The Pig as an Animal Model for Type 1 Diabetes Mellitus – with focus on carbohydrate and fat metabolism

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The Pig as an Animal Model for Type 1 Diabetes Mellitus – with focus on carbohydrate and fat metabolism
Grisen som modelldjur för typ 1 diabetes mellitus – med fokus på kolhydrat- och fettmetabolismen

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SUMMARY

Diabetes mellitus is an endocrine disorder affecting a range of different mammals, including dogs, cats, horses and humans. Worldwide, 347 million people have diabetes, and the disease is an increasing burden on the world's poorest countries. The disease is characterised by chronic hyperglycaemia and afflicted individuals experience increased thirst, polyuria and weight loss, sometimes despite increased appetite. Diabetes mellitus is either insulin dependent or non-insulin dependent. In the present essay, focus is on insulin dependent, type 1 diabetes mellitus (T1DM); a condition where the insulin producing β-cells of the pancreas are destroyed and the insulin producing capacity diminished. Before insulin was discovered and became available as treatment, diabetes was a fatal disease. If T1DM is left untreated, the patient develops diabetic ketoacidosis, a potentially life-threatening condition. During treatment with insulin, there is a risk of insulin-induced hypoglycaemia, also a life-threatening complication.

Animal models for diabetes mellitus are important to enable research to gain more information about the disease and to develop new and improved treatments. Rodent models are widely used for diabetes research. However, large animal models are very important tools for preclinical studies. The pig is a suitable large animal for diabetes studies. The physiology and metabolism of the pig resembles that of humans. Diabetes mellitus is a disease of the pancreas, and the size, shape and position of the porcine pancreas, resembles that of the human pancreas. Blood glucose concentrations range within the same levels as in humans and porcine insulin differ by only one amino acid compared to human insulin.

Diabetes can occur spontaneously in mice and rats, but have to be experimentally induced in pigs. A type 1 diabetes mellitus like state can be induced with pancreatectomy or by injection of chemicals toxic to β-cells such as streptozotocin (STZ) or alloxan. In the present experiment it is showed that T1DM can be safely induced in domestic pigs at an i.v. STZ dose of 150 mg/kg b.w. The pigs develop clinical signs of diabetes mellitus such as polyuria/polydipsia and reduced weight gain compared to healthy individuals. Already two days post STZ treatment the pigs became hyperglycaemic and a significant ($p<0.05$) rise in serum non-esterified fatty acids (NEFA) concentrations and serum triglyceride concentrations was seen. Hyperglycaemia, hypertriglyceridaemia and elevated serum NEFA concentrations are also seen in diabetic humans. One week after initiation of insulin treatment, normoglycaemia is restored. One or three weeks after initiation of insulin treatment, serum concentrations of NEFA and triglycerides, respectively, are lowered to levels no different from that seen in the pigs before the induction of diabetes. This lowering of glucose, NEFA and triglyceride levels are also seen in insulin treated human patients with T1DM. From the present experiment it is concluded that a daily dosage of 1 IU/kg b.w. of short-acting insulin, divided by two times and given in direct connection to feeding is a satisfying treatment regimen. Both non-insulin treated and insulin treated STZ-diabetic pigs are brisk and vivid, and have good appetite.

SAMMANFATTNING

Diabetes mellitus är en endokrin sjukdom som drabbar flera olika däggdjur såsom hund, katt, häst och människa. 347 miljoner människor runt om i världen har diabetes och sjukdomen börjar bli en allt större börda i några av världens fattigaste länder. Sjukdomen karaktäriseras av kronisk hyperglykemi och drabbade individer får ökad törst, ökad urinmängd och rasar i vikt, ibland trots ökad aptit. Diabetes mellitus är antingen insulinberoende eller icke-insulinberoende. I denna uppsats ligger fokus på insulinberoende, typ-1 diabetes (T1DM), en sjukdom där de insulinproducerande β-cellerna i bukspottkörteln förstörs och den insulinproducerande förmågan minskar. Innan insulin upptäcktes och blev tillgängligt som behandling dog diabetiker i ung ålder. Om T1DM lämnas obehandlat utvecklar patienten diabetisk ketoacidos, ett livshotande tillstånd. Under insulinbehandling finns det alltid en risk för insulin-inducerad hypoglykemi, även detta en livshotande komplikation.

Djurmodeller är viktiga redskap i diabetesforskningen för att få mer information om sjukdomen och för att utveckla nya och förbättra behandlingsalternativ. Gnagare används mycket inom diabetesforskning, men stordjursmodeller är mycket viktiga i pre-kliniska studier. Grisen är en lämplig stordjursmodell för diabetesforskning eftersom grisens fysiologi och metabolism liknar människans. Diabetes mellitus är en sjukdom i bukspottkörteln och storleken, utseendet och läget på grisens bukspottkörtel liknar människans bukspottkörtel. Grisens blodglukoskoncentration varierar inom samma intervall som hos människa och porcint insulin skiljer sig bara med en aminosyra från humant insulin.

Möss och råttor kan spontant få diabetes, men när man använder grisar måste diabetes induceras experimentellt. Ett tillstånd som liknar T1DM kan induceras genom pancreasektomi eller genom injektion av substanser som är toxiska för β-cellerna, såsom streptozotocin (STZ) eller alloxan. I den här studien visas att diabetes kan induceras säkert i konventionella svenska grisar med en intravenös dos på 150 mg/kg kroppsvikt av STZ. Grisen utvecklar symtom på diabetes mellitus såsom polyuri/polydipsi och minskad tillväxt jämfört med friska grisar. Redan två dagar efter STZ-behandlingen blir grisarna hyperglykemiska och får en signifikant ($p<0.05$) ökning av serumkoncentration av NEFA och triglycerider. Hyperglykemi, hypertriglyceridemi och ökade nivåer av NEFA i serum ses också hos människor med diabetes mellitus. En vecka efter att insulinbehandlingen startade blev grisarna återigen normoglykemiska. En respektive tre veckor efter insulinbehandlingens påbörjan sjönk serumnivåerna av fria fettsyror (NEFA) respektive triglycerider till nivåer som ej skiljde sig från nivåerna som sågs hos grisarna innan diabetesinduktionen. Dessa sänkningar av blodglukos, NEFA-koncentrationer och triglyceridkoncentrationer ses även hos insulinbehandlade patienter med T1DM. Från den här studien kan konkluderas att en daglig dos på 1 IE/kg kroppsvikt kortverkande insulin, uppdelat på två gånger, givet i direkt samband med utfodring är en tillfredställande behandlingsregim. Både insulinbehandlade och icke-insulinbehandlade STZ-diabetiska grisar är livliga, pigga och har god aptit.

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus (DM) is an endocrine disorder characterised by chronic hyperglycaemia that can affect a range of mammals including dogs, cats, horses and humans. The disease was described already in ancient texts. In an Indian text from the 5th century BC, cases with excessive urination, thirst, emaciation and honeysweet urine are described. The term "diabetes" was introduced in the 1st or 2nd century BC. Diabetes is the Latin meaning of "siphon", the term was chosen due to the large volumes of water that was passing through the body, as if through a tube. In the 17th century Thomas Willis named the disease "diabetes mellitus", mellitus meaning honeysweet. Tasting of the urine was part of regular clinical examination, Avicenna recommended tasting of the urine in texts already in the 1st century (as reviewed by Eknoyan and Nagy 2005).

Diabetes was long believed to be a disease of the urinary tract. Clinical observations of changes in pancreas of diabetic patients were described in the 18th century, however it was not until 1889 that these clinical observations were confirmed when Minkowski and Mering showed that pancreatectomised dogs developed diabetes that could be reversed by implanting pancreatic fragments in subcutaneous tissues (as reviewed by Eknoyan and Nagy 2005).
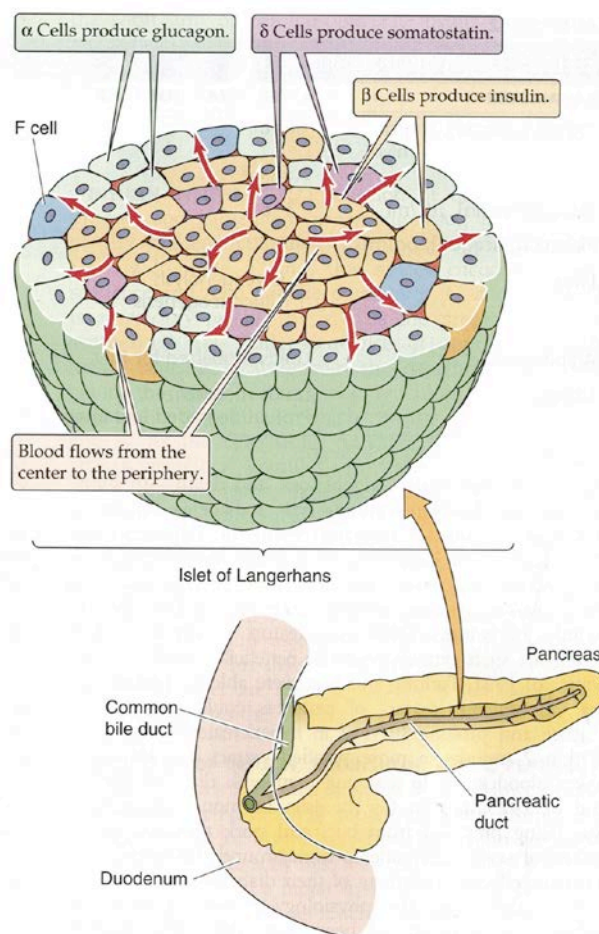

Figure 1. Human islet of Langerhans. From Medical Physiology 2nd edt, Boron, W.F., Boulpaep, E.L., Elsevier Inc. 2005, p 1067.

Pancreas is an abdominal organ closely related to the cranial part of duodenum. It consists of two types of glands, exocrine glands and endocrine glands, the latter called islets of Langerhans (fig. 1). The islets are scattered between exocrine acini and unevenly distributed within the pancreas. In the human pancreas there are 1-2 million islets, each containing 50-300 endocrine cells. The islets constitute only 1-2% of the pancreatic mass. Islets of Langerhans comprise four different cell types, α-cells, β-cells, δ-cells and F-cells. β-cell make up 60-75% of a typical islet and synthesise and secret insulin. α-cells produce glucagon and make up about 20% of a typical islet. δ-cells, considerably less abundant than β-cells and α-cell, produce somatostatin. The F-cell is a rare cell type, producing pancreatic polypeptide that inhibits cells functions of the exocrine pancreas. Pancreatic islets are highly vascular with each cell in direct contact with a capillary. The islets are also richly innervated with both sympathetic and parasympathetic nerve fibers (as reviewed by Goodman 2003).

DM can either be insulin dependent or non-insulin dependent. Insulin dependent diabetes is characterized by a lack of serum insulin due to a reduced mass of the insulin producing β-cells in the pancreas and thereby a reduced production of insulin. Non-insulin dependent diabetes is characterized by a hyperinsulinemia due to insulin resistance. The exact mechanism for insulin resistance is not fully understood, however it has been shown that there is an abnormal response to insulin in tissues such as skeletal muscle, liver and adipose tissue. The tissues reduced capability to respond to insulin result in a reduced uptake of glucose and consequently hyperglycaemia. When non-insulin dependent diabetes debut there might be a small reduction of β-cell mass, however most β-cells are intact. In course of time, however, a progressive reduction of insulin secretion is seen. The pathogenesis for reduction of insulin secretion is not fully understood. Eventually, both insulin dependent and non-insulin dependant DM cause complications with changes in multiple organs (as reviewed by Berne 2006).

Dogs, cats and horses

Although DM can affect a range of different species, there are large differences in both physiology and pathophysiology between species. In dogs, the aetiology and pathogenesis have been poorly characterised. However, genetic predisposition, infection, insulin-antagonistic disease, drugs, obesity, immune-mediated insulitis and pancreatitis have been identified as inciting factors. The disease is rarely reversible in dogs and lifelong insulin treatment is needed in most cases. Bitches in diestrus, when progesterone stimulates secretion of growth hormone and thereby insulin antagonism, can get hyperglycaemia. Afflicted bitches have a significant reduction in β-cell numbers compared to healthy dogs. If an adequate population of β-cells remains and ovariohysterectomy is performed, hyperglycaemia may resolve without the need for insulin treatment (as reviewed by Nelson 2009).

In cats, β-cell destruction is associated with amyloidosis and autoantibodies towards insulin or β-cells have never been identified. This differs from humans where autoimmunity plays a major role in β-cell destruction in insulin dependent diabetes. In cats the severity of amyloidosis determines whether the cat suffers from insulin dependent or non-insulin dependent DM, and the cat can flip back and forth between insulin dependent and insulin dependent DM. In humans, insulin dependent and non-insulin dependent DM have very

different pathogenesis, which will be discussed later. Cats can be easily stressed during blood sampling and develop a stress-induced hyperglycaemia and serum fructosamine concentration, an indirect measurement of sustained hyperglycaemia, needs to be measured to diagnose diabetes in cats (as reviewed by Nelson 2009).

In horses, a non-insulin dependent hyperglycaemia can be seen. Equine metabolic syndrome (EMS) is characterised by adiposity, hyperinsulinemia and insulin resistance, and is associated with laminitis. In patients with EMS, hyperinsulinemia develops in response to insulin resistance (as reviewed by Frank 2011). Glucose cannot diffuse directly into cells, but enters through different transport mechanisms. Transport can be facilitated by glucose transporters (GLUT). GLUT are located in cell membranes, the binding of glucose to the receptor cause a change in the receptor transformation and glucose is transported into the cell across the cell membrane. There are 14 different isoforms of GLUT, GLUT-4 is abundant in adipose tissue and skeletal muscle (as reviewed by Champe et al. 2008). In healthy individuals, insulin cause GLUT-4 to translocate to cell membranes allowing rapid uptake of glucose into cells. In insulin resistant horses, it has been showed that the GLUT-4 translocation is impaired. In patients where β-cells fail to produce enough insulin to compensate for peripheral insulin resistance, hyperglycaemia can be detected (as reviewed by Frank 2011). The pathogenesis of diabetes associated with EMS in horses is somewhat comparable to type 2 diabetes in humans. DM with β-cell dysfunction, low plasma insulin levels and hyperglycaemia have been reported several times in association with pituitary *pars intermedia* dysfunction (PPID) in horses (as reviewed by Durham et al. 2009).

Humans

In human medicine there are mainly to types of diabetes mellitus, type 1 (insulin dependent) and type 2 (non-insulin dependent). The pathogenesis for these two disorders are very different from one another, the common denominator is chronic hyperglycaemia (as reviewed by Berne 2006). Worldwide, 347 million people have diabetes of which 90% have type 2 diabetes. WHO estimated that in 2004 3.4 million people died from consequences of high blood sugar. Diabetes used to be considered a disease of older people in rich countries. However, diabetes is increasing fast in developing countries and the majority of people with diabetes live in Africa. The disease is a large burden on the world's poorest countries (WHO, 2012). In Sweden, diabetes accounts for 6-7% of the total medical costs and the complications due to diabetes stand for the largest part of those costs (as reviewed by Berne 2006). In the present essay, focus is on type 1 diabetes mellitus (T1DM) in humans, with the pig as an animal model.

Type 1 Diabetes Mellitus in Humans

Pathogenesis

The pathogenesis of T1DM is not fully understood. However the disease is caused by an autoimmune destruction of the β-cells in the pancreas (as reviewed by Berne 2006). There is a genetic predisposition for T1DM. This predisposition can be inherited, however it can occur sporadically as well. The development of T1DM can be divided into 6 different stages (as reviewed by Gianani and Eisenbarth 2005).

Stage 1 – genetic predisposition

It is likely that both genetic susceptibility and environmental factors are needed to develop T1DM. If one identical twin develops T1DM, the other twin in the pair does not necessarily develop T1DM too (as reviewed by Gianani and Eisenbarth 2005). Several different alleles, important for diabetes development, have been identified in humans and animal models (Ueda et al. 2003; Bottini et al. 2004; Guo et al. 2004).

Stage 2 – triggering of autoimmunity

For individuals with genetic susceptibility to develop T1DM, environmental factors are needed to trigger autoimmune reactions. Some infectious agents such as enterovirus, coxsackievirus and rubella virus have been suggested as possible triggers of autoimmunity associated with T1DM (as reviewed by Gianani and Eisenbarth 2005). Diet has been shown to participate in development of β-cell autoimmunity, *exempli gratia* cow milk and fruit and berry juices have been shown to be related to the development of T1DM (Virtanen et al. 2012). In recent years, research have been made to investigate the role of gut microbiota and intestinal inflammation in the development of β-cell autoimmunity. Results from animal models have shown that high gut permeability is associated with onset of autoimmune diabetes, and that the composition of the intestinal microbiota and enteral infections also are important for development of β-cell specific immune attacks. Certain bacteria may support the intestinal barrier while others cause increased gut permeability. Some findings suggest that it might be possible to protect individuals from autoimmune diabetes by modulation of the intestinal microbiota. Enterovirus and rotavirus cause a high intestinal permeability; this might be why they have been associated with T1DM. However, enterovirus has also been shown to be able to infect β-cells both *in vitro* and *in vivo* and cause β-cell destruction. Evidence from animal models imply that the gut immune system has a key role in regulation of immune responses leading to β-cell destruction. However, evidence in human beings is yet only suggestive (as reviewed by Vaarala 2012).

The prevalence and incidence of T1DM differ in different parts of the world. The highest incidence is seen in the Nordic countries and the incidence increase fast in all the countries of western Europe (as reviewed by Berne 2006). There is a 'hygiene hypothesis', which suggests that improved sanitation cause an increase in autoimmune disorders. This increase is explained by a loss of regulatory T-cell function due to the fact that children in countries with good sanitation encounter fewer infections during the development of their immune system (as reviewed by Bach 2001 and reviewed by Gianani and Eisenbarth 2005). In many developing countries where improvements in sanitation are made, the incidence of T1DM is increased (as reviewed by Okada et al. 2010).

Stage 3 – activation of autoimmunity

When autoimmunity is triggered, autoantibodies to insulin and other antigens in the β-cells can be detected (as reviewed by Gianani and Eisenbarth 2005). Autoantibodies are used as a marker for autoimmune destruction (as reviewed by Berne 2006). Positivity for two or more autoantibodies that are known to be associated with T1DM correlates with higher risk of

diabetes progression (as reviewed by Gianani and Eisenbarth 2005). Autoantibodies are present years before overt diabetes (as reviewed by Berne 2006).

Stage 4 – progressive loss of insulin secretion

When autoimmune reactions have started, a progressive loss of β-cell mass is initiated (as reviewed by Gianani and Eisenbarth 2005). The destruction of the β-cells is cell mediated, although not fully understood (as reviewed by Berne 2006). When β-cell mass is lost, insulin production declines. Insulin is a hugely important hormone for coordination of the use of fuels by tissues. When blood glucose rise; insulin is secreted from the β-cells. Insulin promotes glucose uptake in tissues. This uptake is most prominent in liver, muscle and adipose tissue and promotes synthesis of glycogen, protein and fat. Insulin secretion ensures that hyperglycaemia is corrected to keep blood glucose on a fairly constant level (as reviewed by Champe et al. 2008). In T1DM when insulin production and secretion declines; in consequence there will be a rise in blood glucose due to the inability to remove glucose from plasma (as reviewed by Goodman, 2003).

Stage 5 – overt diabetes

Clinical symptoms of T1DM are generally not seen until 80-90% of the β-cells have been destroyed. At time of onset, autoantibodies are usually detectable. After a while, however, autoantibodies can no longer be detected. This suggests that autoimmunity is driven by antigens in the β-cells and fades when β-cells are destroyed (as reviewed by Gianani and Eisenbarth 2005).

Stage 6 – insulin dependence with loss of almost all β-cells

Presence of remaining β-cells is estimated by measuring C-peptide. C-peptide is a peptide that is cleaved from proinsulin when insulin is produced. C-peptide is stored in insulin secretory granules and is secreted together with insulin in a ratio of 1:1 molar. A progressive loss of C-peptide is seen in patience with T1DM, although some people retain C-peptide for a long time. Treatment aims to preserve C-peptide because this will decrease episodes of severe hypoglycaemia and slow down progression of complications (as reviewed by Gianani and Eisenbarth 2005). Haemoglobin A1c (HbA1c) is the product of a non-enzymatic reaction between glucose and haemoglobin A. There is a linear correlation between mean plasma glucose concentration and HbA1c; hence HbA1c can be used as an indirect measurement of plasma glucose level over the past four to eight weeks (as reviewed by Berne 2006). Fructosamine is, as stated earlier, widely used in veterinary medicine and can also be used in human medicine to monitor glycaemic control. Like HbA1c, plasma levels of fructosamine do not fluctuate during the day and significantly correlate with daily mean plasma glucose concentrations (Johnson et al. 1982; Baker et al. 1985).

Clinical signs

T1DM is characterized by hyperglycaemia, polyuria, polydipsia and weight loss. In normal individuals blood glucose level is maintained around 5-6 mmol/L. In diabetics, however, blood glucose may be around 17-22 mmol/L and may occasionally increase up to 55 mmol/L. In healthy individuals, renal tubuli reabsorb glucose filtrated in glomerulus; which results in no or very little glucose in the urine. In diabetics with hyperglycaemia, however, the amount of glucose filtrated by glomerulus is so high that the renal capacity for reabsorption is not enough and glucose is spilled into the urine (as reviewed by Goodman, 2003).

When glucose remains in renal tubular lumen due to the kidneys inability to reabsorb it, glucose exerts an osmotic hinder for water reabsorption. In consequence, abnormally high volume of water is excreted in the urine. This phenomenon is called osmotic diuresis and the afflicted individual experience polyuria (as reviewed by Goodman, 2003).

Polyuria causes dehydration, which stimulates thirst. Excessive drinking, polydipsia, is often the first symptom recognized by patience with T1DM. Diabetics may also show excessive food consumption, polyphagia. The mechanism for increased appetite is not fully understood but might be the body's attempt to compensate for urinary loss of glucose (as reviewed by Goodman, 2003).

Insulin deficiency reduces all anabolic processes and accelerates catabolic processes. Despite increased appetite, in diabetics increased protein degradation and utilization of stored fats is seen, which results in rapid weight loss (as reviewed by Goodman, 2003).

Long-term complications of T1DM include microangiopathy, retinopathy, nephropathy, neuropathy and macrovascular disease. In T1DM with microvascular changes, thickening of the basal membrane of the capillaries is seen. Later, thrombosis causes loss of capillaries and consequently impaired perfusion of tissues. Patients with T1DM have increased risk of vision impairment and blindness; this is due to thickening of the capillaries in the retina causing hypoxia in the tissue. In diabetic nephropathy, primarily changes in the glomeruli are seen. In glomeruli, thickening of basal membrane and sclerosis is present; causing a progressive loss of filtration capacity and renal insufficiency. Many diabetic patients suffer from neuropathy. Changes such as axonal degeneration and pathologic changes in the myelin are seen in motor neurons, sensory neurons and autonomous neurons and cause impairment of signal conduction. Patients with diabetes have a higher risk of atherosclerosis. Hyperglycaemia increases the risk of disease in coronary vessels and heart attack (as reviewed by Berne 2006).

Treatment

Treatment of T1DM aims to minimize glucose concentration variability, to minimize the risk of nocturnal and severe hypoglycaemia and to prevent or delay long-term complications. Untreated T1DM inevitably leads to the development of diabetic ketoacidosis, a condition causing death. Hence, before the Nobel price awarded discovery of insulin by Banting and Best in 1921 T1DM was a fatal disease. The finding of insulin was made using dogs, but the first insulin preparations intended for human use came from bovine and porcine pancreas and hugely increased the life expectancy of diabetics. There were, however, some side effects

such as allergic reactions to insulin, insulin-antibodies development and lipodystrophy at site of injection in the early preparations made of animal insulin (as reviewed by Tibaldi, 2012).

Synthetic insulin was, as the first protein synthesized *in vitro*, produced in the 60s to provide a more effective and more readily available insulin treatment. In the late 70s, development of recombinant DNA enabled large-scale synthesis of human insulin in *Escherichia coli*. The use of synthetic human insulin decreases the risk of allergic reactions and insulin-antibody development and is therefore a good alternative to animal insulin. Over the years several types of insulin and insulin analogs, long-, short- and rapid acting, have been developed to help diabetics improve glycaemic control. The use of insulin analogs have improved glycaemic control and reduced the risk of nocturnal hypoglycaemia. However, many patients still have difficulty gaining and maintaining glycaemic control and therefore further improvements are necessary and alternatives to insulin treatment need to be investigated (as reviewed by Tibaldi, 2012).

Transplantation of either whole-pancreas or islets could potentially cure T1DM. Successful transplantation would keep the patient normoglycaemic. However; a transplantation that result in partial endogenous production should not be totally discarded as it can lead to a need for smaller doses of exogenous insulin and reduce the risk of hypoglycaemic episodes (Ryan et al. 2005). A lot of research has been done in the area for the last three decades but satisfying results have not been reached and many obstacles remain (as reviewed by Bretzel et al. 2007).

Pancreas transplantation can result in independence of exogenous insulin and improve the quality of life for the patients. Whole-pancreas transplants are generally less prone to graft rejection compared to islet transplants. However, the procedure of pancreas transplantation is associated with a significant risk of mortality that cannot be discounted. Transplantation of cell islet is a less perilous procedure. It is, however, difficult to get the islets to survive in their new host. In animal experiments, approximately 50% of transplanted cells will not engraft, this may be due to low functional capacity after β-cell isolation, local inflammation, apoptosis, blood clotting and hypoxia before revascularization (as reviewed by Robertson et al. 2006 and reviewed by Bretzel et al. 2007). In humans, islet are transplanted into the portal vein, as this is the only method that has repeatedly lead to insulin independence in humans (as reviewed by Lundgren 2010). Intraportal islet transplantation elicits instant blood mediated inflammatory reaction (IBMIR). IBMIR activates both inflammatory and coagulation pathways that can severely damage transplanted islets; this has been shown both *in vitro* and (Bennet et al. 1999) *in vivo* in clinical transplantations (Moberg et al. 2002; Johansson et al. 2005). IBMIR is triggered by tissue factor (TF) that is present in the islet of Langerhans, *in vitro* IBMIR can be inhibited if human islets are perfused with blood containing factor VIIa, an efficient inhibitor of TF (Moberg et al. 2002). Transplanted patients with a strong IBMIR reaction show no increase in endogenous insulin production 7 days after transplantation (Johansson et al. 2005). Finding ways to avoid IBMIR is crucial to develop well functioning methods for transplantation of pancreatic islets.

When transplanting pancreatic islets to patients with T1DM, one has to keep in mind the autoimmune aetiology of the disease and the possibility that grafted islets will be destructed

by autoimmune reactions in their new hosts. Grafted islet cells seem to be more prone to autoimmune destruction than whole-pancreas transplants. In diabetics receiving islet cells, usually a gradual decline, and often depletion, of graft function is seen (as reviewed by Bretzel et al. 2007). In a large five-year follow-up study of patients who had received islet transplantation it was seen that 62% had been insulin independent after transplantation, but only close to 10% of those remained insulin independent after five years (Ryan et al. 2005).

After transplantation of either whole-pancreas or islets, life-long immunosuppressive treatment is needed to avoid graft rejection and autoimmunity. Traditionally glucocorticoids have been used as immunosuppressive treatment. However, in recent years the use of glucocorticoids as immunosuppressive treatment for transplanted patients has declined (as reviewed by Meier-Kriesche et al. 2006). Glucocorticoids can cause diabetes inasmuch they cause insulin resistance and inhibit glucose uptake in muscle, fat and liver. The glucocorticoids potential to cause hyperglycaemia is of a large concern when treating patients that are already diabetic. Successful islet transplantations have been performed with a glucocorticoid-free immunosuppressive regimen (Shapiro et al. 2000).

Immunosuppressive drugs have general side effects related to the level of immunosuppression. This involves all types of infections and are most commonly seen early after transplantation when levels of immunosuppressive drugs are high. In addition to that, different immunosuppressive drugs have different undesirable side effects. Many immunosuppressive drugs cause an increased risk of malignancy; especially skin malignancies and lymphoma/lymphoproliferative malignancies but also different solid organ tumours. Transplant recipients have got a three to four-fold increased risk of malignancy related to chronic immunosuppression (as reviewed by Buell et al. 2005). The relative risk may increase 100-fold for specific malignancies compared with the general population. In transplant recipients a 100-fold increase in vulvar and anal carcinomas and a 400- to 500-fold increase in Kaposi´s carcinomas have been seen (as reviewed by Penn 2000). Most immunosuppressive protocols in islet transplantation include calcineurin inhibitors, such as tacrolimus. However, tacrolimus has been shown to be both diabetogenic, by impact on insulin secretion (Vincenti et al. 2007), and nephrotoxic (Nankivell et al. 2003). Usually a combination of immunosuppressive drugs is used to lower the risk of undesirable side effects (as reviewed by Lundgren 2010).

When progresses in transplantation research are made, a demand for a huge number of islets will come. To meet this demand, xenotransplantation could possibly be a solution to donor shortage (as reviewed by Bretzel et al. 2007). The pig is a suitable animal to use for xenotransplantation while porcine insulin differs in only one amino acid from human insulin and blood glucose concentrations are within the same range in pigs and humans. More research is needed in the area to overcome obstacles such as hyperacute xenogenic rejection that is seen when organs are transplanted between species. The possible transmission of retroviruses from pigs to humans is also a big concern that needs to be overcome.

Animal models for Type 1 Diabetes Mellitus

Animal models are important tools in the process of gaining more information about the aetiology and pathogenesis of T1DM, and in developing new treatments for the disease. Good animal models have been developed to provide an opportunity for such research. These animal models enable one to gain a better understanding of diabetes in humans. It is desirable that the phenotype of the animal model mimics the one of diabetic humans. Animal models are especially important when studying early steps in the pathological process. Such investigations cannot be conducted in humans. Large animal models are particularly important when developing new and improving treatments for T1DM. Large animals, such as pigs and primates, have a more similar physiology and are more genetically close to humans compared to for example rodents (as reviewed by Larsen and Rolin 2004; Jin et al. 2010). The animal model of choice must depend on which aspect of the human disease that is being investigated.

Rodents

Rodents as animal models for T1DM in humans are widely used inasmuch there is a long tradition of using these animals as models for humans and they are cheap to breed and to house compared to larger animals. In 1974 a laboratory rat colony that spontaneously developed diabetes was discovered, in 1978 a detailed characterisation of the syndrome was published. These rats are called BB-rats and the syndrome developed is similar to T1DM. The onset of diabetes is extremely rapid, occurring over hours to a few days. Within a day, hyperglycaemia, decreased plasma insulin levels and elevated plasma NEFA and ketone body levels are seen. Somewhat later in the disease process, a rise in plasma glucagon level is also seen. Post-mortem examinations of severe cases reveal small pancreatic islets with almost no remaining β-cells. Post-mortem examinations of BB-rats with milder metabolic derangements show insulitis with leucocyte infiltration in and around islets (Nakhooda et al. 1978). Islet cell surface antibodies (ICSA) and antibodies to splenic lymphocytes are present at the time for or after the diagnosis of diabetes but may not be present before the diagnosis in all rats (Dyrberg et al. 1984). The animals show a marked decrease in the population of T-lymphocytes which result in a dysfunction of the lymphocyte mediated immune response. The BB-rat's predisposition to lymphopoietic malignancy and to infection is not seen in humans with T1DM. A line of non-lymphopenic diabetic rats has been developed. Acutely diabetic BB-rats die from ketoacidosis within 2 weeks of onset of the disease unless insulin is given (as reviewed by Buschard and Thon 2003). Diabetic ketoacidosis is very dangerous complication of diabetes mellitus that will be discussed in more detail later.

The non-obese diabetic (NOD) mice model is somewhat equivalent to the BB-rat model. The model is genetically determined and all NOD-mice are considered to be genetically identical. However, not all individuals develop diabetes. In the original NOD strain, 80% of the females and 10% of the males developed diabetes. This suggests that it is the susceptibility that is inherited rather than the expressed disease, which is also the case for BB-rats and humans (as reviewed by Buschard and Thon 2003). Insulin is a major autoantigen for NOD mice but autoimmunity to other islet antigens may be necessary for islet destruction and disease development to occur (as reviewed by Gianani and Eisenbarth 2005). In contrast to humans mice have not only one but two insulin genes, insulin 1 and insulin 2. The insulin molecules

differ in sequence by 2 amino acids (Paronen et al. 2003). NOD mice with a knockout in the insulin 2 gene have a higher incidence of diabetes than wildtype NOD mice and could be due to a defective central tolerance for insulin (as reviewed by Gianani and Eisenbarth 2005) while a decreased amount of insulin is presented in the thymus of insulin 2 knockout NOD mice (Paronen et al. 2003). *Id est* a fail in discrimination of self versus non-self occur and insulin may be regarded as non-self eliciting an immune response against insulin, a major autoantigen in T1DM (Wong et al. 1999). The clinical features of the diabetes syndrome in NOD mice include hypoinsulinemia, glycosuria, hypercholesterolemia, ketonuria, polydipsia, polyuria and polyphagia, *id est* quite similar to human T1DM. In contrast to the BB-rat the NOD mouse can survive without insulin treatment for quite a long time (as reviewed by Buschard and Thon 2003).

The BB-rat and the NOD mouse spontaneously develop diabetes quite similar to T1DM in humans (as reviewed by Buschard and Thon 2003). However, a diabetes like syndrome can be created by injection of streptozotocin or alloxan, or by surgical pancreatectomy in a range of different species including rodents (Gäbel 1983; Kobayashi et al. 2004; Strauss et al. 2012; Hara et al. 2008; Jensen-Waern et al. 2009). These experimental methods are discussed in more detail later.

Pigs

Large animal models are a good complement to rodents when studying T1DM and the pig is a very useful species in preclinical studies. The pig is an omnivore and even though there are some anatomical differences, its physiology regarding nutritional requirements is very similar to those of human beings. Pigs can be fed well defined meals at certain times, which is an advantage compared to the more continuous feeding pattern needed for rodents. Many of the organ systems and the physiology and pathophysiology in pigs resemble those of the humans. When studying T1DM, subcutaneous injections are frequently used, especially for insulin treatment. There is a high degree of similarity between the skin and subcutaneous tissues in humans and pigs and the pharmacokinetics after subcutaneous injections of compounds are very similar (as reviewed by Larsen and Rolin 2004).

Pigs, in contrast to rodents, can be trained to allow experiments being carried out stress free when the pigs are conscious. Blood sampling is a potential stressful procedure, however permanent vein catheters can be implanted to the pig which reduces stress to minimum. Due to the larger size of the pig compared to rodents, it is possible to obtain larger volumes of blood and also easier to take biopsies from different tissues (as reviewed by Larsen and Rolin 2004).

Pigs are easy to handle, especially when they are small in size. The weight gain could be a limiting factor in studies using domestic pigs. For this reason, models for diabetes induction in the Göttingen minipig has been developed and are widely used (as reviewed by Larsen and Rolin 2004). Models for diabetes induction in domestic pigs have also been developed (Kobayashi et al. 2004, Hara et al. 2008, Jensen-Waern et al. 2009). However, the minipigs might be more suitable for long-term studies due to its inherited small size.

The size, shape and position of the porcine pancreas (fig. 2) are similar to the human pancreas. In young pigs the structure of the islets of Langerhans is more diffuse than in adult pigs. The islets in adult pigs are more like human islets in structure. The insulin produced by the porcine β-cells differs by only one amino acid from human insulin (as reviewed by Larsen and Rolin 2004).
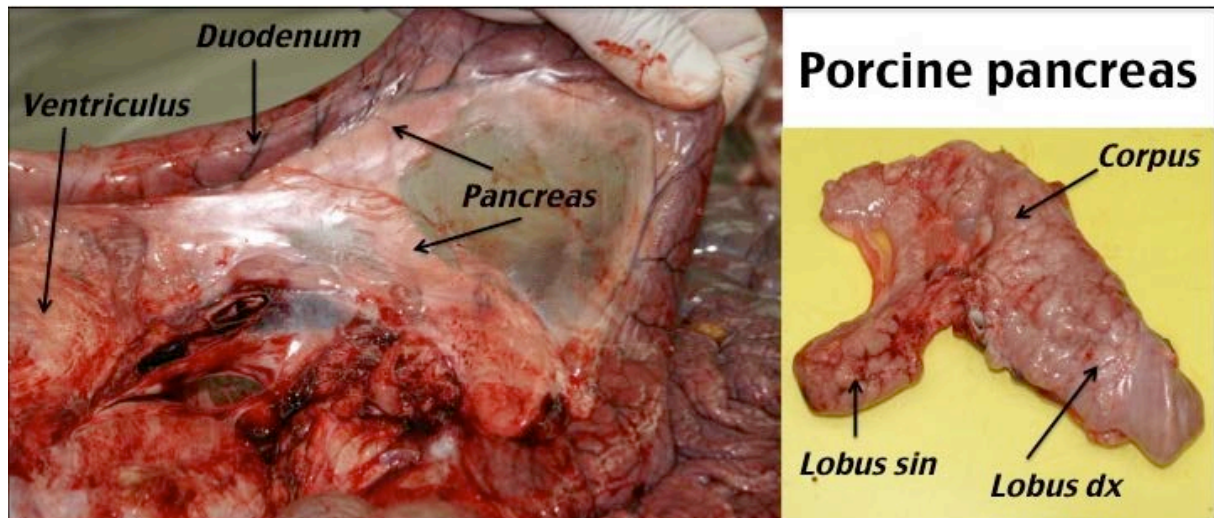

Figure 2. Porcine pancreas.

Induction of diabetes

Although spontaneous diabetes in pigs has been reported (Biester. 1925) a type1-like diabetes mellitus is very rare in pigs. Thus, when pigs are used in diabetes research the disease needs to be induced experimentally. Diabetes can be induced either by surgical pancratectomy (Gäbel 1983; Kobayashi et al. 2004; Strauss et al. 2012) or chemically by streptozotocin injection (Gäbel 1983; Strauss et al. 2012; Hara et al. 2008; Jensen-Waern et al. 2009).

Surgical pancreatectomy has been widely used as a method for inducing diabetes in laboratory animals. The impairment of glucose tolerance in pigs is related to the extent of pancreas removed. Total pancreatectomy completely stops secretion of endogenous insulin inasmuch all β-cells are removed (Kobayashi et al. 2004); this results in severe hyperglycaemia. When diabetes is surgically induced, both the endocrine and exocrine parts of the pancreas are removed, *id est* this is not characteristic for T1DM in humans. Pancreatectomised pigs, not receiving any treatment, develop diarrhoea associated with the lack of exocrine pancreatic function, loose weight, show lower levels of activity than control pigs and die within less than 12 days from surgery (Gäbel 1983, Kobayashi et al. 2004). Pigs supplemented with exogenous insulin and exogenous pancreas enzymes can survive without complications for at least 28 days (Strauss et al. 2012).

Streptozotocin is an antibiotic substance produced by *Streptomyces achromogenes*. Even though streptozotocin is active against a range of both gram-positive and gram-negative bacteria (Varva et al. 1959-1960) it has never been used as antibiotic treatment to patients because of its capacity to selectively cause destruction of the β-cells in the pancreas (Elsner et al. 2000). This selective destruction of the β-cells makes STZ diabetogenic and can be used as

a tool in diabetes research. STZ has also, since the 60s been used to treat patients with insulinoma (Stanely and Marks 1971) and is still used today.

STZ is transported into the cells through the GLUT2-receptor without disturbing the function of the receptor (Elsner et al. 2000). Inside the cell, STZ cause DNA-strands to break. The magnitude of the DNA fragmentation is dose-dependent. When DNA strands break, the DNA repair mechanism is activated. In cells treated with streptozotocin, the activity of the nuclear enzyme poly ADP-ribose polymerase (PARP) is increased (Yamamoto et al. 1981) to participate in the DNA repair mechanism. Massive DNA damage elicits a major activation of PARP. The processes catalysed by PARP rapidly consume nicotinamid adenine dinucleotide (NAD) and adenosine triphosphate (ATP), which leads to an energy crisis in the cell. The energy crisis culminates in cell death. This cellular suicide is presumably a safety mechanism to hinder cells to survive with highly mutant phenotypes. PARP is a very important molecule in the pathogenesis of STZ-diabetes. PARP -/- mice are completely protected from STZ-induced diabetes and β-cell death (as reviewed by Pieper et al. 1999). In models for type 2 diabetes, a combination of NAD and STZ can be given. NAD partially protects β-cells from the damaging effects of STZ; which results in a mild diabetes (Larsen et al. 2002).

The GLUT-2 receptor is not only found in the β-cells of the pancreas, it is also found in the liver and kidneys (as reviewed by Champe et al. 2008). In STZ-treated animals, hepatic changes are seen within less than six hours after the STZ treatment (Kume et al. 2004). Any STZ treatment of animals leads not only to diabetes, but also cause liver and kidney damage (Elsner et al. 2000). Inducing diabetes with a combination of partial pancreatectomy with low-dose STZ in Rhesus money has shown to reduce liver and kidney damage, compared to using only high-dose STZ (Jin et al. 2010). Jensen-Waern et al. (2009) showed that STZ-diabetes can be safely induced in Swedish pigs (Yorkshire x Landrace, high health certified). Those pigs were given a STZ dose of 150 mg/kg b.w.

Inducing diabetes with STZ cause a reduction in insulin secretion and a decreased β-cell mass, which is two important characteristics in human T1DM. In STZ-induced diabetes in pigs however, there is no inflammatory process involved – this is not the case for T1DM in humans (as reviewed by Larsen and Rolin 2004).

Inducing diabetes by pancreatectomy is associated with higher costs than STZ-induced diabetes. When performing pancreatectomy, more personnel and longer work time is needed for the procedure. Pancreatectomised pigs also need longer intensive care treatment and monitoring by higher qualified people post surgery compared to STZ-treated pigs (Strauss et al. 2012).

STZ selectively destroy β-cells and preserve glucagon producing α-cells and the exocrine function of the pancreas; this preservation is not possible at pancreatectomy (Gäbel 1983). The preserved α-cells and exocrine pancreas function is an advantage when trying to create a state similar to T1DM in humans. Even though C-peptide levels can remain low for a month in pigs treated with 150 mg/kg b.w. STZ, some residual β-cells and regeneration have been seen (Hara et al. 2008, Strauss et al. 2012). However, Gäbel (1983) showed that diabetes that is irreversible for at least seven months can be induced with STZ dosage of 150 mg/kg b.w.

More recently Jensen Waern et al. (2009) also showed that STZ dosage of 150 mg/kg b.w. can induce diabetes in pigs with only very little C-peptide production for at least three weeks, mimicking the state of diabetic human patients. Hara et al. (2008) showed that at a dosage of 200 mg/kg b.w. STZ, pigs remain completely diabetic for at least 20 weeks without evidence of any residual β-cell function.

Alloxan is another substance that can be used for diabetes induction in a range of different experimental animal including rodents, rabbits and pigs (Gäbel 1983; Federiuk et al. 2004; Misra and Aiman 2012). Like streptozotocin, alloxan enters the pancreatic β-cells via the GLUT-2 receptor and produces DNA strand breaks (Yamamato et al. 1981) causing cell death (as reviewed by Szudelski 2001). Using alloxan is cheaper than STZ (Federiuk et al. 2004), however some researchers report difficulties in maintenance of a diabetic state and high mortality rates for animals treated with alloxan (Misra and Aiman 2012 and reviewed by Jain and Arya 2011). Some even propose alloxan to be a very doubtful method for diabetes induction and that there are no evidence that alloxan-induced diabetes is related to human diabetes mellitus (as reviewed by Jain and Arya 2011). Other claims that alloxan can be used for safe induction of diabetes if paying careful attention to species differences in susceptibility, dosage, route of administration and post-treatment management (Federiuk et al. 2004). Alloxan has been shown to cause kidney damage with necrotic changes seen already within 48 hours from alloxan injection (Jacob and Barer 1972). The β-cell toxicity of STZ is considered to be more specific than that of alloxan. Alloxan more commonly produce a transitory diabetic state compared to STZ that, at appropriate dosage, produces a complete diabetic state (Gäbel 1983).

Primates

Non-human primates are genetically more close to humans compared to pigs. There is a safe method for inducing diabetes by combining partial pancreatectomy and low-dose STZ in Rhesus macaques (Jin et al. 2010). Diabetes can also be created with only STZ injections in Baboons (McCulloch et al. 1988). However, using non-human primates is associated with more ethical considerations, more personnel needed and higher costs compared to using pigs (Strauss et al. 2012). A major disadvantage in using primates as an animal model is the potential transmission of infectious diseases from primates to humans. Especially *Cercopithecine herpesvirus* 1, also know as B virus (McHV1) is of a large concern. Non-human primates can harbour McHV1with only mild symptoms such as vesicular lesions or more commonly as an asymptomatic latent infection in sensory ganglia (as reviewed by Estep et al. 2010). In Rhesus Macaque, a primate commonly used in research, seroprevalence of McHV1 range from 70% to 100% in adult populations (as reviewed by Elmore and Eberle 2008). Infected primates shed virus in saliva, urine and genital secretions. Humans become infected through contact with contaminated body fluids though scratches, bite wounds or needle sticks and around 90% of afflicted individuals develop encephalitis with symptoms such as pain, fatigue, headache, nausea, ataxia, ascending flaccid paralysis and agitation (as reviewed by Estep et al. 2010). If not promptly treated, McHV1 infection in humans has a fatality rate greater than 70% and many survivors have marked neurological deficits. Almost all afflicted humans have either been bitten or scratched by primates (as reviewed by Elmore and Eberle 2008).

In the European Union legislation, Directive 2010/63/EU that took full effect from 1 January 2013 the use of non-human primates for scientific purposes is very restricted. There are high requirement for housing, social stimulation and activity. It is stated in the directive that all Member States should act to replace, reduce and refine the use of laboratory animals. In particular non-human primates should be a matter of priority where it is possible. Non-human primates can only be used in experiment that are necessary for humans and where the purpose of the procedure cannot be achieved by the use of any other species (Directive 2010/63/EU of the European Parliament and of the Council).

Metabolism of carbohydrates, amino acids and fat in healthy and type 1 diabetic subjects

As stated earlier, insulin is a hugely important hormone for coordination of the use of fuels by tissues. Blood glucose concentration is a major regulator of insulin secretion. Insulin promotes glucose uptake in tissues to ensure that hyperglycaemia is corrected to keep blood glucose on a fairly constant level (MacLeod 1922).

Glucagon is a hormone that counteracts insulin and is the most important hormone for restoration of normoglycaemia in insulin-induced hypoglycaemia (Rizza et al. 1979b). In normal subjects, glucagon is suppressed by insulin. In untreated or poorly controlled T1DM with insulin deficiency, plasma glucagon levels are high. However, in patients with controlled T1DM with blood glucose concentration within reference range, a meal elicits a glucagon response that cause a prolonged hyperglycaemia compared to healthy subjects; probably because of the limited β-cell function and thereby inability to get an appropriate rise in endogenous insulin secretion (Rizza et al. 1979a). In healthy subjects, plasma glucagon levels are elevated in the presence of hypoglycaemia to promote glycolysis and gluconeogenesis so that normoglycaemia is restored. In patients with T1DM an abnormal glucagon response to hypoglycaemia is seen, the response is blunted or sometimes even absent (Gerich et al. 1973). When the glucagon response to hypoglycaemia is impaired, catecholamines become hugely important for recovery from hypoglycaemia (Rizza et al. 1979b). Intrahepatic islet transplantation does not restore normal glucagon response to hypoglycaemia (Paty et al. 2002).

During the fasting state in normal subjects, blood glucose concentrations are maintained on a fairly constant level by controlled hepatic glucose output. During an overnight fast, approximately 75% of hepatic glucose output is achieved by glycogenolysis. 25% is achieved by gluconeogenesis from various substrates, mainly lactate, alanine, glycerol and pyruvate. The hepatic glucose output is controlled by plasma levels of insulin and glucagon (as reviewed by Taylor and Agius 1988).

During the postprandial state in normal subjects, circulating glucose is taken up by peripheral tissues and about one third is cleared by the liver. Food intake stimulates a sharp increase in insulin secretion from the β-cells; which in turn inhibits lipolysis and promote storage of glucose and fatty acids as glycogen in liver and muscle and as triglycerides in adipose tissue. Subjects with T1DM fail to release enough insulin to the circulation after food intake. In state of insulin deficiency, hepatic release of glucose is uninhibited and uptake of glucose by peripheral tissues is decreased. Hence, postprandial hyperglycaemia is seen in diabetic

subjects (as reviewed by Taylor and Agius 1988). Although in untreated or poorly controlled T1DM, constant hyperglycaemia is seen.

Insulin deficiency cause rapid mobilisation of fatty acids from adipose tissue and subsequently a rise in plasma NEFA (Laurell 1956). Insulin lack in T1DM cause excessive mobilisation of fatty acids that are converted into ketone bodies by the liver, insulin deficit also cause a reduced rate of fatty acid synthesis in the liver (as reviewed by Taylor and Agius 1988). In healthy adults, serum non-esterified fatty acid (NEFA) concentrations are approximately 0.5-0.6 mmol/L (Munkner 1959). Young children, < 8 years old, have higher serum concentrations of NEFA (0.55-0.75 mmol/L) compared to adults. During the adolescent years, NEFA levels are lowered and are around 0.4 mmol/L when the adolescents are 18 years old (Heald et al. 1967). In untreated or poorly controlled T1DM plasma NEFA concentrations are elevated (Laurell 1956). Furthermore, elevated NEFA concentration stimulates glycogenolysis and gluconeogenesis (Staehr et al. 2003) and inhibits insulin secretion (Zmyslowska et al. 2011) that contributes to augmented hyperglycaemia. In healthy individuals, an increase in blood NEFA concentration is seen in the fasting state (Laurell, 1956).

Glucagon stimulates lipolysis in adipocytes and release of NEFA to the circulation (Hagen 1960). The uptake of NEFA by the liver is proportional to the serum concentration of NEFA and not dependant on glucagon. However, glucagon acts on the liver to inhibit release of triglycerides and to stimulate ketogenesis (Heimberg et al. 1969). In diabetic subjects with insulin deficiency endogenous glucagon contributes to the maintenance of ketogenesis and plays an essential role in the development of diabetic ketoacidosis (Gerich et al. 1975).

Triglycerides are synthesised in the liver by esterification of fatty acids, the latter is either synthesised *de novo* in the liver or derived from adipose tissue. Triglycerides are transported from the liver to adipose tissue packed with apoprotein, very low-density lipoprotein (VLDL). Synthesis of triglycerides in the liver seems to never be saturated. Synthesis of VLDL, however, is limited by substrate availability of the components making up the VLDL particle. When synthesis of VLDL is saturated, triglycerides accumulate in the liver (as reviewed by Taylor and Agius 1988). Insulin acts antilipolytic by inhibiting hormone-sensitive lipase in adipose tissue. As consequence, storage of triglycerides in adipose tissues is promoted and the release of fatty acids to the circulation is inhibited. In healthy individuals, insulin reduces plasma concentrations of triglycerides by inhibiting VLDL production in the liver and by activating lipoproteinlipase (LPL), which promotes catabolism of triglyceride-rich lipoproteins (as reviewed by Vergès 2009).

In diabetic patients with good glycaemic control, serum triglyceride concentrations are within reference range or slightly decreased due to augmented plasma insulin levels (as reviewed by Vergès 2009). Serum triglyceride concentrations vary in different ages. Children, 0-9 years old, have triglyceride levels of 0.3 ± 0.02 mmol/L. Triglyceride concentrations initially increase with age and the highest concentrations are seen in patients 40-49 years old, which have concentrations of 1.1 ± 0.02 mmol/L. From 50 years on, serum triglyceride concentrations decrease again. Obese subjects have higher triglyceride concentrations compared to individuals with normal body weight (ideal body weight ± 10%) and lean

patients have lower concentrations of triglycerides (Kudo 1969). In patients with untreated or poorly controlled T1DM hypertriglyceridaemia is seen (Bagdade et al. 1968).

Amino acids are building blocks in protein synthesis. Higher animals require nine different amino acids (lysine, histidine, leucine, isoleucine, valine, methionine, threonine, tryptophan, phenylanine) in their diet; these are essential amino acids, as they cannot be synthesised *in vivo* in these animals. Some species have additional amino acid requirements. Amino acids that the animals are able to synthesise *in vivo* are termed non-essential. Some amino acids can, by degradation of their carbon skeleton, be used for gluconeogenesis and ketogenesis, table 1 (as reviewed by D'Mello 2003).

Table 1. Glucogenic and ketogenic amino acids (From Amino Acids as Functional Molecules in Amino Acids in Animal Nutrition 2nd edt, Cambridge, M.A., CABI Publishing 2003, p 3.)

Glucogenic	Ketogenic	Glucogenic and ketogenic
Threonine	Leucine	Isoleucine
Arginine	Lysine	Phenylalanine
Methionine		Tyrosine
Valine		Tryptophan
Histidine		
Cysteine		
Glutamate		
Glutamine		
Aspartate		
Asparagine		
Glycine		
Serine		
Proline		
Alanine		

Insulin is one of the major hormones involved in protein metabolism in skeletal muscle. Insulin exerts an anabolic effect on muscle and lack of insulin in T1DM cause muscle protein breakdown due to decreased insulin stimulation and increased glucocorticoid stimulation. Glucocorticoids, in contrast to insulin, are catabolic hormones (Odedra et al. 1981). In STZ-diabetic rats with polyphagia, eating 55% more than control animals, body weight gain is still reduced compared to control animals (Rodríguez et al. 1997).

T1DM can metabolically be compared to starvation, as tissues cannot in the presence of insulin deficiency utilise glucose to the same extent as healthy individuals can. In starvation an increased amino acid release from muscle is seen. Predominantly alanine, which is a rate-limiting step in gluconeogenesis, is released to the circulation (Felig et al. 1970). Branched chain amino acids (BCAA), which include leucine, isoleucine and valine, are important nitrogen sources for alanine formation. Oxidation of these amino acids takes place in muscle and not primarily in the liver, which is the case for other amino acids. After a protein meal, BCAA escape hepatic uptake and constitute most of the amino acid uptake by muscle tissue. Release of BCAA from muscle tissue is less than expected on the basis of their concentration

in muscle tissue; thus, this shows that BCAA also have an important task in acting as fuel for muscle (as reviewed by Felig et al. 1977).

A diabetic state does not change the liver's overall protein turnover (Rodríguez et al. 1997). However, significant changes are seen in serum concentrations of the BCAA. Serum concentrations of BCAA are increased in diabetic individuals. Serum concentrations of glucogenic amino acids such as glutamate, glutamine, alanine, proline, glycine, serine and threonine, on the other hand, are decreased in diabetic individuals and in the fasting state of healthy individuals (Blackshear and Alberti 1975; Rodríguez et al. 1997). The decrease of these gluconeogenic amino acids can be associated with increased gluconeogenesis in the liver. Jensen-Waern et al. (2009) showed that the amino acid metabolism in STZ-diabetic pigs resemble that of the diabetic human with elevated serum BCAA and decreased serum concentration of alanine.

Diabetic ketoacidosis

Diabetic ketoacidosis (DKA) develops in patients with severe insulin deficiency. DKA is the most dangerous acute metabolic complication of diabetes mellitus and is a potentially life-threatening condition. DKA was first described in 1886, *id est* 35 years before the discovery of insulin (Dreschfeld 1886). As described earlier, insulin deficiency leads to increased blood concentrations of counterregulatory hormones (Rizza et al. 1979b). In developed ketoacidosis, catecholamines, cortisol and growth hormone are markedly elevated; these hormones activate hormone sensitive lipase that cause breakdown of triglycerides and release of NEFA to the circulation (as reviewed by Flier et al. 1983). The highest levels of plasma concentrations of NEFA are seen in patients with the most severe ketoacidosis (Bagdage et al. 1968) Ketone bodies are utilized as fuel by tissues, especially when blood glucose concentrations are low and not sufficient to provide energy for tissues (as reviewed by Taylor and Agius 1988). In DKA an exaggerated production of the ketone bodies β-hydroxybutyric acid and acetoacetic acid is seen. The ketogenesis in the liver is stimulated by high glucagon levels. The ketoacids dissociate at physiological pH and excess hydrogen ions are bound to bicarbonate, consequently a decrease in serum bicarbonate levels is seen and metabolic acidosis, with arterial pH ≤ 7.3, develops. DKA is usually associated with profound total body potassium depletion due to urinary loss of potassium, reduced oral intake and loss through vomiting. However, plasma potassium concentrations are typically normal (as reviewed by Chiasson et al. 2003).

Patients with DKA often presents with polyuria/polydipsia, abdominal pain, nausea and vomiting. Due to the abdominal pain, the condition might be mistaken for acute surgical abdomen. Rapid and deep respiration with acetone breath is also typical of DKA (as reviewed by Chiasson et al. 2003). The mental status can in mild cases be unaffected. In severe cases, however, stupor and coma in seen (as reviewed by Eledrisi et al. 2006). In the late 19th century, when DKA was first described; about 90% of the afflicted people died, often within a couple of hours to a few days (Dreschfeld 1886). Nowadays, in experienced centers the fatality rate of DKA is less than 5% (as reviewed by Kitabchi et al. 2003).

In patients with DKA, fluid therapy should be promptly initiated to rehydrate the patients and thereby improve renal perfusion. Measurement of serum electrolytes is crucial in DKA treatment. There is usually a substantial total body deficit of sodium, chloride, potassium and phosphorous. Potassium must inevitably be measured before any insulin treatment. If the patient has low serum potassium concentration, insulin therapy will further lower the potassium serum levels as potassium are taken up into cells, this could produce a life-threatening hypokalaemia. Patients with low serum potassium concentrations must be given supplemental potassium. Insulin therapy is extremely important to revert the state of diabetic ketoacidosis. A bolus dose should be given intravenously followed by continuous infusion. After a while, insulin therapy will cause blood glucose concentration to normalise. When this happens it is extremely important not to withdraw insulin. Instead, glucose should be given in addition to insulin to maintain glucose at a level of 6-12 mmol/L. Insulin therapy should be continued until venous pH is greater than 7.3. In mild cases of DKA, rapid acting insulin can be given subcutaneously (as reviewed by Eledrisi et al. 2006).

AIM OF THE PRESENT EXPERIMENT

Jensen-Waern et al (2009) showed that Swedish high health certified domestic pigs can safely be made diabetic by intravenous injection of STZ at a dose of 150 mg/kg b.w. The glucose and amino acid metabolism of those pigs mimicked that of patients with untreated T1DM. Furthermore, the pigs had clinical sign of diabetes such as, polyuria/polydipsia and reduced daily weight gain compared to conventional pigs. In the present experiment we aim to further develop the T1DM-model in Swedish high health certified domestic pigs as previously described by Jensen-Waern et al (2009). The aim of the study is to make the pigs free from clinical signs of disease (free from polydipsia and polyuria and to achieve a good growth rate) by insulin treatment, and to investigate how much insulin is needed to reach these goals. To our knowledge, insulin treatment of Swedish high health certified domestic pigs have not been previously described and there is at present no protocol for such treatment. Developing a protocol for insulin treatment of STZ-diabetic pigs will enable transplantation studies to be performed, as glycaemic control is needed to allow the STZ-diabetic pigs to undergo anaesthesia for transplantation of islets of Langerhans.

In the present experiment, the fat metabolism of both untreated and insulin treated STZ-diabetic pigs will be investigated to find out weather the metabolism mimics that of untreated and insulin treated diabetic humans or not, inasmuch it is desirable to have an animal model that mimics the condition investigated in humans. To our knowledge, the fat metabolism of insulin treated STZ-diabetic pigs has not previously been described.

Jensen Waern et al (2012) developed a protocol for immunosuppressive treatment of Swedish high health certified domestic pigs for the purpose of using this type of pigs in transplantation research. Results from the present experiment in combination with previous studies regarding STZ-diabetes (Jensen Waern et al 2009) and immunosuppressive medication in pigs (Jensen Waern et al 2012) will hopefully result in a disease model suitable for pancreatic islet transplantation research in Swedish domestic pigs.

MATERIALS AND METHODS

Animals

Eight high health certified SPF pigs (Yorkshire x Landrace, Serogrisen, Ransta; Wallgren and Vallgårda 1993) of both sexes were used. After the acclimatisation period of two weeks, the pigs weighed 32.5 ± 2.6 kg. They were housed at the Department of Clinical Sciences in individual pens measuring approximately $3m^2$, within sight and sound of one another. Straw and wood shavings were used as bedding. A 12:12 h light/dark schedule (lights on at 07:00) was used and an infrared lamp (24h) was provided in the corner of each pen. The room temperature was 18 ± 2°C. The pigs were fed commercial finisher diet without growth promoters (SOLO 330 P SK, Lantmännen, Sweden) twice daily (07:00 and 18:00), amount depending on body weight according to the SLU Norm (Göransson and Lindberg 2011). Water was provided *ad libitum*.

Acclimatisation and social training

When the pigs arrived at the Department of Clinical Sciences and placed in individual pens they were very nervous and frightened when staff members came in to the pens to clean. The first two days, the pigs were given time to adjust to their new environment. On the third day social training begun, each pig was assigned 15 minutes of social training each day. At the beginning of social training the pigs had become calmer in their environment but were still very nervous around people. The first step for the trainer was to just sit down in the pen. Initially the pigs were running around like crazy in the presence of a person in the pen. However, progressions were made rapidly and already on day one of social training; a few minutes into the first session the pigs calmed down and ate pieces of apples that were offered by the trainer. As the pigs got calmer around people and dared to eat apple pieces from the hand, attempts to brush the pigs were made. The pigs were also introduced to a piece of canvas with velcro that later would be put on their back after surgery to protect the indwelling vein catheter. Already on the second day of social training 6 out of 8 pigs allowed the trainer to brush them, and all the pigs ate apple pieces. On the fourth day of social training the pigs were very confortable around people. However, 3 out of 8 pigs were still a bit scared of the canvas piece and the sound of the velcro. The social training continued for a total of 9 days. When social training was finished, the pigs were very tame and not bothered by having people around (fig. 3). During the acclimatization period the pigs were also trained to step onto an electronic scale.
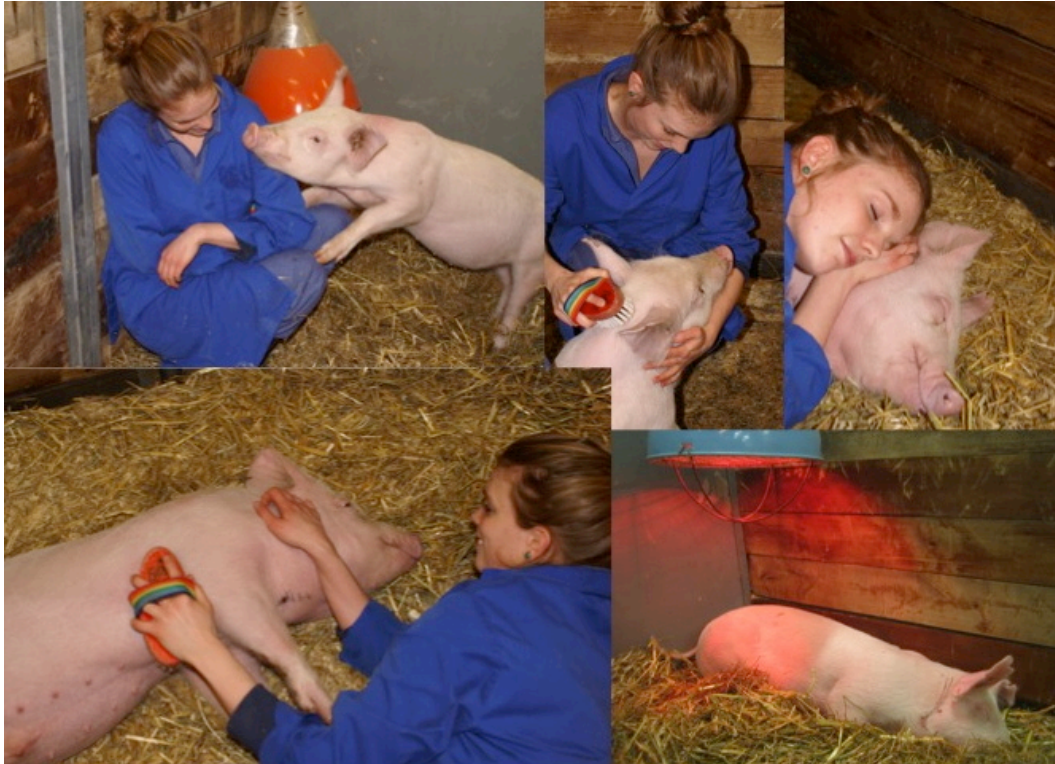
Figure 3. After 9 days of social training, the pigs were very calm around people.

Experimental design

All procedures were approved by the Ethical Committee for Animal Experimentation, Uppsala, Sweden. The protocol ran for a total of seven weeks (fig 4).
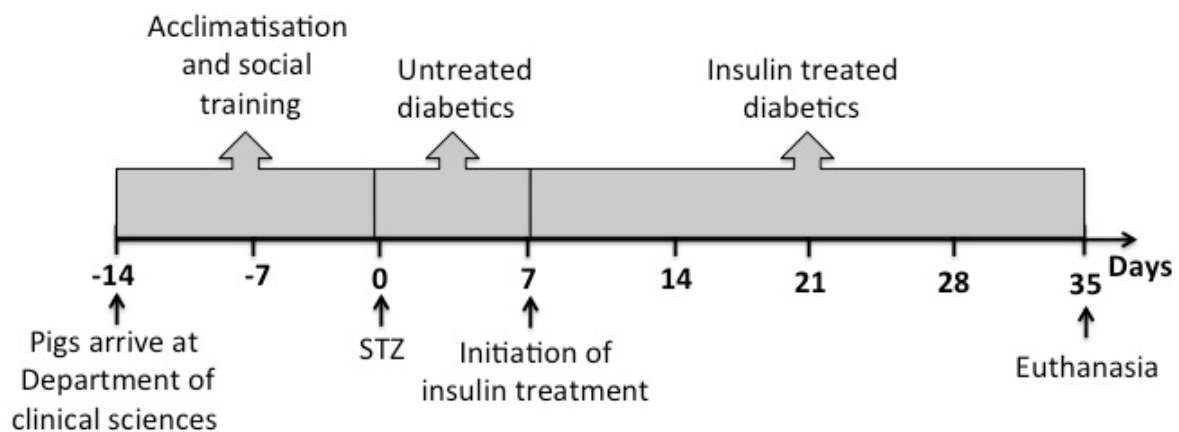

Figure 4. Time chart of the experimental design of the present diabetes study. Pigs were acclimatised and socially trained, diabetes was induced with streptozotocin (STZ) and diabetic pigs were treated with insulin.

After acclimatisation the pigs underwent surgery for insertion of catheters and diabetes induction. Anaesthesia was induced in the stable with a combination of medetomidine (Domitor® vet 1mg/mL, Orion Pharma Animal Health, Sollentuna, Sweden) at a dose of 0.05 mg/kg body weight (b.w.) i.m. and tiletamine and zolazepam (Zoletil® 250 mg/mL, Carros, France) at a dose of 5 mg/kg b.w. i.m. Buprenorphine (Temgesic® 0.3 mg/mL, Schering Plough, Brussels, Belgium) was given i.m. for additional analgesia at a dose of 0.01 mg/kg b.w. The pigs were moved to a preparation room where they were intubated and general

anaesthesia was maintained with isoflurane vaporized in oxygen. The concentration of isoflurane was continuously adjusted to achieve adequate depth of anaesthesia. The pigs were kept on controlled breathing and ventilated to Et CO_2 of approximately 5 kPa. During anaesthesia, non-invasive blood pressure, saturation O_2, ECG, Fi/Et CO_2, Fi/Et O_2, Fi/Et AA (Isoflurane), heart rate, respiratory rate, rectal body temperature, tidal volume, minute volume and positive end-expiratory pressure were monitored. Five minutes prior to end of anaesthesia the pigs were moved to spontaneous breathing and oxygen was increased to approximately Fi 80%. Ceftiofur (Excenel vet.®, 50 mg/ml, Pfizer Oy, Helsinki, Finland) was given pre-operative at a dose of 1mg/kg b.w.

Surgical areas were shaved and thoroughly cleaned with soap and chlorhexidine solution before the pigs were moved to the surgical theater. The pigs were initially placed in dorsal recumbency. Legs were fixed with straps and hoofs covered with socks to prevent heat loss. A silicon catheter (Silicon Cath with round tip, Instec Solomon, PA) was placed in *vena jugularis dexter* under aseptic conditions according to Rodriguez and Kunavongkrit (1983). With the silicone catheter in place, diabetes was induced with STZ i.v.

The pigs were moved to lateral recumbency. The silicone catheter were brought through a subcutaneous tunnel and exteriorised on the back between the scapulas, and finally covered with a piece of canvas (fig 5). The surgical procedure was finished within one hour.

Figure 5. Pig with a silicon catheter placed in vena jugularis dexter, brought through a subcutaneous tunnel and exteriorised on the back between the scapulas to enable frequent blood sampling and intravenous injections.

Catheters were flushed once daily with 2 ‰ heparinised saline (5000 IU/mL, Leo, Denmark). During the first week post surgery, blood samples were taken at least once daily for measurements of glucose concentrations. After one week, insulin treatment begun and blood samples for measurement of glucose concentration was taken at least four times daily. Blood samples were taken once a week for the other blood analyses. Clinical examination was carried out at least once daily throughout the study. Body weight was measured three times a week.

One week post STZ, subcutaneous treatment with short acting porcine insulin (Caninsulin 40 IU/ml, Intervet, Netherlands) was initiated. The initial dose of 2/3 IU /kg b.w. was gradually increased to 1 IU/kg b.w. divided by two times daily. The pigs were injected with insulin in direct connection with feeding.

At the end of the experiment, the pigs were euthanized with an overdose of pentobarbital sodium (Pentobarbital vet. 100 mg/ml, Apoteksbolaget, Sweden). The pigs underwent gross examination post mortem.

Induction of diabetes

Due to the short half-life of STZ (Sigma S0130, Stockholm, Sweden) it was dissolved in 100 mmol/L disodium citrate buffer solution, pH 4.5, at a concentration of 80 mg/mL, and administered i.v. within 5 minutes, while the animals were under general anaesthesia. The total amount of STZ administered per individual corresponded to 150 mg/kg b.w.

Postoperative care

After surgery, the animals were moved back to the stable where they were extubated and carefully monitored during wakeup and for 13 hours post STZ injection. Blood glucose concentrations were repeatedly measured by test strips (Accu-Chek, Roche Diagnostics, Basel, Switzerland; this test has previously been validated for porcine samples at the department of Clinical Chemistry, SLU) before and 1, 3, 7, 9, 11 and 13 hours after administration of STZ. Hypoglycaemia was promptly treated with an i.v. bolus of glucose (Glukos APL, 500 mg/ml, Apoteksbolaget, Sweden) corresponding to 0.5 g/kg b.w.

Blood analyses

Blood glucose concentrations were measured as described above. Concentrations ranging from 0.2 mmol/L to 33.3 mmol/L could be measured by the Accu-Chek device. Blood ketone bodies concentrations were measured by test strips (FeeStyle Precision β-ketone, Abbot/ADC, Chicago, U.S.) when clinically indicated and at time for euthanasia. Serum NEFA concentrations were measured at SLU by use of a kit (96-well Serum/Plasma Fatty Acid Kit Non-Esterified Fatty Acids Detection 100 point kit, Zenbio, NC, U.S.) and serum triglyceride concentrations were analysed at SLU using automated equipment (EKTACHEM DT60II, Medinor, Stockholm, Sweden). Triglyceride concentrations ranging from 0.17 mmol/L to 4.52 mmol/L could be measured with the automated equipment. Serum C-peptide concentration was determined at the Department of Immunology, Genetics and Pathology, Uppsala University by use of Porcine C-peptide ELISA (Mercodia, Uppsala, Sweden).

EDTA-preserved blood was analysed for total and differential white blood cell counts, and haematology (Hb, EPK, EVF, MCH, MCHC, reticulocytes, morphology) at SLU with an electronic cell counter validated for porcine blood (Advia 2120, Siemens, Erlangen, Germany). Serum samples were analysed for enzyme activities of aspartate amino transferase (ASAT), alanine aminotransferase (ALAT), γ-glutamyltransferase (GT) and glutamate dehydrogenase (GLDH) and Creatinine (CREA), Total Bilirubin, Magnesium (Mg), Sodium (Na), Potassium (K) and Chloride (Cl) at SLU using automated equipment (Architect C4000, Abott Diagnostics, North Ryde, Australia).

Statistical analyses

All values are expressed as mean ± SD and the statistical significance of differences was determined by Wilcoxon matched-pairs signed rank test. P values <0.05 were considered statistically significant.

RESULTS

Induction of diabetes

Pre-STZ blood glucose concentration ranged within 2.2-7.6 mmol/L. Within 9 hours post streptozotocin injection the blood glucose concentration fell to a range of 2.5-4.7 mmol/L in 6 out of 8 pigs. Two pigs became hypoglycaemic within 11 respectively 13 hours post STZ with blood glucose values of 4.7 respectively 1.3 mmol/L. All pigs were hyperglycaemic with blood glucose concentration >23 mmol/L within 48 hours post STZ.

Postoperative care

The animals regained swallowing reflex and were extubated within 15 minutes post anaesthesia. Time until the animals were awake ranged from 5 minutes to 2 hours post surgery.

Pig no 8 was found comatose with muscle spasms the day after STZ injection when the personnel arrived in the stable at 07.00. The pig had, at the day of surgery, become hypoglycaemic and treated with glucose intravenously. When the veterinary surgeon left the pigs on operation day, the blood glucose of no 8 had started to rise. However, during the early morning it must have started to fall again. The day after diabetes induction, blood glucose and general condition of pig no 8 was assessed repeatedly and the pig was treated for hypoglycaemia by intravenous glucose injections. The coma and spams were initially reverted by the glucose injections, however the pig became spasmic again later in the afternoon. The following day, the pig was calm and started to eat. From then on, he had no more clinical signs of hypoglycaemia.

Within seven day postoperative, the central vein catheter of pig number 3 and 6 did not function and it was determined that these pigs would act as non-treated controls. Seven days postoperative pig no 3 presented with high fever and inappetence. The central vein catheter of pig no 3 was removed and intramuscular treatment with ceftiofur (Excenel vet.®, 50 mg/ml, Pfizer Oy, Helsinki, Finland) was initiated since the catheter upon removal was covered with fibrin. However, the general condition of the pig was not improved and the pig was euthanized four days later and withdrawn from the study. Post-mortem examination revealed thrombophlebitis of *vena jugularis dexter*.

General appearance

The general appearance of the pigs was unremarkable throughout the study. They were brisk and vivid and had good appetite. No clinical signs of disease were apparent, other than polyuria/polydipsia that was seen during the period when the pigs were diabetic but not treated with insulin.

The weight gain of each pig is presented in figure 6. The insulin treated pigs had a daily weight gain of 532 ± 96 g. This corresponds to the weight gain of conventional high health herd pigs of the same age, which is approximately 500 g (personal communication Anna Wallenbeck, Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences). Pig no 6, who did not receive any insulin treatment, had a daily weight gain of 186 g, which corresponds to 37% of the weight gain of conventional pigs.
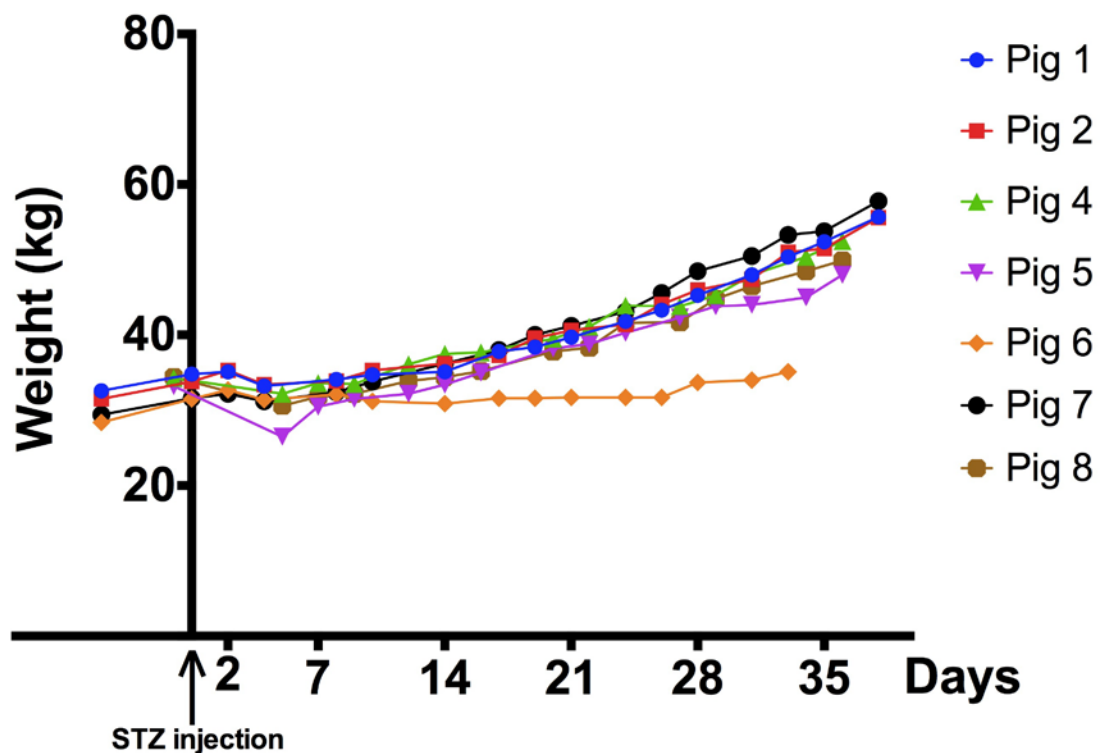
Weight gain

Figure 6. Weight in kg during the study period for pig no 1, 2, 4, 5, 6, 7 and 8. Weight measured thrice a week.

Blood analyses

Haematology

Haemoglobin, erythrocyte particle count, erythrocyte volume fraction, mean corpuscular volume, mean corpuscular haemoglobin concentration, reticulocyte particle count and erythrocyte morphology were within reference values in all pigs at all sampling occasions throughout the study. In five pigs, slight neutrophilia was seen on one sampling occasion either at two or three weeks post STZ. Other than that, total and differential white blood cell counts was within reference range for all the pigs during the study period.

Biochemical analyses

Serum creatinine, electrolytes (Na, K, Cl, Mg) and total bilirubin were within reference range during the entire study period in all pigs. In pig no 6 a slight increase in serum GLDH (39

nkat/L) was seen at time for euthanasia (5 weeks post STZ). The other liver enzyme activities (ALAT, GT, GLDH) were within reference range at all sampling occasions for all pigs. In pig no 8 a sevenfold increase in ASAT was seen at time for euthanasia (five weeks post STZ). Other than that ASAT was within reference range at all sampling occasions for all pigs.

Blood glucose

During the first week post STZ injection, when the pigs did not receive any insulin treatment, blood glucose concentrations were more than 17 mmol/L; *id est* the pigs were hyperglycaemic. After initiation of insulin treatment blood glucose concentrations were somewhat lowered and after 9 days of insulin treatment (with insulin dose of 1 IU/kg b.w.) pigs were normoglycaemic (blood glucose concentrations ranging within 4-14 mmol/L), but with periods of both hyperglycaemia and hypoglycaemia. In each individual, blood glucose concentrations fluctuated a lot (range within 3 mmol/L to >33,3 mmol/L) throughout the days and hyperglycaemic peaks were seen after feeding. Blood glucose concentrations were significantly higher 1.5 h post feeding compared to fasting values ($p < 0.0001$), figure 7. On two occasions in pig no 5, blood glucose concentrations fell below 0.2 mmol/L. Pig no 6, who did not receive any insulin treatment had blood glucose concentrations above 24 mmol/L throughout the study period.
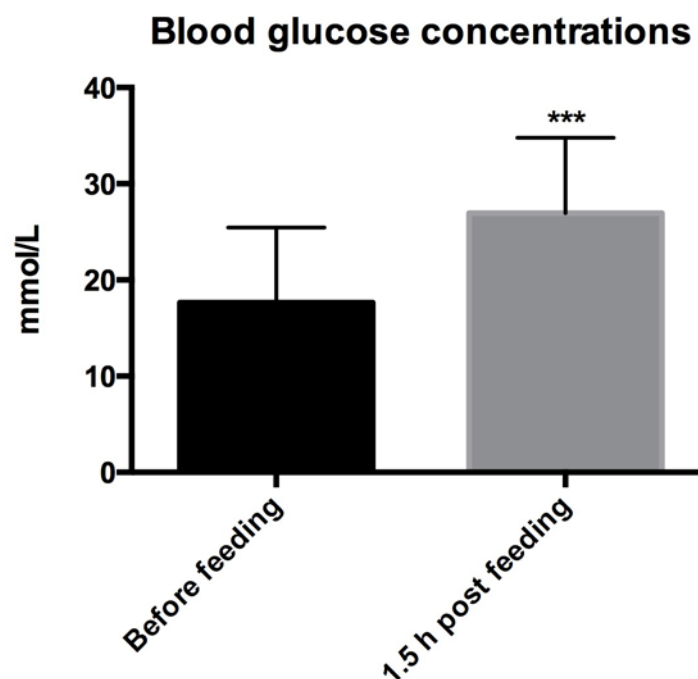
Figure 7. Blood glucose concentrations before and 1.5 h post feeding in pig no 1, 2, 4, 5, 7, 8 treated with insulin twice daily. p <0.001.

C-peptide

Two days after STZ injection the serum C-peptide concentrations were below 60 ρmol/L, values below 100 ρmol/L are seen in human patients with T1DM. Five out of eight pigs had serum C-peptide concentrations below the assay range, *id est* <20 ρmol/L, throughout the study period. In three pigs serum C-peptide concentration ranged from <20 to 96 ρmol/L.

Ketone bodies

Five days post STZ treatment pig no 5 showed inappetence. Blood ketone bodies were measured and showed hyperketonemia with a concentration of 4 mmol/L. Another STZ treated pig was used as reference and had blood ketone concentration of 0.4 mmol/L. Pig no 5 was given 2.5 IU of rapid acting insulin (Actrapid® 100 IU/ml, Novo Nordisk A/S, Bagsvaerd, Denmark) and treatment with Caninsulin was initiated. Two days later, appetite and general appearance was normal.

At time of euthanasia the blood ketone concentrations were 0.04 ± 0.05 mmol/L for all pigs.

NEFA and Triglyceride measurements

Serum NEFA concentrations before STZ treatment was 0.22 ± 0.09 mmol/L. Two days post STZ treatment serum NEFA concentrations were significantly higher than before treatment ($p<0.05$). 14 days post STZ treatment, *id est* seven days after initiation of insulin treatment, there was no significant difference in serum NEFA concentration before and after STZ-treatment (figure 8). In pig no 6, who did not receive any insulin treatment, an elevation of serum NEFA concentrations was seen on day two. The concentration was somewhat lowered during the study, but was higher than the NEFA concentration of insulin treated pigs from day 14 onwards.

*Figure 8. Serum triglyceride and NEFA concentrations in pig no 1,2,3,4,5,7,8 before and after streptozotocin treatment. From day 7 onwards, the animals were treated with insulin twice daily. * indicate a significant difference (p <0.05) from before streptozotocin treatment.*

Serum triglyceride concentrations before STZ treatment was 0.21 ± 0.05 mmol/L. Two days post STZ treatment serum triglyceride concentrations were significantly higher than before treatment ($p<0.05$) and peak TG values were observed seven days post STZ treatment. 28 days post STZ treatment, *id est* 21 days after initiation of insulin treatment, there was no significant difference in serum triglyceride concentration before and after STZ-treatment (figure 8). In pig no 6, who did not receive any insulin treatment, an elevation of serum

triglyceride concentrations was seen on day two. The concentration was somewhat lowered but was higher than the triglyceride concentration of insulin treated pigs throughout the study.

Post-mortem examination

Three out of seven pigs had sub-acute thrombophlebitis in *v. jugularis dx*, whereof one with secondary acute purulent endocarditis. Pig no 8 had a small, well-encapsulated abscess in the right thigh. Post mortem examination of pig no 6 revealed skeletal muscle wasting, subcutaneous fat reduced to minimum and no visible fat reserves in the abdominal cavity. In two pigs no pathological findings were observed.

DISCUSSION

In the present study glucose metabolism in STZ-diabetic pigs was investigated. Stress triggers the release of epinephrine from the adrenal glands and increased serum levels of epinephrine promotes glycogenolysis, lipolysis and inhibits the insulin mediated uptake of glucose in peripheral tissues, *id est* causing hyperglycaemia (as reviewed by Champe et al. 2008). It is hugely important to be able to draw blood samples under stress free conditions in diabetes studies to eliminate the risk of stress-induced hyperglycaemia affecting the measurements of blood glucose concentrations. For this reason acclimatisation is crucial to accustom the pigs to being handled by and calm around humans. In this study, acclimatisation was successful and the pigs were very tame and never stressed during blood sampling. It is also important for the welfare of the animals to feel safe and calm and not to be stressed. The animal welfare aspect should be considered in any type of experiments when pigs are used.

The acclimatisation period is also important to eliminate the risk that infections may impact on the results in the study. The stress that the pigs are experiencing during transport from the breeder to the research facility may trigger subclinical diseases to develop. An acclimatisation period of 2 weeks, as used in the present experiment, covers the incubation period of most infectious diseases of swine in Sweden. The intestinal microflora is also adjusted to the environment within two weeks (Melin et al. 1997). Furthermore, the two weeks acclimatisation period allows for daily clinical examination, ensuring that the pigs are healthy when going into the experiment.

STZ causes β-cell death (Yamamoto et al. 1981, Pieper et al. 1999). When β-cells are necrotized, serum insulin levels increase due to leakage of insulin from disintegrating cells (Kume et al. 2004). Insulin stimulate glucose uptake in cells and inhibit glycogenolysis and gluconeogenesis (as reviewed by Champe et al. 2008). Hence, when serum insulin levels increase, serum glucose concentration decrease. In this study, all pigs became hypoglycaemic within 13 hours of STZ injection. The brain, erythrocytes and renal medulla are dependant on glucose for energy and can be severely damaged under hypoglycaemic conditions (as reviewed by Sjaastad et al. 2003). When inducing diabetes with STZ in pigs, frequent monitoring of blood glucose post STZ injection and treatment of hypoglycaemia with glucose is crucial to avoid damage to vital organs, animal suffering and ultimately death of the animals.

After the initial rise in serum insulin concentration a decline is seen when insulin is cleared from the systemic circulation mainly by liver and kidneys, but also by other insulin-sensitive cells such as muscle cells, adipocytes, fibroblasts, monocytes, lymphocytes and gastrointestinal cells. All cells carrying insulin receptors and containing degradation mechanism can degrade insulin (as reviewed by Duckworth et al. 1998). Treatment with STZ leads to a decreased or discontinued production of insulin depending on the dose administered and the magnitude of β-cell death (Yamamoto et al. 1981, Pieper et al. 1999). When creating STZ-induced diabetes the goal is to give the animals a STZ-dose sufficiently high to cause a reduction or lack of endogenous insulin and thereby creating hyperglycaemia. When serum insulin concentration declines hyperglycaemia develops. In this experiment all pigs became hyperglycaemic within 48 hours post STZ injection.

Two days post STZ treatment, a significant rise in serum NEFA concentration and serum triglyceride concentration were seen. This mimics the fat metabolism of untreated diabetic humans (Laurell 1956; Bagdade et al. 1968). 14 days post initiation of insulin treatment, NEFA concentration was lowered to that of healthy individuals. For triglyceride concentration to decrease to levels comparable to healthy individuals, however, it took 21 days from initiation of insulin treatment. Ergo, 21 days after initiation of insulin treatment, the fat metabolism of insulin treated STZ-diabetic pigs mimics the fat metabolism of diabetic humans with god glycaemic control. In the present study young pigs (about 5 months old at time for euthanasia, *id est* in the transition to sexual maturity) were used. The serum triglyceride concentrations seen in the pigs prior to the STZ injection and 21 days after initiation of insulin treatment correspond to that of healthy children and adolescents (0-19 years old) (Kudo 1969). In the present experiment, the serum NEFA concentrations before STZ treatment and 14 days after initiation of insulin treatment corresponds to levels seen in healthy adult and older adolescent humans (Munkner 1959; Heald et al. 1967).

In the present study, the pigs were hyperglycaemic with blood glucose concentrations above 17 mmol/L before the initiation of insulin treatment. *Id est,* the pigs were in a diabetic state. After initiation of insulin treatment, blood glucose levels were lowered and a normoglycaemic state was reached at a dosage of 1 IU/kg b.w. divided by two times daily. However, the blood glucose concentrations fluctuated a lot throughout the days with undesirable episodes of hyperglycaemia and hypoglycaemia. Less fluctuation and a more constant normoglycaemic state could perhaps be reached by dividing the daily insulin dose and feeding by three times instead of two. Another approach could be to combine the short-acting insulin with a long-acting insulin. It is not possible to give the pigs higher dosage of short-acting insulin when treating the pigs twice daily. The present experiment shows that at a dose of 1 IU the pigs experience periods of hypoglycaemia, though without any clinical signs of disease. If the dosage was to be increased, however, the pigs might experience severe hypoglycaemia; which could cause illness and a potentially life-threatening condition.

When developing an animal model for research purposes, it is desirable to create a model that mimics the state investigated in humans and to reduce the personnel and working hours needed. As stated earlier, the blood glucose concentrations of the pigs fluctuated a lot in the present experiment. This can, however, be tolerated as domestic pigs are usually only held in

experiment for a couple of months. During insulin treatment, the pigs had no clinical signs of diabetes, their weight gain was at least as good as conventional pigs and the metabolism of NEFA and triglycerides mimicked the metabolism of insulin treated diabetic humans. However, one has to keep in mind that it takes three weeks for serum triglyceride concentrations to decline to levels comparable to healthy individuals. In the present experiment the amino acid metabolism was not investigated. Hence, one cannot for sure say that the amino acid metabolism was comparable to healthy individuals three weeks after initiation of insulin treatment. However, as the metabolism of carbohydrates, fat and amino acids is so closely linked, it is plausible to speculate that the metabolism of amino acids would be normalised when the metabolism of carbohydrates and fat is. Jensen-Waern et al. (2009) showed that the amino acid metabolism in non-insulin treated STZ-diabetic pigs mimics that of untreated humans with T1DM.

The non-insulin treated control pig had a weight gain much lower than conventional pigs, the weight gain corresponded to only 37% of that of conventional pigs. The control pig had polyuria/polydipsia that did not resolve during the course of the study. However, the general appearance of the pig was unremarkable. The pig was just as vivid and brisk as the insulin treated pigs. As expected, the metabolism of the control pig differed from the insulin treated pigs. The control pig was hyperglycaemic throughout the experiment and the serum levels of NEFA and triglycerides were not lowered to the same extent as it was in the insulin treated pigs. Furthermore, during the period when the STZ-diabetic pigs were not treated with insulin complications such as hyperketonemia developed, which is also seen in diabetic humans that do not receive any insulin or have poor glycaemic control.

In the present experiment, young domestic high health certified SPF pigs were used. The reasons to why these animals were chosen include that there are data from previous diabetes studies with these types of pigs. Furthermore, the present experiment aimed to develop a model for islet transplantation and pancreatic islets have been isolated from domestic pigs and are available for further experiments. Young animals were chosen for practical reasons, as adult pigs are large in size and thereby difficult to handle. Onset of T1DM occurs in childhood or adolescence, thus this age group of pigs is suitable. Young pigs grow very fast; this might have contributed to the difficulties in adjusting insulin treatment and to avoid large fluctuations in blood glucose concentrations.

In the present study one pig was found to have hyperketonemia five days post STZ treatment, *id est* before the start of insulin treatment. However, hyperketonemia could be reverted before development of diabetic ketoacidosis since it was discovered early in the process. Hyperketonemia in this pig shows that model of diabetes used in this experiment mimics T1DM also regarding development of ketoacidosis. The afflicted pig in the present experiment had a blood ketone concentration of 4 mmol/L. Humans with diabetic ketoacidosis usually have blood ketone concentrations of 12-30 mmol/L (as reviewed by Berne 2006). The risk of developing hyperketonemia is one reason to why daily clinical examination of diabetic pigs used in research is so important.

When analysing serum insulin, endogenous insulin cannot be differed from exogenous insulin. Furthermore, insulin half-life in plasma is very short, about 4 minutes (Turner et al.

1971; Starr and Rubenstein 1974). The metabolic clearance rate of insulin is three times that of C-peptide (Katz and Rubenstein 1973). The large difference in clearance rate is partly due to the large uptake of insulin by the liver. C-peptide is not excreted from the circulation by the liver (Stoll et al. 1970). The half-life of C-peptide in plasma is approximately 35 minutes (Faber et al. 1978). The longer half-life of C-peptide compared to insulin in addition to the fact that exogenous insulin does not interfere with measurements of C-peptide makes C-peptide an accurate indirect measure of insulin secretory activity in the β-cells.

C-peptide is cleaved from pro-insulin and secreted together with insulin in a 1:1 molar ratio from β-cells (as reviewed by Gianani and Eisenbarth 2005). It is desirable that serum C-peptide concentrations remain low in animal models that are meant to mimic human T1DM. Non-diabetic humans usually have a serum C-petide concentration of 300-700 ρmol/L and diabetic patients <100 ρmol/L (Personal communication Bo Nilsson, Department of Immunology, Genetics and Pathology, Uppsala University). In humans, the β-cells' regenerative capacity is limited. In animal models, however, mitotic activity of the β-cells have been observed in both chemically induced diabetes and in genetically determined diabetes (as reviewed by Risbud and Bhonde 2002). In STZ-diabetic mice it has been shown that β-cell regeneration is stimulated by insulin treatment and glycaemic control (Grossman et al. 2010). Jensen Waern et al. 2009 showed that serum C-peptide concentrations remain below 100 ρmol/L for at least three weeks when using same method for diabetes induction as used in this study; however, those pigs did not receive any insulin treatment. In the present study we showed that C-peptide concentrations remain below 100 ρmol/L for at least 5 weeks also in insulin treated STZ-diabetic pigs.

Porcine insulin only differs in one amino acid from human insulin (as reviewed by Larsen and Rolin 2004). Porcine C-peptide, however, differs from human C-peptide by 39% (Chance et al. 1968; Oyer et al. 1971). In the present study, an ELISA kit developed and validated for porcine C-peptide was used. Figure 9 shows the amino acid sequence of porcine and human C-peptide. In many articles porcine C-peptide concentrations are presented in μg/L, 1μg/L equals 364ρmol/L.
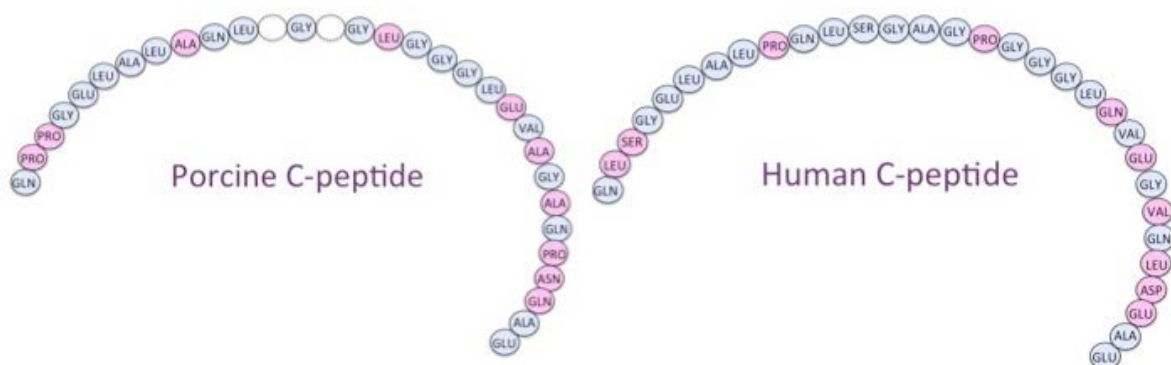

Figure 9. Amino acid sequence of porcine and human C-peptide. Similarities shown in blue and differences in pink. Crosshatched circles represent amino acids that are missing in the porcine C-peptide compared to human C-peptide. Drawn by the author on the basis of information in articles by Chance et al. (1968) and Oyer et al (1971).

Since STZ is transported into the cells through the GLUT2-receptor, any treatment with STZ causes liver and kidney damage (Elsner et al. 2000). When inducing diabetes with STZ the aim is to use as low dose of STZ as possible to minimize liver and kidney damage but still produce a diabetic state in the animal. In this study, serum creatinine values were within reference value during the entire study period in all pigs, this shows that the kidney function was not significantly impaired by the STZ treatment in this experiment. In one pig, a mild increase in serum GLDH was seen at the last sampling occasion. However, this mild increase is very unspecific and probably not related to any liver damage while the activities of GT and ALAT were within reference range at this occasion. Furthermore, increased liver enzyme concentrations does not necessarily mean that the hepatic function will be impaired inasmuch more than two thirds of the hepatic parenchyma can be removed without any significant impairment of hepatic function (as reviewed by McGavin and Zachary 2007). Clinical signs of liver injury include vomiting, diarrhoea, anorexia and polydipsia/polyuria (as reviewed by Nelson 2009). No clinical signs of liver failure were apparent. Consequently, diabetes can be safely induced in pigs at an STZ-dose of 150 mg/kg b.w.

STZ is an excellent tool for diabetes induction. However, opinions whether to use pancreatectomy or STZ for diabetes induction in laboratory animals diverge. Some people argue that pancreatectomy should only be considered when no other method is possible to use due to the high mortality and welfare considerations associated with the invasive surgery (Gäbel 1983; and reviewed by Larsen and Rolin 2004). Others argue that only pancreatectomy should be used in transplantations research to be certain that all ensure that insulin measured is produced by the transplant and not by remaining β-cells (Kobayashi et al. 2004, Strauss et al. 2012). However, in clinical transplantation insulin is given to patients after transplantation to give the grafted islet some rest (Johansson et al. 2005). If transplanted cells were to be placed in a hyperglycaemic environment it would probably be disadvantageous to the graft. The very low C-peptide concentrations seen in this experiment are not a disadvantage but rather an advantage in pre-clinical transplantation studies preformed with the pig as an animal model.

Post-mortem examination revealed thrombophlebitis in jugular veins and haematologically spread infections. The rises in blood leukocyte count and neutrophil count are likely to be due to these infections. Infections could have been acquired either during surgery or through the central vein catheter. To avoid surgery related infections it is crucial to maintain aseptic or sterile condition through preparation and surgery. In this study, the aseptic conditions during and in connection with surgery could be improved. The animals were not washed in the stable before they were moved from their pens to the preparation room, this might have contributed to the fact that aseptic conditions were not optimal in this experiment. The surgical areas were thoroughly washed in the preparation room. However, the pigs were initially placed in dorsal recumbency and later placed in lateral position when the catheters were exteriorized between the scapulas. The surgical area between the scapulas was not wash a second time after the pigs were moved to lateral position. A second wash before exteriorizing the catheter may improve aseptic conditions. Furthermore, there was no permanent operating room available, and the room used could probably have been more thoroughly cleaned before surgery was performed. In this study antibiotics (ceftiofur) was given only as a single injection prior to

surgery. Jensen-Waern et al. (2009) performed the same surgical procedure, but with antibiotics (penicillin) given for three consecutive days post surgery and did not see infections at post mortem examination. This indicates that antibiotics should be given for three consecutive days and not only as a single injection prior to surgery.

Central vein catheters enable frequent blood sampling and intravenous injections in pigs (Rodriguez and Kunavongkrit 1983; Mortiz et al. 1989). There is a risk of catheter related and potentially lethal infections (Moritz et al. 1989), this must kept in mind when keeping animals with central vein catheters. Infection can occur through migration of skin organisms at the insertion site into the cutaneous catheter tract and along the surface of the catheter to the blood stream. Infection can also be caused by direct contamination of the catheter or catheter hub by contact with contaminated hands, fluids or devices (as reviewed by O'Grady et al. 2011). Several hygiene procedures have been shown to help preventing catheter related infections, this includes:

- Maintaining aseptic technique during insertion of intravascular catheter.

- Washing the skin with >0.5% chlorhexidine solution with alcohol before insertion of central vein catheters.

- Washing the skin around the catheter daily with 2% chlorhexidine solution.

- Scrubbing the access port with antiseptics (for example chlorhexidine or 70% alcohol) to minimize contamination risk.

- Preforming hand hygiene procedures, either by washing hands with soap and water or alcohol-based hand rubs, before accessing, repairing or dressing an intravascular catheter.

- Wear either clean or sterile gloves when accessing, repairing or dressing an intravascular catheter.

Some researchers would flush catheters with antibiotics to prevent catheter related infections; this however should be avoided if possible due to increasing antibiotic resistance. Improving hygiene procedures should always be the first intervention in preventing catheter related infections.

Hyperglycaemia predispose for infections and improved hygiene procedures and asepsis when handling the central vein catheters will be applied in forthcoming experiments.

CONCLUSION

The pig is an excellent animal model for T1DM in humans. The physiology and metabolism of pigs is similar to that of humans. Similarities that are especially important in diabetes studies include the similarity in size, shape and position of the pancreas, the similar blood glucose range in healthy and diabetic humans and pigs, and the amino acid sequence of insulin, which only differ in one amino acid between the two species. In addition, pigs are easy to handle and can be handled under stress free conditions if proper social training has been performed.

Diabetes can be safely induced with STZ at a dose of 150mg/kg b.w. i.v. and the diabetic state developed mimics T1DM in humans regarding clinical signs and metabolism. Within three weeks of insulin treatment, the metabolism of glucose and fat mimics that of healthy individuals and diabetic patients with good glycaemic control. The general appearance of insulin treated diabetic pigs is unremarkable and there are no clinical signs of disease. The recommended daily dosage of short acting insulin is 1IU/kg b.w. s.c., divided by two times daily. Insulin should be given in direct connection with feeding. Using this model, pigs can be kept diabetic for at least 5 weeks without any signs of β-cell regeneration.

With the information from the present experiment and previous studies by Jensen-Waern et al. 2009 and 2012 we can conclude that there is now a model with STZ-diabetic high health certified Swedish domestic pigs that mimics T1DM in humans regarding carbohydrate, amino acid and fat metabolism in non-insulin and insulin treated animals. Clinical signs of disease seen in STZ-diabetic pigs are the same as those seen in diabetic humans. Furthermore there is now satisfying protocols for both immunosuppression and insulin treatment of these pigs. The information from these experiments enables islet transplantation studies with the pig as an animal model.

ACKNOWLEDGEMENTS

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