests that a short course of warm ischaemia in NHBDs may not have a significant deleterious effect on isolated islets in terms of islet function.

In summary, none of the in-vitro biochemical tests developed was able to predict the curative potential of islet material when transplanted into NOD-SCID mice. Also, islets from type 2 diabetic donors were unable to restore normoglycaemia in NOD-SCID mice. In addition, normally functioning pancreatic islets can be isolated successfully from the pancreata of NHBDs with a short warm ischaemia time and a single donor transplant from a NHBD resulted in a state of stable insulin independence in a type 1 diabetic recipient. In light of the current shortage of donors and the islet mass requirements of an increasing number of potential recipients, procuring pancreata from NHBDs might provide an additional source of isolated islets for clinical transplantation.

Chapter 4

The Effect of the Immunosuppressant Rapamycin on MIN-6 Cells, Rat and Human Islets

Chapter 4 The Effect of the Immunosuppressant Rapamycin on MIN-6 Cells, Rat and Human Islets

4.1 Introduction

Rapamycin (sirolimus) is a macrolide fungicide with potent antimicrobial, immunosuppressant and anti-tumour properties. As an immunosuppressant it has a mechanism of action distinct from that of cyclosporine, tacrolimus, corticosteroids and azathioprine. In humans, it has been used successfully as an immunosuppressant in islet (206), combined kidney-pancreas (263), renal (264) and liver (265) transplantation and as rescue therapy in lung and heart transplantation (266). Rapamycin has also found a role as a tool to suppress neointimal hyperplasia of rapamycin-eluting coronary stents in humans with coronary artery disease (267) and in the porcine coronary model (268). Indeed gene expression profiling of human stent-induced neointima by cDNA array analysis of microscopic specimens, reveals upregulation of FK506-binding protein 12 (FKBP12), the intracellular binding protein of rapamycin (269). Furthermore, CCI-779 (a water-soluble ester analogue of rapamycin) has significant activity against a wide range of in-vitro human cancers. It is currently undergoing clinical evaluation as an anti-tumour agent (270).

The anti-proliferative effects of rapamycin can be explained by understanding the intracellular mechanism of action of rapamycin. Rapamycin binds intracellular FKBP12 to form a complex, which binds to and inhibits the serine/threonine kinase activity of the mammalian target of rapamycin (mTOR) (271). Inhibition of mTOR blocks downstream phosphorylation of several proteins. A reduction in the kinase activity of $p70^{s6k}$ results in reduced phosphorylation of the 40S ribosomal protein S6 (which is essential for mRNA translation and hence protein synthesis) (272). Also, there is increased binding of the dephosphorylated eukaryotic initiation factor 4E binding protein-1 (4EBP1), or phosphorylated heat- and acid-stable protein (PHAS-1), to the mRNA cap-binding subunit of the eukaryotic initiation factor-4 (eIF-4F) complex, which inhibits its activity. This blocks the translation of mRNAs required for cyclin D1 synthesis, protein synthesis and cell cycle progression from G_1 to S phase (273). In addition, there is increased turnover of cyclin D1 at the protein and mRNA level. This

effect, in addition to the decreased translation of cyclin D1 (due to 4EBP1/ eIF-4F inhibition) results in a relative deficiency of cyclin D1, which is required for G_1 to S phase transition (270).

Rapamycin causes cell death by apoptosis in BKS-2 lymphoma cell lines. Tacrolimus (which competes with rapamycin for FKBP12 binding sites) inhibits rapamycin-induced apoptosis, suggesting that, in this cell line, rapamycin binding to FKBP12 is essential for programmed cell death (274). In addition, rapamycin induces apoptosis in the Rh1 and Rh30 rhabdomyosarcoma cell lines, and it is likely that mTOR has a critical role in rapamycin-induced apoptosis (275). Rapamycin enhances apoptosis and increases sensitivity to cisplatin in the human promyelocytic leukaemia cell line HL-60 and the human ovarian cancer cell line SKOV3 (276). Rapamycin can evoke apoptosis in human dendritic cells, in a time- and dose-dependent manner. The caspase inhibitor ZVAD-fmk only partially inhibits rapamycin-induced apoptosis. Monocytes, macrophages (either monocyte-derived or freshly isolated alveolar macrophages) and myeloid cell lines are resistant to the apoptotic effect of rapamycin (277). In addition, interleukin-7 prevents apoptosis of T-cell acute lymphoblastic leukaemia cells by down-regulating the cyclin-dependent kinase inhibitor $p27^{kip1}$ and up-regulating bcl-2 expression. The effect on $p27^{kip1}$ is inhibited by rapamycin, suggesting that in its phosphorylated form, $p27^{kip1}$ act as a tumour suppresser gene (278). In addition, rapamycin inhibits mitochondrial-based $p70^{s6k}$, which prevents phosphorylation of serine-136 on the pro-apoptotic BAD (the phosphorylated form is inactive) and blocks cell survival induced by insulin-like growth factor. Furthermore, IGF-1-induced phosphorylation of BAD Ser-136 is abolished in $p70^{s6k}$-deficient cells. This suggests that $p70^{s6k}$, by phosphorylating and hence inactivating BAD is crucial to continued cell survival (279). Although rapamycin inhibition of mTOR and subsequent dephosphorylation of $p70^{s6k}$ resulted in a significant reduction in proliferation of BxPC3 and Panc-1 human pancreatic adenocarcinoma cell lines, rapamycin alone did not induce apoptosis in this cell line (280). In summary, rapamycin has been shown to induce apoptosis in some cell lines, but not others.

In the Edmonton study, blood trough rapamycin levels were monitored to maintain them in the range of 12 to 15 ng/mL for the first three months and in the range of 7 to 10 ng/mL (or 7.66 to 10.9 nM) thereafter (206). Other authors describe a putative

rapamycin therapeutic window in a renal transplant regime that includes cyclosporine, as 5 to 15 ng/mL (or 5.47 to 16.4 nM) (281).

The Edmonton study was not designed to assess the effect of rapamycin on islet function. A previous study demonstrated that rapamycin, with or without cyclosporine, is not associated with adverse impact on islet function or glucose metabolism in the canine model of pancreatic transplantation (282). Another study demonstrated that rapamycin induces primary non-function of islet xenografts in a dose-dependent manner suggesting that rapamycin may be diabetogenic (283). Yet another study demonstrated that rapamycin reduces insulin secretion of HIT-T15 cells after 48 hours culture, but rapamycin had no effect on insulin secretion of Wistar rat islets after 24 hours culture (284). The effect of rapamycin on the MIN-6 cells and islets is not clear. Thus, the aim of our study was to address whether rapamycin has any effect on MIN-6 cells and rat and human islets in-vitro.

4.2 Materials and Methods

4.2.1 MIN-6 cell culture

MIN6 insulinoma cells were cultured in T175 cm flasks in 25 mM glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 15% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37°C under conditions of 95% air, 5%CO_2. Medium was changed every 3 or 4 days. Cells were trypsinised weekly and passages 35-50 were used exclusively.

4.2.2 Isolation of pancreatic islets

The Children's Hospital of Philadelphia guidelines for the use and care of laboratory animals were followed. Male Sprague-Dawley rats (Charles River Laboratories, Boston, MA, USA) were injected with Nembutal (0.05 mg/g rat). After the rats were anaesthetised, the bile duct was cannulated, and the pancreas was inflated with approximately 20 mL of Hanks' balanced buffer. The inflated pancreas was removed, and cleaned of its lymph nodes, fat, blood vessels and bile duct. Tissue was digested with collagenase P (Roche Molecular Biochemicals) as previously described and purified on a discontinuous Ficoll gradient (285,286). Isolated islets were washed and cultured in complete CMRL-1066 (supplemented with 10% fetal bovine serum, 2 mM L-glutamine,

100 U/mL penicillin, 100 μg/mL streptomycin) culture medium at 37°C (95% air, 5%CO_2).

4.2.3 MIN-6 cell viability determination

1. MTT: The MTT assay is an indirect measure of cell viability. The assay is based on the ability of viable cells (in particular mitochondrial succinate dehydrogenase) to reduce MTT (C,N-diphenyl-N'4-5-dimethyl thiazol-2-yl tetrazolium bromide) to insoluble coloured formazan crystals. 1.6 x 10^6 MIN-6 cells from one T175 flask were plated in one 24-well plate in 25 mM glucose DMEM for 3-4 days. Thereafter, the medium was removed and replaced with different glucose concentrations and different rapamycin concentrations in DMEM for either 1, 2 or 4 days. After culture with or without rapamycin, cells were washed twice with 1 mL Krebs-HEPES buffer (115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES, pH 7.4 and 1% bovine serum albumin) no glucose and incubated in 1 mL Krebs-HEPES buffer (0 mM glucose) with 0.5 mg/mL MTT for 60 min at 37°C. The supernatant was discarded, and cells in each well were lysed with 500 μL of 2-propanol and incubated for 60 minutes at room temperature. The optical density of the resultant coloured 2-propanol was measured at 560 nm on a MicroKinetics plate reader.

2. Fluorescent live/dead cell assay: A two-colour fluorescence cell viability assay was used based on the ability of calcein-AM to be retained within live cells, inducing an intense uniform green fluorescence and ethidium homodimer (EthD-1) to bind the nuclei of damaged cells, thus producing a bright red fluorescence in dead cells. 1.6 x 10^6 MIN-6 cells from one T175 flask were plated in one 24-well plate in 15 mM glucose DMEM for 3 to 4 days. Thereafter, the medium was removed and replaced with different rapamycin concentrations in 15 mM glucose DMEM for either 2 or 4 days. After culture with or without rapamycin, cells were washed twice with 0.5 mL sterile PBS. The wash solution was then centrifuged, the supernatant removed, and the pellet resuspended in 200 μL PBS containing 2 μM calcein-AM and 4 μM EthD-1. This solution was then returned to the appropriate well of the 24-well plate and incubated at room temperature for 45 minutes in the dark. The green fluorescence of the live cells was measured by the Wallac 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Gaithersburg, MD, USA). Excitation was at 488 nm and live cells were detected at

wavelength of 510 nm with a bandpass filter. Images of the stained cells were captured by digital fluorescence microscopy using the SimplePCI software (Compix Inc. Imaging Systems, Cramberry, PA, USA).

4.2.4 Cell viability in human islets

Human islets were obtained from the Islet Isolation Core of the JDFI-Penn Islet Transplantation Center, with the approval of the local Ethics Committee. Consent was obtained in accordance with accepted guidelines. Purity was assessed by staining the preparation with dithizone and calculating the percentage of all tissue that stained positive for insulin. Human islets were cultured in CMRL (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin) at 37°C under conditions of 95% air, 5%CO_2 in a T75 flask. All human islets were removed from the flask, washed, and resuspended in 12 mL of CMRL. A volume of islet suspension was added to each well of a 6-well plate. Either vehicle control (0.1% ethanol) or rapamycin was added to each well. After 4 days culture, 0.2 mL of supernatant was removed from each well and 0.2 mL of Krebs-HEPES buffer (0 mM glucose) with 0.5 mg/mL MTT was added and incubated for 120 minutes at 37°C. Thereafter, all well contents were removed, spun and the supernatant removed. The islets were then washed with 1 mL Krebs-HEPES buffer. The supernatant was again discarded and 300 µL of 2-propanol was added and incubated for 60 minutes at room temperature. After further centrifugation, 200 µL of supernatant was removed for MTT analysis. The optical density of the resultant coloured 2-propanol was measured at 560 nm on a MicroKinetics plate reader.

4.2.5 Rat islet insulin secretion

Rat islets were cultured in10-cm tissue-culture dishes, containing 10 mL of supplemented CMRL-1066 and 1, 10 or 100 nM rapamycin or the vehicle control (0.1% ethanol). After 4 days culture, islets were removed and washed twice in RPMI medium. Within each dish, rat islets were divided into 3 groups depending on their size; small, medium or large. Five rat islets per well were counted into a 6-well plate, picked sequentially, starting from the group of largest islets to the group of smallest islets and then back to the group of largest islets and incubated in 1.5 mL of either a low-glucose RPMI solution (3 mM glucose) or a high-glucose RPMI solution (20 mM glucose) with 1 mM carbachol at 37°C for 2 hours. After incubation, the supernatant was removed and

analysed for its insulin content by radioimmunoassay (Penn Diabetes Center RIA Core, USA).

4.2.6 Cell viability in rat islets

Propidium iodide (PI) is a highly polar dye, which penetrates cells with damaged membranes and stains the nuclei red. Fifty rat islets were cultured in 3 ml of supplemented CMRL-1066 and either 10 or 100 nM rapamycin or the vehicle control (0.1% ethanol). After four days culture, PI solution was added to each well (final concentration 15 μg/mL) and incubated for 45 minutes in the dark at room temperature. Islets were then removed, centrifuged and the supernatant removed and discarded. The islets were resuspended in 60 μL Krebs-HEPES buffer and transferred to a 384-well plate. The intensity of PI fluorescence was measured by a Wallac 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Gaithersburg, MD, USA), with excitation at 485 nm and emission at 630 nm. Fluorescent images were captured by confocal fluorescence microscopy at pixel size of 0.546μm and 20× magnification with excitation at 488 nm and emission at 610 nm (Penn Diabetes Center Biomedical Imaging Core, USA). For each well, a minimum of 10 random size-matched islets were acquired under bright and fluorescence microscopy.

4.2.7 Identification of apoptosis by transferase-mediated dUTP nick-end labeling assay

An in-situ Cell Death Detection Kit (Boehringer Mannhein, Indianapolis, IN, USA) was used to detect apoptotic cells. In this method, terminal deoxynucleotidyl transferase was used to catalyse the polymerisation of fluorescein-labeled nucleotides to free 3-OH termini of DNA strand breaks. After 19 hours culture DMEM with vehicle control (0.1% ethanol), 10 or 100 nM rapamycin, MIN-6 cells were trypsinised and washed twice with cold PBS (Phosphate-Buffered Saline)/1%BSA. Cells were then fixed with 200 μL 2% paraformaldehyde and incubated for 60 min at room temperature. After rinsing with PBS, cells were resuspended with 250 μL of permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) and incubated for 30 min at room temperature. Then, 50 μL of TUNEL reaction mixture was added to samples and the positive controls, (50 μL of label solution only was added to the negative controls), and cells were incubated at 37°C for 1 h. Apoptotic cells were identified by FITC staining,

and analysed by flow cytometry. A Coulter EPICS Elite Flow cytometer (Beckman-Coulter, Hialeah, FL, USA) equipped with a 5-watt argon laser operated at 488 nm and 260 milliwatts output was used for all studies. Monomeric forms of the MIN6 cells were electronically gated based on forward and side scatter measurements to exclude cell aggregates from evaluation. Fluorescence signals were collected with a photomultiplier tube configured with 550 nm dichroic and 525 nm band pass filters. 10,000 events were collected into a 4 decalog single parameter histogram for each sample. Percent positive cells were determined based on the evaluation of cells treated with TUNEL reagents lacking TdT using a cursor setting that yielded less than 2% positive cells.

4.2.8 Islet cell apoptosis identified by electron microscopy

Rat islets were cultured in complete CMRL-1066 in 6-cm dishes with vehicle or with rapamycin 100 ng/ml for 4 days. Islet preparations were then washed briefly with pre-warmed, serum-free medium, and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer for 4 hours. The islets were collected from the dish to make a pellet. After washing, osmication, and dehydration with ethanol and propylene oxide, the pellets were embedded in EM-Bed 812, and polymerized at 70°C for 48 hours. Semithin sections (1 micron) were stained with Toluidine blue to screen general cell morphology. Ultrathin sections (80 nm) were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL-1010 TEM operated at the accelerated voltage of 80 kv.

4.2.9 Statistical analysis and data presentation

Data are presented as mean ± S.E.M. Statistical significance of differences between groups was analysed by one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison tests. A p value of <0.05 was considered statistically significant. The molecular weight of rapamycin is 914.2, thus a rapamycin concentration of 10 ng/mL is actually 9.142 nM. The rapamycin stock solutions used in this study were 0.01, 0.1, 1, 10 and 100 ng/mL. However, to case comparisons with the units used in clinical therapeutic drug monitoring, the rapamycin concentration data in the graphs is labelled as 0.01, 0.1, 1, 10 and 100 nM.

4.3 Results

4.3.1 Dose-dependent effect and time-course of rapamycin on MIN-6 cell viability using MTT

To determine whether rapamycin has a deleterious effect on MIN-6 cells, MIN-6 cells were incubated with rapamycin concentrations of 0 (vehicle control), 0.01, 0.1, 1, 10 and 100 nM in DMEM for 1, 2 or 4 days and then evaluated for cell viability using the MTT method. Results are expressed as mean (% of control mean) ± SEM of 3 experiments performed in quadruplicate (n=12). As early as day 1 (Figure 22A), rapamycin started to reduce MIN-6 cell viability in a dose-dependent manner. At day 1, the threshold concentration causing decreased MIN-6 cell viability was 0.01 nM rapamycin (83 ± 6% of control, $p<0.05$), the maximal effect was at 10 nM rapamycin (63 ± 5% of control, $p<0.001$) and IC_{50} was 0.02 nM rapamycin. At day 2 (Figure 22B), the threshold concentration was 0.01 nM rapamycin (68 ± 6% of control, $p<0.001$), the maximal effect was at 10 nM rapamycin (52 ± 2% of control, $p<0.001$) and IC_{50} was 0.01 nM rapamycin. At day 4 (Figure 22C), the threshold concentration was again 0.01 nM rapamycin (77 ± 4% of control, $p<0.001$). The maximal effect was not reached at day 4 and hence the IC_{50} could not be calculated. The threshold concentration at day 1, 2 and 4 was the same (0.01 nM rapamycin), although the level of significance increased from day 1 to day 2 and 4. The IC_{50} fell from 0.02 nM rapamycin at day 1 to 0.01 nM rapamycin at day 2. These results suggest that rapamycin has a dose-dependent deleterious effect on MIN-6 cell viability, evident at 1 day, by using the MTT method.

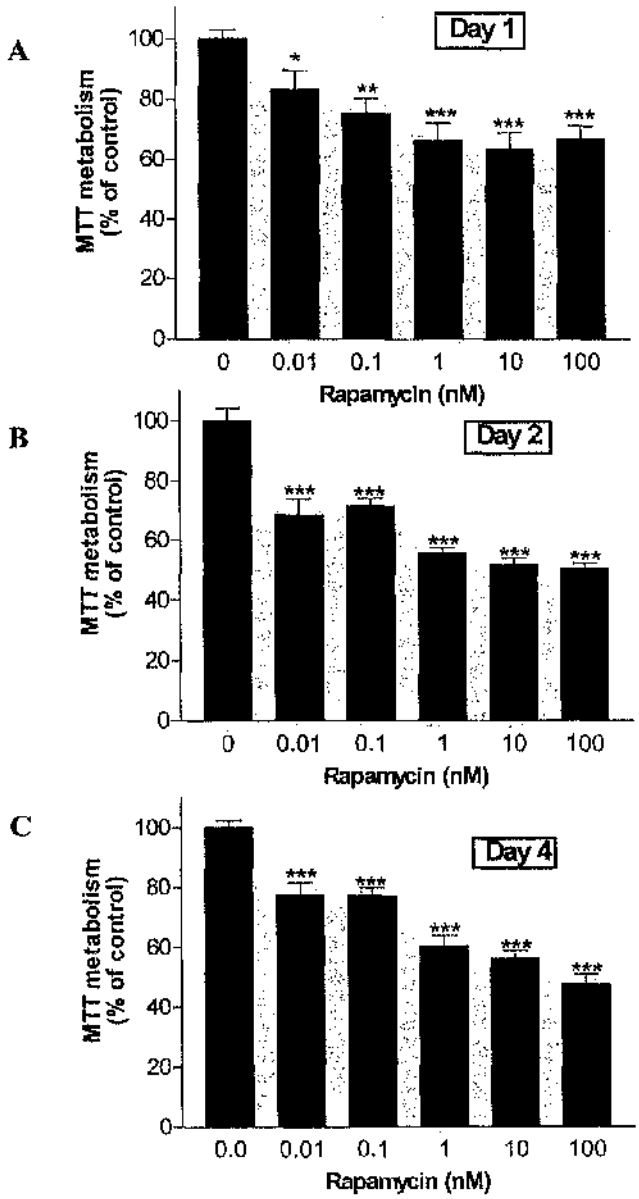


Figure 22. Dose-dependent effect of rapamycin on MIN-6 cell viability measured by MTT metabolism. MIN-6 cells were plated in 24-well plates and cultured for 3-4 days in 25 mM glucose (G25) DMEM (15% fetal bovine serum). Supernatant was removed and cells were further cultured with various concentrations of rapamycin or vehicle control (0.1% ethanol) in G25 DMEM (15% FBS) for 1 day (A), 2 days (B) or 4 days (C). Cell viability was measured by MTT metabolism. Results are expressed as mean (% of control mean) ± SEM of 3 experiments performed in quadruplicate (n=12). * $P<0.05$, ** $P<0.01$, ***$P<0.001$.

4.3.2 Glucose-dependency of rapamycin effect on MIN-6 cell viability

These experiments were designed to assess whether the rapamycin effect on MIN-6 cell viability, as measured by the MTT method, was glucose-dependent after 4 days culture (Figure 23). Results are expressed as mean (normalised to control mean) ± SEM of 3 experiments from 8 to 12 observations. MIN-6 cells cultured in 3 or 15 mM glucose in DMEM had a threshold concentration of 0.1 nM rapamycin (78 ± 5% of control, $p<0.05$ and 76 ± 1% of control, $p<0.05$ respectively). MIN-6 cells cultured in 25 mM glucose DMEM had a threshold concentration of 0.01 nM rapamycin (77 ± 4% of control, $p<0.05$). The maximal effect was not reached for 3 or 25 mM glucose, but MIN-6 cells cultured in 15 mM glucose had a maximal effect at 10 nM rapamycin (49 ± 5% of control, $p<0.05$) and an IC_{50} of 0.09 nM rapamycin. At each rapamycin concentration (0.01 nM to 100 nM) there was no significant difference between the cell viability of MIN-6 cells cultured for 4 days in 3, 15 or 25 mM glucose. These results suggest that the dose-dependent effect of rapamycin on MIN-6 cell viability is glucose-independent.

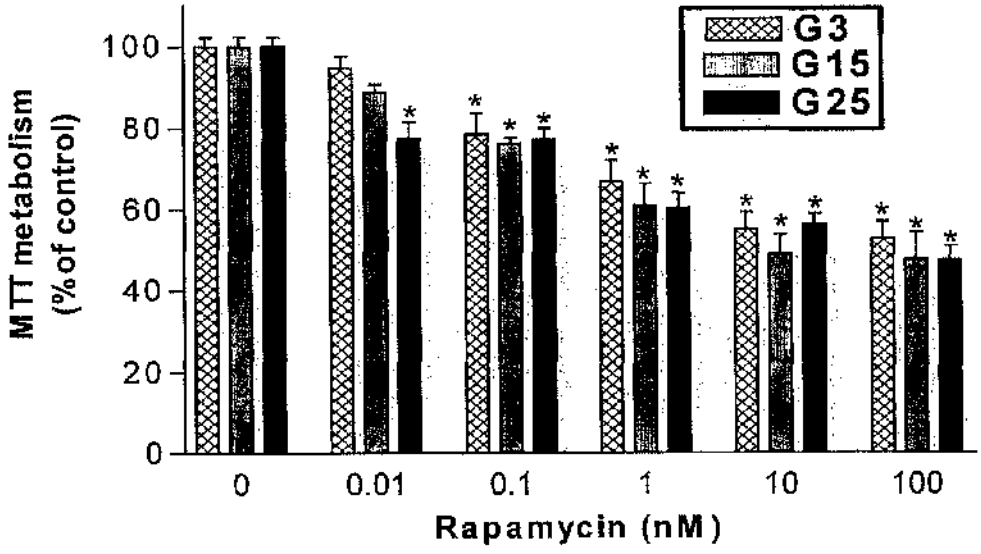


Figure 23. Glucose-dependency of rapamycin effect on MIN-6 cell viability measured by MTT metabolism. MIN-6 cells were plated in 24-well plates and cultured for 3-4 days in 25 mM glucose (G25) DMEM (15% FBS). Supernatant was removed and cells were further cultured with various concentrations of rapamycin or vehicle control (0.1% ethanol) each in 3 mM glucose (G3- hatched bars), 15 mM glucose (G15-stippled bars) and 25 mM glucose (G25-solid bars) DMEM (15% FBS) for 4 days. Cell viability was measured by MTT metabolism. Results are expressed as mean (normalized to control mean in the absence of rapamycin) ± SEM of 3 experiments from 8 to 12 observations. * $P<0.05$.

4.3.3 Dose-dependent effect and time-course of rapamycin on MIN-6 cell viability using calcein AM

The aforementioned MTT assay is an indirect measure of cell viability – it actually measures formazan, which is a purple substance formed by the oxidation of yellow C,N-diphenyl-N'4-5-dimethyl thiazol-2-yl tetrazolium bromide (MTT) by the action of mitochondrial succinate dehydrogenase. Hence, the more mitochondrial succinate dehydrogenase present, the greater the purple intensity of the sample. However, it is possible that the overall activity of succinate dehydrogenase is not only related to the number of viable cells present. It is difficult to exclude an intracellular metabolic effect of rapamycin, which may be having an inhibitory effect on succinate dehydrogenase. It is for this reason, we attempted to demonstrate reduced MIN-6 cell viability by a different method; a two-colour fluorescence cell viability assay. This assay is based on the ability of calcein-AM to be retained within live cells, inducing an intense uniform green fluorescence and ethidium homodimer (EthD-1) to bind the nuclei of damaged cells. Thus the more green fluorescence present, the more live cells are present in the sample. Results are expressed as mean fluorescence (normalized to control mean) ± SEM of 3 experiments performed in quadruplicate (n=12). At day 2 (Figure 24A), the threshold effect was at 10 nM rapamycin (73 ± 5% of control, $p<0.001$), with no maximal effect achieved. At day 4 (Figure 24B), the threshold effect was at 0.1 nM rapamycin (54 ± 5% of control, $p<0.001$), with a maximal effect at 10 nM rapamycin (40 ± 4% of control, $p<0.001$) with an IC_{50} of 0.01 nM rapamycin. Fluorescent images are of MIN-6 cells cultured with 0.1% ethanol (Figure 24C) or 100 nM rapamycin (Figure 24D) for 4 days, then stained with calcein AM and ethidium homodimer (EthD-1). The images demonstrate increased bright red fluorescence in those cells treated with rapamycin compared to control. These results confirm the previous findings, using the MTT method, that rapamycin does indeed have a dose-dependent deleterious effect on MIN-6 cell viability.

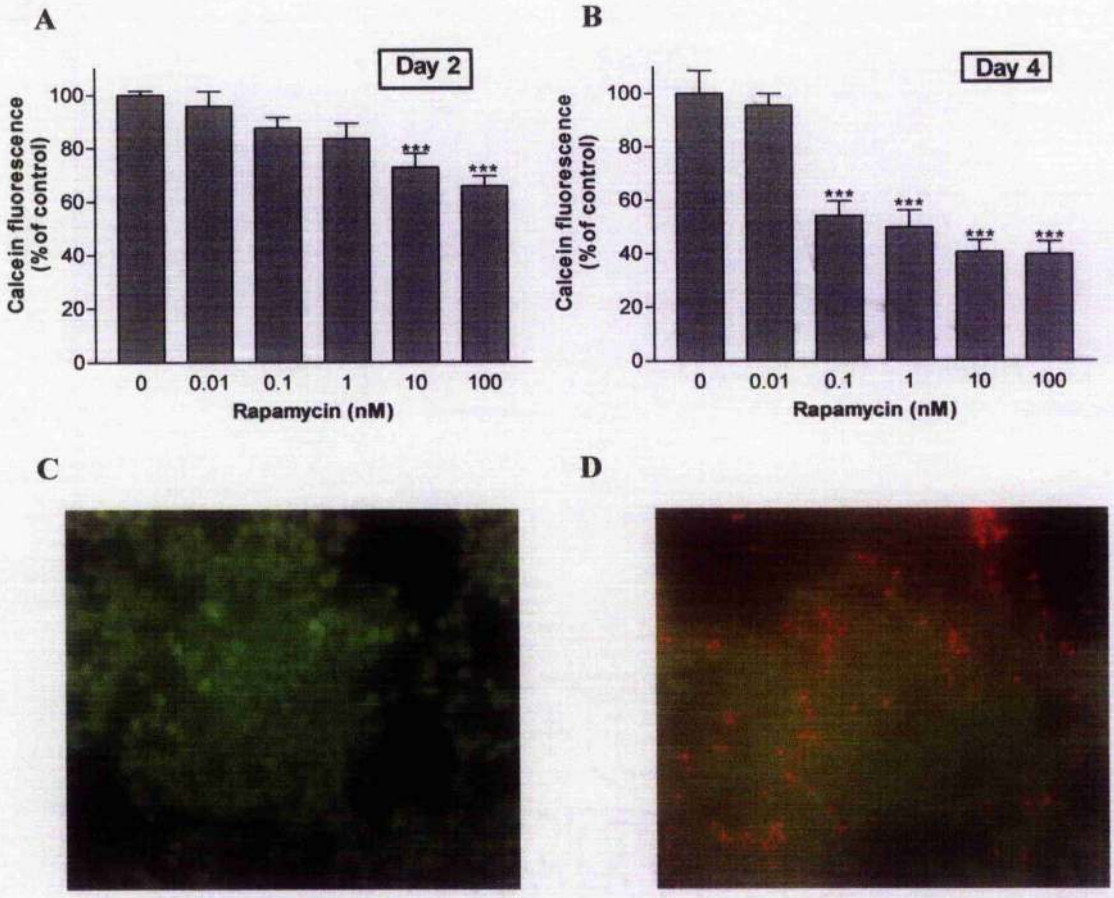


Figure 24. Dose-dependent effect of rapamycin on MIN-6 cell viability measured by calcein fluorescence. MIN-6 cells were plated in 24-well plates and cultured for 3-4 days in 15 mM glucose (G15) DMEM (15% FBS). Supernatant was removed and cells were further cultured with various concentrations of rapamycin or vehicle control (0.1% ethanol) for 2 days (A) or 4 days (B). Cell viability was quantitated by Calcein fluorescence. Results are expressed as mean fluorescence (normalised to control mean) ± SEM of 3 experiments performed in quadruplicate (n=12). ***P<0.001. Fluorescent images are of MIN-6 cells cultured with 0.1% ethanol (C) or 100 nM rapamycin (D) for 4 days, then stained with Calcein AM and ethidium homodimer.

4.3.4 Effect of rapamycin on rat islet insulin secretion

To determine whether rapamycin has any effect on glucose-stimulated insulin secretion of rat islets, islets were cultured with vehicle control or different concentrations of rapamycin for 4 days, and thereafter a 2 hour static incubation test was performed on each group of islets. Results are expressed as mean insulin secretion (μU/islet/2 hours) ± SEM in 4 experiments from 8 to 12 observations. The rat islets cultured in vehicle control had a four-fold increase in insulin secretion in

response to 20 mM glucose and 1 mM carbachol (CCH) compared to 3 mM glucose control (Figure 25). At rapamycin concentrations of 1 and 10 nM there was a reduction in glucose-induced insulin secretion, which was not statistically significant. At a supra-therapeutic rapamycin concentration of 100 nM, however, there was a 54% reduction (from 198 ± 25 to 90 ± 8 μU/islet/2 hours) in glucose and carbachol-induced insulin secretion ($p<0.01$). Basal insulin secretion (3 mM glucose) was not affected by rapamycin at any concentration tested. These results suggest that a supra-therapeutic rapamycin concentration of 100 nM significantly impairs glucose-induced insulin secretion.

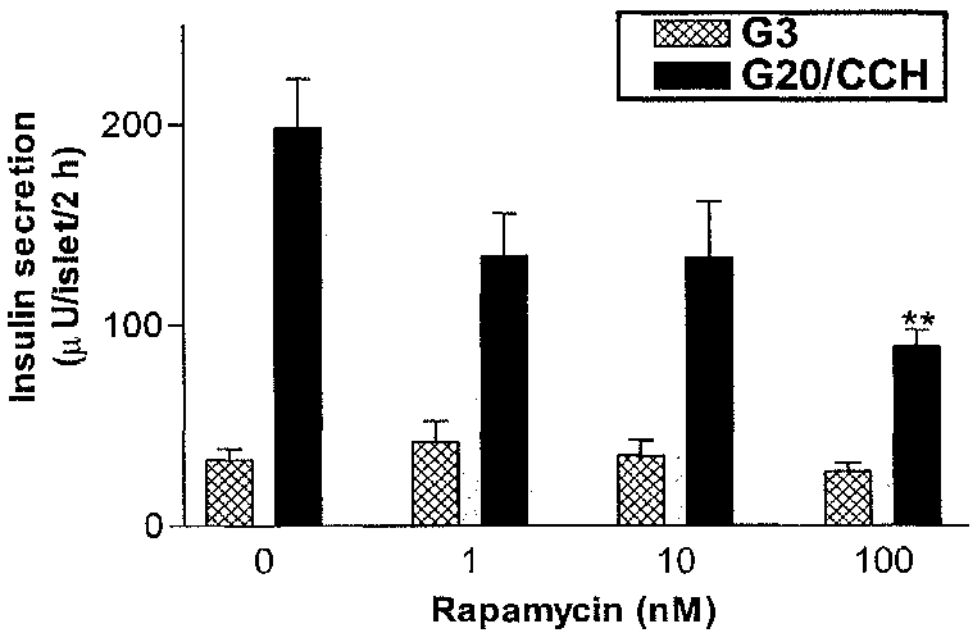


Figure 25. Effect of rapamycin on rat islet insulin secretion after 4 days culture. Rat islets were cultured with either 1, 10 or 100 nM rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Islets were washed then incubated for 2 hours with 3 mM glucose or 20 mM glucose and 1 mM carbachol, then samples were taken for insulin RIA. Results are expressed as mean insulin secretion (μU/islet/2 hours) ± SEM in 4 experiments from 8 to 12 observations. ** $P<0.01$.

4.3.5 Effect of rapamycin on rat and human islet cell viability

To determine whether rapamycin has any effect on human and rat islet cell viability, islets were cultured with vehicle control (0.1% ethanol) or rapamycin for 4 days and then cell viability was measured by different methods. Results are expressed as mean

fluorescence, normalized to control mean ± SEM in 6 experiments. After 4 days culture in different rapamycin concentrations, rat islets were stained with propidium iodide to quantify rat islet cell death (Figure 26A). At 1 and 10 nM rapamycin there was a small, insignificant increase in PI fluorescence, but at a supra-therapeutic rapamycin concentration of 100 nM there was a 5.3 fold increase in PI fluorescence ($p<0.01$). The images of rat islets (a bright field image was first acquired followed by a confocal image to detect PI fluorescence and overlaid) demonstrate increased PI fluorescence from the islet cultured with rapamycin (Figure 26C), compared to control (Figure 26B). Human islets were cultured for 4 days with a supra-therapeutic rapamycin concentration of 100 nM or control and then human islet cell viability measured using the MTT method (Figure 27). Results are expressed as mean (normalised to control mean) ± SEM of 4 experiments with 11 observations. There was a 55% reduction in MTT metabolism compared to control islets ($p<0.001$). These results suggest that a supra-therapeutic rapamycin concentration of 100 nM has a deleterious effect on rat and human islets.

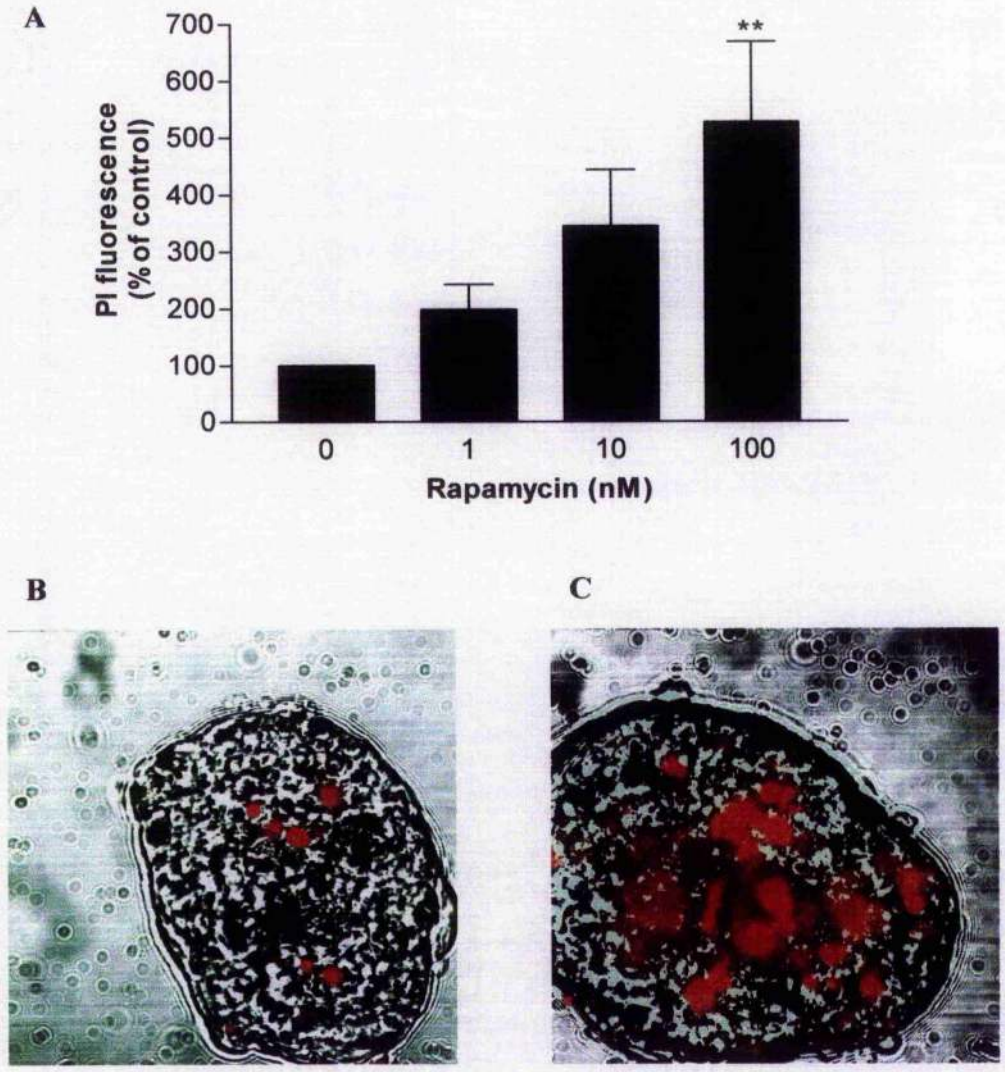


Figure 26. Effect of rapamycin on rat islet cell viability. (A) Rat islets were cultured with either 1, 10 or 100 nM rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Islets were then stained with propidium iodide and the fluorescence intensity measured. Results are expressed as mean fluorescence, normalised to control mean ± SEM in 6 experiments. ** $P<0.01$. Rat islets were cultured with 0.1% ethanol (B) or 100 nM rapamycin (C) for 4 days and then stained with PI. A bright field image was first acquired followed by a confocal image to detect PI fluorescence and overlaid.

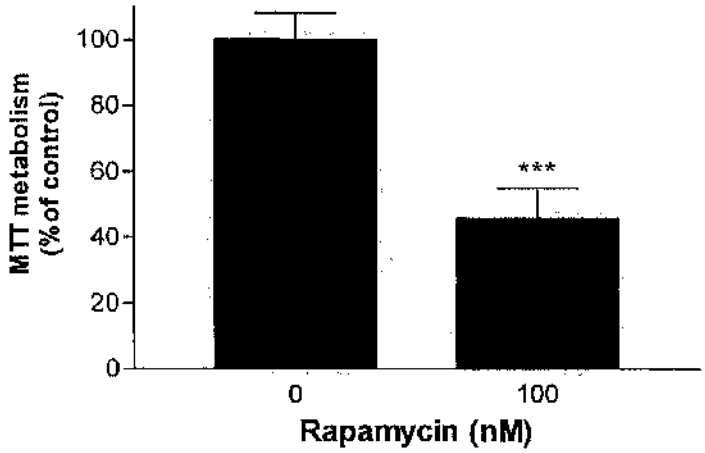


Figure 27. Effect of rapamycin on human islet cell viability after 4 days culture. Human islets were cultured with either rapamycin or vehicle control (0.1% ethanol) in supplemented CMRL for 4 days. Cell viability was measured by MTT metabolism. Results are expressed as mean (normalised to control mean) ± SEM of 4 experiments with 11 observations. ***P<0.001.

4.3.6 Rapamycin induces apoptosis in MIN-6 cells

To assess whether rapamycin induces apoptosis, MIN-6 cells were treated with either vehicle control (0.1% ethanol), 10 or 100 nM rapamycin for 19 hours and then the degree of apoptosis was measured by the TUNEL assay (Figure 28). Results are expressed as mean (normalised to control mean) ± SEM of 4 experiments from 7 to 8 observations. Compared to control, there was a 3.1 ± 0.6 fold increase ($p<0.01$) in apoptosis in the MIN-6 cells treated with 10 nM rapamycin and a 3.4 ± 0.4 fold increase ($p<0.01$) in apoptosis in the MIN-6 cells treated with a supra-therapeutic rapamycin concentration of 100 nM. These results suggest that 10 and 100 nM (a supra-therapeutic concentration) rapamycin induces apoptosis in MIN-6 cells.

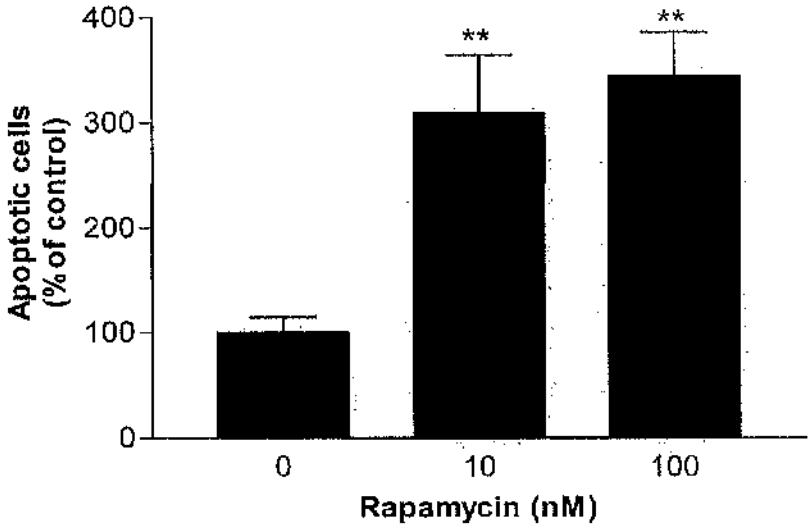


Figure 28. Effect of rapamycin on MIN-6 cell apoptosis. MIN-6 cells were cultured with either 10 or 100 nM rapamycin or vehicle control (0.1% ethanol) in 5 mM glucose DMEM for 19 hours, then apoptotic cells were measured by flow cytometric TUNEL assay. Results are expressed as mean (normalised to control mean) ± SEM of 4 experiments from 7 to 8 observations. **P<0.01

4.3.7 Rapamycin induces apoptosis in rat islets

Rapamycin causes cell death of rat islet-cells as assessed by electron microscopy. Rapamycin treatment of rat islets resulted in numerous apoptotic β-cells (Figure 29B) as well as α-cells (Figure 29C). β-cells were identified by their specific square shape granules, and α-cells were recognized by their round dark granules by electron microscopy. Vehicle-treated islets cells had a normal ultrastructure (Figure 29A). In contrast, rapamycin-treated islet-cells showed typical apoptotic morphologic changes including nuclear condensation (a), nuclear envelopes with irregular dilation (b), granule dilution (c), clumped and condensed mitochondria (d), as well as some typical apoptotic bodies (solid arrow).

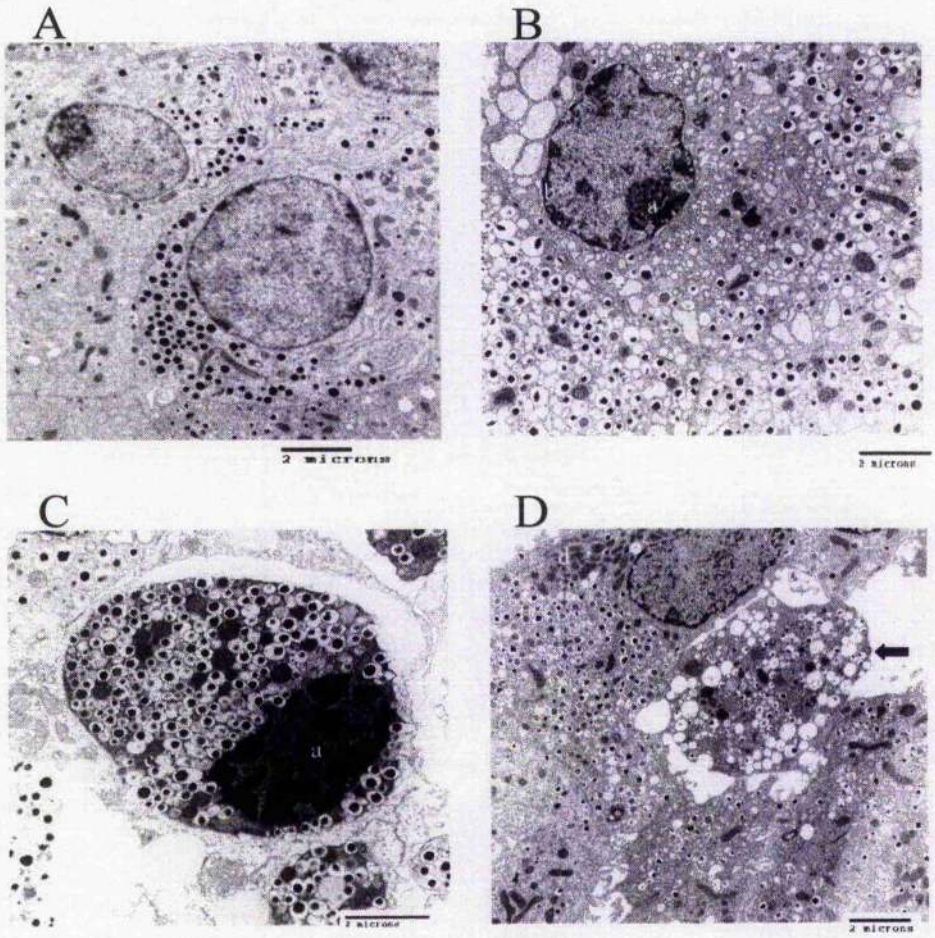


Figure 29. Apoptotic effect of rapamycin on islet cells. Rat islets were cultured in CMRL1066 (10% FBS). Islets were treated with vehicle (A) or with rapamycin 100 ng/ml (B, C, D) for 4 days. Cell apoptosis was assessed by electron microscopy. Apoptotic cells (β-cells, panel B, α-cells, panel C) in islets were detected by nuclear condensation (a), nuclear envelope irregular dilation (b), granule dilution (c), clumped and condensed mitochondria (d) and apoptotic bodies (solid arrows, panel D). Results are representative of two independent experiments.

4.4 Discussion

We have demonstrated for the first time, by several methods, that a therapeutic concentration of rapamycin causes cell death of the transformed MIN-6 cell line and that a supra-therapeutic rapamycin concentration has a deleterious effect on primary islet cells. In MIN-6 cells, this effect starts to occur as early as day 1 with a very low threshold concentration (0.01 nM), as measured by the MTT method. Why the MTT method appears to be more sensitive than the calcein method (at day 2 the threshold concentrations is 10 nM) is not easily explained. This may reflect the fact that calcein staining is indicative of cell death only, whereas MTT measurement reflects two cellular processes, namely cell viability and mitochondrial metabolism. As such, rapamycin may not only reduce cell viability, but also have an additional inhibitory effect on mitochondrial metabolism. The effect of rapamycin does not seem to be modulated by glucose concentration in the medium.

To confirm the findings observed in the clonal insulin-producing MIN-6 cells, the effect of rapamycin was also studied in rat and human islets. The rat islet PI fluorescence experiment was designed to assess whether rapamycin causes rat islet cell damage. However, there was only a significant effect at a supra-therapeutic rapamycin concentration of 100 nM. We also showed that this concentration of rapamycin reduces human islet cell viability, by the MTT method. These results confirm that rapamycin toxicity at therapeutic concentrations is limited to the MIN-6 cell line and only at supra-therapeutic concentrations does rapamycin have a deleterious effect on rat and human primary islet cells. The TUNEL assay data demonstrates that rapamycin-induced MIN-6 cell death occurs by apoptosis. Electron microscopy indicates that rat islet α- and β-cell death occurs by apoptosis at a supra-therapeutic rapamycin concentration of 100 nM.

In addition to causing MIN-6 cell apoptosis, rapamycin also impairs insulin secretion in the static-incubation insulin-secretion test. In this study, the observed reduction in insulin secretion may be attributed in part to the reduced rat islet cell viability and in part to an intracellular signalling impairment, such as inhibition of mitochondrial glucose oxidation as possibly demonstrated indirectly by the effect of rapamycin on MTT metabolism.

The mechanisms of rapamycin-induced reduction of insulin secretion and MIN-6 cell viability are currently not completely understood. The apoptotic effects may involve the inhibition of mTOR and its down-stream effectors. This may result in increased levels of phosphorylated pro-apoptotic factors (and tumour suppressor genes) and/or reduced levels of anti-apoptotic factors. An alternative hypothesis might be a disruption of normal translation of proteins, which are essential for continued cell survival (the mTOR cell-survival pathway) (279).

The successful normalisation of glucose control in brittle type 1 diabetic patients using the Edmonton protocol has provided a potential cure for this disease. However, the long-term side effects of this protocol are still not clear, such as whether the immunosuppressants used can cause β-cell toxicity. In the Edmonton Study patients had trough blood rapamycin levels monitored to maintain them in the range of 12 to 15 ng/mL for the first three months after transplant and in the range of 7 to 10 ng/mL thereafter (206). It is suggested by various authors that the target therapeutic range for whole blood trough concentrations of rapamycin is 5 to 10 ng/mL or 5 to 15 ng/mL with concomitant cyclosporin treatment (287). The rapamycin concentrations required to cause in-vitro MIN-6 cell death and apoptosis are similar to the range of whole-blood trough rapamycin concentrations desired in patients receiving rapamycin as an immunosuppressant. The rapamycin concentration required to evoke deleterious effects on rat and human islets in our in-vitro study is supra-therapeutic; nonetheless, transplanted islets are also exposed to higher whole blood peak rapamycin concentrations. Furthermore, human islets transplanted into the portal vein are exposed to higher peak rapamycin concentrations, following gastrointestinal absorption of rapamycin, than the concentrations present in the circulating peripheral whole blood. Indeed, peak rapamycin levels in portal blood have been observed to be double the levels found systemically (288). Also, unpublished data from one patient, reveals that the intra-portal rapamycin concentration 2 hours post-dosing was 54 ng/mL, whereas the peripheral whole-blood concentration was 42 ng/mL, both levels significantly higher than the target peripheral whole-blood rapamycin trough concentration.

A previous study demonstrated that 10 nM and 100 nM rapamycin had no effect on rat islet insulin secretion after 24 hours culture (284). We found that culturing rat islets

with a supra-therapeutic rapamycin concentration of 100 nM for 4 days did indeed reduce insulin secretion. This difference may be explained by the insufficient duration of exposure of rat islets to rapamycin in the previous study.

In summary, this is the first report to demonstrate that therapeutic concentrations of rapamycin can cause in-vitro MIN-6 cell apoptosis, and that supra-therapeutic concentrations of rapamycin in-vitro, can have a deleterious effect on rat and human islets and reduce rat islet insulin secretion. More work needs to be done on rat and human islets to establish whether therapeutic concentrations of rapamycin have any effect on primary β-cells and whether there are any consequent clinical implications.

Chapter 5
Conclusions and Future Directions

Chapter 5 Conclusions and Future Directions

Currently we have evidence that human islet transplantation, in a select group of type 1 diabetics, can result in insulin-independence for more then two years (207). Furthermore, at present, the acute risks of the procedure and risks of the immunosuppressant regime are less than the pre-existing risks of labile diabetes and hypoglycaemia. In view of the short period of follow-up, it is not possible to comment on the long-term risks nor benefits. The unanswered questions include;

- does islet transplantation have any influence on pre-existing micro- or macrovascular diabetic disease?
- does islet transplantation have any influence on the development of micro- or macrovascular diabetic disease?
- how long does the period of insulin-independence post-transplant last?
- are there any long-term effects of the immunosuppressants used?

If indeed the acute risks of the procedure and the risks of long-term immunosuppression are shown to be minimal, or the improvement in glycaemic control reduces the frequency of diabetic complications and the grafts continue to function, it could be argued that islet transplantation should be extended to a wider, less restrictive cohort of type 1 diabetic patients. If this comes to fruition, then the yield of high-quality islets will need to be increased. Even if there is no relaxation in the inclusion criteria for transplantation, the experience of transplant physicians is that organ demand always outstrips supply.

Thus, it is essential to increase the pool of potential donors to increase the overall number of pancreata available for islet isolation. In addition, the islet isolation procedure should be optimised such that there is as great a yield of high-quality islets as possible. In addition, the immunosuppressant regime should be as non-toxic to the transplanted islets as possible, while at the same time preventing the islets from host immunological damage.

This thesis has demonstrated that it might be possible to increase the supply of pancreata by using islets from a source not previously considered, namely non-heart-

beating donors. On the other hand, according to our data, islets from type 2 diabetic donors would not be considered useful.

Our aim when developing our in-vitro quality assessment tests was to characterise the islet preparation quality and subsequently correlate the results of the in-vitro tests with the in-vivo function of the islets in the diabetic mouse model. This did not prove to be successful. It may be that this is because the in-vitro tests do not reflect the in-vivo function of the transplanted islets in any way, or perhaps the diabetic mouse model was not optimised for use in these types of experiments. Nonetheless, these in-vitro tests continue to be performed in the current stage of the HUP Islet Transplantation Program. Five patients with type 1 diabetes have already received human islets and are all insulin-independent. All patients are undergoing sophisticated post-transplant assessments of their glycaemic control and insulin reserve by performing the same tests used in the previous studies (206,207,253). These results will then be compared to the in-vitro tests to determine whether any correlation exists. If a correlation does exist it may be possible to characterise the islet preparation according to the in-vitro tests and predict the efficacy of the islet preparation when transplanted.

The ideal immunosuppressant regime should provide maximal immunological protection, whilst at the same time having minimal (especially islet) toxicity. There have been no substantive reports of a diabetogenic effect of rapamycin. Indeed one of the perceived benefits of the current immunosuppressant regime is that it is essentially islet-neutral. The disadvantage of the previous regimes that used cyclosporin and corticosteroids was that these drugs have the potential to cause diabetes either by increasing peripheral insulin resistance or by toxicity to the transplanted islets. Currently, we have no evidence that therapeutic concentrations of rapamycin have any in-vitro effect on rat or human islets, although further work and close observation of current rapamycin-treated islet transplant recipients is required before concluding that therapeutic concentrations of rapamycin have no detrimental effect on transplanted human islets.

In light of the clinical success of islet transplantation programs, and the realisation that the main current limiting factor if these programs are to be expanded is the inadequate

supply of islets, alternative donor and cell sources for β-cell replacement has become an area of intense research interest.

The foetal pancreas develops from the endoderm. The most primitive pancreatic cell is undifferentiated and under the influence of transcription factors will develop into either endocrine or exocrine cells (289,290). In humans, pancreatic cells containing insulin are present from 8 weeks of foetal life (291), whilst those containing exocrine enzymes are not present until 12 weeks (292). Beta and other endocrine cells form from undifferentiated cells and initially remain in their midst together with developing exocrine cells. In the human foetal pancreas obtained early in the second trimester, 29% of cells are undifferentiated, 48% contain exocrine enzymes and 16% endocrine hormones (7% insulin) (293). Beta and other endocrine cells eventually form buds on the periphery of the mixed cell clusters and break away to coalesce and form islets (294).

Thus it is theoretically possible, that given the right environment, embryonic pancreatic undifferentiated stem cells can be stimulated to differentiate into insulin-secreting cells. This approach has been successful in reversing diabetes in mice (295). However, the use of aborted human embryonic tissue for research is controversial with many groups ethically opposed to it. Nonetheless research has taken place (mainly in animals) not only with foetal stem cells, but with foetal pancreatic tissue. Islet-cell clusters from porcine foetal and neonatal pancreata have been transplanted into human recipients with type 1 diabetes and renal failure, however, although the grafts continued to function there was no reduction in exogenous insulin requirements (296). Furthermore, although a porcine supply of islets for transplant into humans could possibly satisfy demand there are many that are concerned that this would allow the transmission of pig endogenous retrovirus to humans. Thus this avenue of research has diminished in recent years.

Instead of using foetal stem cells for reversal of diabetes much research has been devoted to using adult stem cells to reverse diabetes. Peck et al claimed to have reversed diabetes in non-obese diabetic (NOD) mice by transplanting islets generated in vitro from pancreatic adult stem cells, which had not been previously isolated (297). Furthermore Bonner-Weir et al have been working on expanding human pancreatic

differentiated duct cells ('functional stem cells') in vitro, then turning them into insulin-producing islet cells (298).

Other avenues being explored in an attempt to engineer insulin-secreting cells include genetic engineering of hepatocytes to secrete a single-chain insulin analogue (299), genetic engineering of intestinal mucosal cells to secrete insulin in physiological response to hyperglycaemia (300) and using adenovirus to induce islet neogenesis within the liver of mice (301).

In summary, there are numerous approaches being investigated to optimise the current regimes based on the Edmonton experience and intense research activity in techniques designed to uncover alternative sources of β-cells.

Of course the goal of all researchers working in this area is to replace the failing native β-cells with alternative insulin-secreting cells to effect a 'cure for diabetes'.

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