# Isolation of neonatal porcine islet tissue and transplantation into diabetic mice. A methodological evaluation.

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## Introduction

Diabetes mellitus type I can be a disabling disease with a high risk of complications (i.e. neuropathy, atherosclerosis and nephropathy). Furthermore, the treatment with insulin can be complicated with severe episodes of hypoglycaemia.

As diabetes type 1 is caused by an autoimmune destruction of the islets of Langerhans, replacement of these islets could be the treatment of choice for this disease provided the immunological process is halted. Centres world wide perform islet allotransplantation (i.e. transplantation within species) as an experimental treatment of diabetes *(Ricordi et al. 1992, Secchi et al. 1997, Warnock et al. 1992, Birkeland et al. 1995)*. However the results are poor with only 8 % of cases having more than one year of insulin independence after transplantation (international islet transplant registry, 1999).

Islet transplantation faces two major obstacles : rejection and shortage of human donors. Rejection is avoided by immunosuppression and in experimental models by immunoprotection of the grafts with micro-encapsulation or larger encapsulating devices *(Lanza et al. 1995, Lacy et al. 1991, Monaco et al. 1991)*. Shortage of human donors has necessitated the search for alternative donors, and in this context the pig has drawn most attention, because the physiology of the pig is comparable to that of humans. Indeed, pig insulin has been successfully used for decades in the treatment of human diabetics. Pigs breed fast, have large litters and can be bred under standardised conditions, which is important, as factors such as strain and age influence the outcome of porcine islet isolations *(Socci et al. 1990, Heiser et al. 1994)*.

By small modifications of the automated method developed for human islet isolation, it is possible to isolate large numbers of adult porcine islets *(Ricordi et al. 1990)*. These islets are well functioning in vivo after transplantation to rodents. However, unlike human islets, the adult porcine islets have a very thin peri-insular capsule and are therefore very fragile disintegrating easily in overnight culture *(van Deijnen et al. 1992, Warnock G.L. et al. 1995)*.

Islet tissue from foetuses possess a potential for growth, and has been extensively examined in the rat *(Yderstraede et al. 1995), pig (Korsgren et al. 1988)* and humans *(Tuch et al. 1991)*. Foetal porcine islet tissue has even been transplanted to eight diabetic humans with histological signs of graft survival after three weeks, and porcine C-peptide production detectable up to eight months after transplantation, but without any detectable metabolic improvement in the patients *(Groth et al. 1993)*.

Recently, a method has been developed for the isolation of islet tissue from neonatal pigs *(Korbutt et al. 1996)*. The neonatal islet-like cell clusters (NICC's) can be isolated in large numbers, and are able to differentiate and maybe even proliferate in vitro and in vivo.

The purpose of this study is to describe the methods for isolation, transplantation and in vivo evaluation of porcine neonatal islet-like cell clusters. Focus is on the transplantation procedure, but aspects of isolation and functional outcome in vitro and in vivo are also included.

*Materials and Methods*

Hams F10 (Gibco) containing 10 mmol/l glucose, 50 mmol/l IBMX (iso -butyl methyl xanthine, Sigma), 0,5% Bovine Serum Albumin (Gibco), 10 mmol/l nicotinamide (Sigma), 100 microgram/ml Streptomycin and 100 U/ml penicillin (Gibco). Hanks Balanced Salt Solution (HBSS, Gibco) supplemented with 0.25 % Bovine Serum Albumin , 10 mmol/l Hepes, 100 U/ml penicillin and 100 mg/ml Streptomycin. Collagenase A and streptozotocin were obtained from Boehringer Mannheim.

Animals : We obtained the pigs from a local SPF farmer. Mice were kept in type II cages (267x207x140mm), one mouse per cage, in a hood (Scantainer, Scanbur). Temperature was kept at $23^oC \pm 3^oC$ and humidity 55% $\pm$ 15 %. The animals had free access to autoclaved tap water and radiation sterilised pelleted food (Altromin, Brogaarden, Denmark). When handling the mice, single use coats, caps, mouth & nose cover and single use rubber gloves were worn. Gloves were disinfected with ethanol between handling of the individual mice.

Isolation of porcine neonatal isletlike cellclusters (NICC's): The neonatal pigs 1-3 days old, are sedated with 0.15 ml hypnorm (Fentanyl citrate 0.315 mg/ml and Fluanisone 10 mg/ml) and 0.15 ml stresnil (40 mg Azaperon + 0.5 mg P-hydroxybenzoemethylster + 0.05 mg P-hydroxybenzoepropylester / ml) i.m. Propofol 0.5 ml (10 mg / ml) is injected i.v. in a superficial abdominal vein (Fig. 1). The pig is sprayed in the oropharynx with lignocaine (10 mg / dose), orally intubated and put into general anaesthesia with halothane. Under sterile surgical conditions a laparotomy is performed, and the pigs are exsanguinated by cannulating the abdominal aorta (Fig. 2). The pancreata are carefully dissected from the surrounding tissues and the portal vein. After removing the pancreata (Fig. 3) they are placed in $4^oC$ HBSS (supplemented as described earlier) until processing in the laboratory less than 1 hour later.

The glands are cut into 1-2 mm$^3$ pieces by a pair of scissors, then put into tubes containing HBSS with collagenase (2.5 mg/ml), and shaken in a $37^0C$ waterbath for 15 minutes. Thereafter, the digest is filtered through a 500 μm metal filter, washed 3-4 times in HBSS and incubated in Petri-dishes containing Ham's F10 (supplemented as described). The NICC's are cultured for 9 days in humidified air ($37^oC$, 5% CO2 / 95% atmospheric air) with media change the first day and every two days thereafter.

The number of NICC's can be counted by staining them with dithizone which stains the zinc molecules in insulin granules red. The NICC's are sized using a calibrated grid and converted into equivalents of 150 μ m.

Transplantation of NICC's: Mice are made diabetic by injection of Streptozotocin (275 mg/kg) in a tail vein. Only animals with at least 2 measurements of a blood glucose (BG) > 20 mmol/l are used as recipients. The mice are anaesthetised by a subcutaneous injection of 0.011 ml Diazepam (5mg/ml) and 0.011 ml Hypnorm (Fentanyl citrate 0.315 mg/ml and Fluanisone 10 mg/ml). Access to the left kidney is obtained by a lumbar incision, a capsulotomy is performed, and a small amount of air is injected under the kidney capsule using a 25 G venflon (Fig. 4). With a microsyringe, NICC's are aspirated into a polythene tube (ID 0.58 mm, Portex), spun down and carefully injected under the kidney capsule (Fig. 5). After removing the tube, the capsule is cauterised.

Follow up: In the postoperative period, BG is measured 2-3 times the first day, and injections of glucose and saline are administered when needed to avoid hypoglycaemia and dehydration. The animals are followed with BG measurement once a week .

Following normalisation of the BG, the in vivo function can be further evaluated by an oral or an intraperitoneal glucose tolerance test : After a 2 hour fasting, glucose (3 mg/g body weight, in a 50 % solution) is administered either intragastrically through a gastric tube or intraperitoneally by injection. BG is measured at time 0, 15, 30, 60 and 120 minutes.

To make sure, that the grafts are responsible for the normoglycaemia, the grafts can be removed by nephrectomy: the mice are anaesthetised and access to the kidney is obtained as mentioned earlier. At this time the kidney often have to be carefully dissected free from surrounding fibrotic tissue. The kidney stalk is localised and ligated using a Ligaclip (Ethicon). The animals are
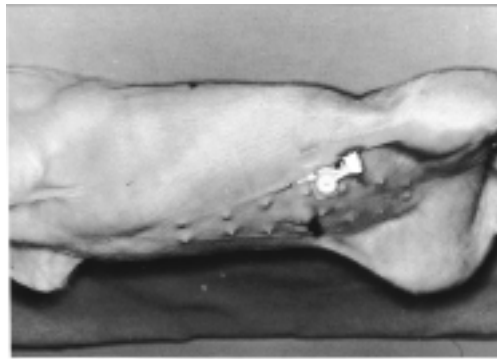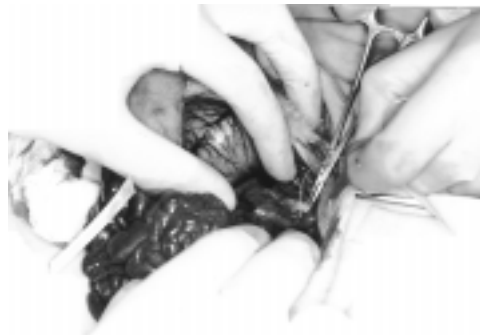
Fig. 1 : Anaesthesia is induced by injection of Propofol in a superficial abdominal vein.



a)



b)

Fig. 2 : The pig is exsanguinated by placing a suture under the abdominal aorta (a), inserting a venflon in the aorta (b) and fixing it with the suture.
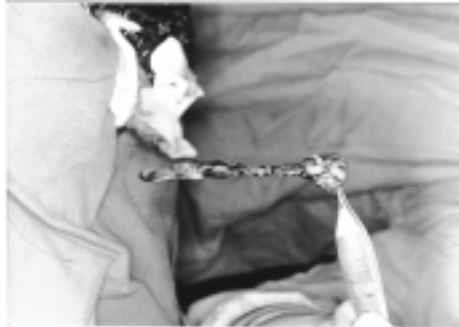
Fig. 3 : Excised pancreas from a 1-3 day old pig.





Fig. 4 : Capsulotomy is performed by injection of a small amount of air under the kidney capsule (a+b) using a syringe connected with a tube to a 25 G venflon.

Fig. 5 : Tissue is gently placed and spread under the kidney capsule.

followed for another two days with daily BG measurements to make sure they return to hyperglycaemia.

*Results*

To examine the morphology of NICC's in vitro at the day of transplantation, tissue from a series (n=8) of NICCs cultured for 9 days was fixed in formalin, spun down into a gel (Cytoblock, Shandon inc.) and embedded in paraffin. Sections were immunohistochemically stained for insulin, glucagon and somatostatin and the cellular distribution was evaluated by point counting morphometry. The number of cells staining positive for insulin amounted to 16 %, 30 % stained positive for glucagon and 11% stained positive for somatostatin.

In another series (n=21) 2000 NICC's cultured for 9 days were transplanted to BALB-C nu/nu mice (Bomholtgaard, Denmark). The mice were followed for up to 20 weeks (Fig. 6). 13 mice became normoglycaemic within an average of 10 ± 3 weeks (means ± 95% CI). 8 mice were sacrificed between 9 and 20 weeks after transplantation (means 14 weeks) because of persistent diarrhoea or dilated intestines. However, none of these mice had shown signs of graft function (i.e. decreasing BG - level). Six mice were graftectomized and returned to hyperglycaemia. Two mice were bled for serum insulin analysis (Auto-Delfia, Wallac, Denmark). They had non fasting circulating porcine insulin levels of 970 and 581 pico-mol/l respectively, clearly indicating graft function. To exclude cross reactivity with endogenous mouse insulin, two normal, non transplanted mice were also bled and no porcine serum-insulin was measured.

*Discussion*

Normally xenogeneic transplantation results in hyperacute rejection of the graft within minutes to hours after transplantation, at least as far as vascularised grafts are concerned. Hyperacute rejection is caused by naturally occurring preformed antibodies against foreign determinants primarily situated on endothelial cells. If hyperacute rejection is avoided, acute vascular rejection will take place within 24 hours, possibly also triggered by antibodies *(Hamelmann et al. 1994)*. If the graft is still not destroyed, a T-cell dependent rejection will take place within a few days *(Platt, 1998)*. As NICC's are not vascularised they contain very few endothelial cells and are therefore not hyperacutely rejected nor subjects to acute vascular rejection *(Mandel et al. 1995)*. This opens for the sequential study of cellular transplants for ongoing immunological reactions and furthermore enables evaluation of the effect of
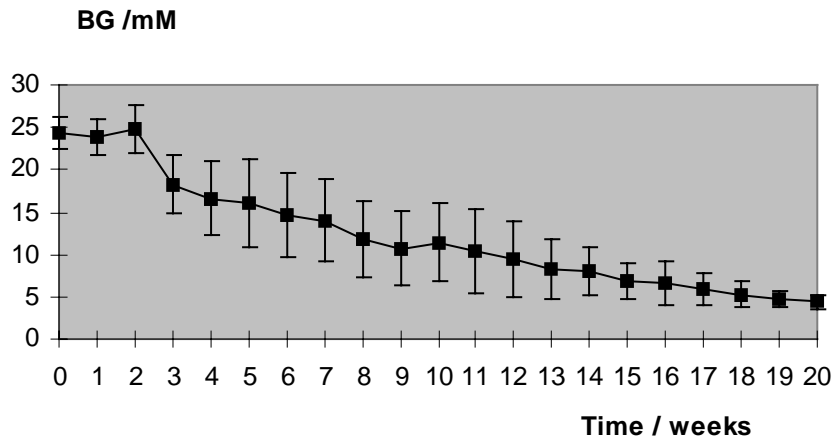
**BG /mM**



Fig. 6: BG after transplantation of 2000 NICC's to diabetic athymic nude mice (n=13) which became normaglycaemic (means ± 95% CI)

different modifications modalities. We used the athymic mice to study the functional and immunohistochemical outcome from transplantations, as the lack of T-cell response prevented rejection. However, the immune-deficient mice are also prone to catch infections. We have employed a strict sterility policy (as previously described) radically reducing the rate of infections.

The mice were made diabetic by intravenous injection of streptozotocin. It has been suggested, that streptozotocin is taken up in the beta cells via the GLUT2 glucose transporter, and causes fragmentation of the cellular DNA by reducing the level of NAD+, thereby destroying the beta-cells of the pancreas *(Thulesen et al. 1997)*. When streptozotocin is administered as a single dose the islets are virtually free of inflammation but when administered as multiple low doses the immune system is activated which results in severe insulitis and diabetes. However, some strains of nude mice are less susceptible to multiple low dose administration of streptozotocin *(Hagemann et al. 1994)*. The NOD (non obese diabetic) mouse is probably a better model in future immunological

studies as the pathogenesis of the spontaneous diabetes in these animals is closer to that of humans *(Hagemann et al. 1994)*.

In human islet transplantation the liver is the preferred site of transplantation. The islets are injected into the portal vein and embolises in the liver. This ensures sufficient blood supplies in the early period after transplantation until the new vascular supplies have been established. Islets can survive for some time by diffusion from the surroundings, and after transplantation vascular supplies will be restored within 14 days by ingrowth from the recipients own vascular bed *(Vajkoczy et al. 1995)*. In this model the islet tissue is transplanted beneath the kidney capsule as it gives the opportunity to remove the graft in toto for further examination. Furthermore the return of hyperglycaemia, after total graft removal, excludes the possibility of normoglycaemia originating from spontaneous recovery of streptozotocin induced diabetes. The kidney is, however not the optimal site for transplantation as it results in systemic delivery of insulin with hyperinsulinaemia and insulin resistance *(Guan et al. 1998)*. The physiologically optimal site of

transplantation will probably be a site with portal delivery of insulin i.e. a parahepatic omental pouch *(Ueki et al. 1994)*. An omental pouch may also be an advantage when larger volumes are to be transplanted e.g. when the islets are micro-encapsulated, as it will still be possible to remove the graft in toto. However, the peritoneal cavity is not the optimal site for transplantation because nutrition and oxygenation is less sufficient and the number of islets needed to restore normoglycaemia in diabetic rodents is more than doubled *(Siebers et al. 1993)*.

By a method developed by Korbutt (1996) it is possible to isolate a substantial number of NICC's from neonatal pig pancreas. We report that 2000 porcine NICC's can restore normoglycaemia in athymic nude mice. Non function of the grafts may have been caused by poor batches of NICC's *(Yoon et al. 1999)*, perhaps caused by non discovered infection of the tissue in culture. Mice sacrificed because of illness, early in the follow up period, may have normalised if they had been followed for a longer period.

In the foetal pig pancreas, endocrine cells are present as early as 4 weeks of gestation, but at that time the beta cells are scattered around the exocrine tissue. The endocrine cells gather into isletlike cell-clusters and are not organised into real islets until months after birth *(Alumets et al. 1983)*. In this model IBMX and nicotinamide is added to the culture media as it increases replication *(Rabinovitch et al. 1980)* and DNA - synthesis in the beta cells (Sandler et al. 1986). Still we found, that after 9 days of culture the insulin positive cells only make up 16 % of the total. We found a relatively high number of glucagon positive cells. However, many of the insulin positive cells may stain for glucagon as well because the endocrine cells originate from the same pluri-potent precursor *(Ramiya et al. 2000)*. As found by others *(Korbutt et al. 1996, Yoon et al. 1999)* the BG level is not normalised until 4-20 weeks after transplantation. During this period further proliferation and maturation is taking place *(Yoon et al. 1999)*. The in vivo function after normalisation of the BG level is highly sufficient as judged by glucose tolerance tests *(Korbutt et al. 1996)*. As found by others, non-fasting BG is normalised to a level of 4-5 mmol/l which is normal for the donor (pig) but not the mouse (6-8mmol/l). Humans and pigs share the same basic glucose level which is an important aspect in xenotransplantation.

Unlike the fragile adult porcine islets, NICC's can be cultured for at least 9 days without disintegrating. This makes it possible to perform gene modulation and alter the immunogenecity before transplantation *(Knechtle, 1996)*. Culture is also a rather gentle way of purifying the NICC's from exocrine tissue, as exocrine tissue usually does not survive in prolonged culture. Furthermore, culturing gives more space for microbiological examinations before transplantation. Recently, there has been increasing concerns for transferring zoonoses from pigs to humans, and especially the porcine endogenous retro viruses (PERVs), situated in the genome of porcine cells, has drawn a lot of attention.

We thus demonstrate a feasible technique for transplanting isolated islet tissue at the renal subcapsular space. Technical aspects are discussed, the methods are simple and can be used routinely to evaluate morphologically and functionally, different islet tissue sources.

*Summary*

Shortage of human donors for islet of Langerhans transplantation has given attention to the pig as an alternative donor in xenotransplantations. We describe and discuss a method for isolation of neonatal porcine islet tissue and its transplantation beneath the renal capsule of streptozotocin diabetic mice. The transplantation procedures are applicable in many similar models including different cell types.

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