PREPARATION AND XENOTRANSPLANTATION OF PORCINE ISLETS

INTRODUCTION

The present invention relates to improvements in and/or relating to the treatment of diabetes using xenotransplantation. More particularly but not exclusively the present invention relates to the preparation of viable xenotransplantable porcine islets and/or the treatment of a mammalian patient (including humans) suffering from diabetes involving the transplantation into the mammal of viable porcine islets capable of producing insulin within the host.

BACKGROUND

Type 1 (insulin-dependent) diabetes mellitus is a common endocrine disorder that results in substantial morbidity and mortality, and leads to considerable financial costs to individual patients and healthcare systems.

Treatment with insulin, while life-saving, often does not provide sufficient control of blood glucose to prevent the feared complications of the disease, which has provided the impetus for intensive research into better methods of sustaining normoglycaemia.

Among the newer treatment strategies that have been proposed, transplantation of pancreatic β islet cells, obtained either from other humans or animals, has received the most attention worldwide. This is because transplantation can restore not only the insulin-secreting unit, but also the precise fine tuning of insulin release in response to multiple neural and humoral signals arising within and beyond the islets of Langerhans.

Human islet cell transplantation is limited by the shortage of human islet tissue. The use of pig islet cells is currently viewed as the most promising alternative since:

(a) the supply of pig cells can be readily expanded by optimising the supply of donor animals;

(b) pig and human insulin have close structural similarities; and

(c) physiological glucose levels in pigs are similar to those in humans.

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The rationale for this treatment approach (termed 'xenotransplantation') is that the implanted pig islets have the potential to mimic the normal physiological insulin response in type 1 diabetics such that near-normal blood glucose levels may be achievable without insulin administration or with a reduced requirement for it. As a consequence, long-term diabetes complications may be prevented and patients should experience less hypoglycaemia than they do with the currently recommended 'intensive' insulin regimens.

OBJECT

It is an object of the present invention to provide a method of preparing porcine islets which produces islets viable for xenotransplantation into a mammalian patient the islets being capable of producing insulin within a mammalian host, as well as the islet preparation so produced, or irrespectively or how produced, or a similar form.

Alternatively or additionally, it is a further object to provide a method of treating a mammalian patient suffering from diabetes which involves the xenotransplantation of porcine islets into the mammalian patient.

Alternatively or additionally, it is a further object to at least provide the public or medical community with a useful alternative approach to diabetes treatment.

STATEMENTS OF INVENTION

In a first aspect the invention consists in **a method of preparing a xenotransplantable porcine islet preparation capable upon xenotransplantation of producing porcine insulin in an appropriate recipient mammal,** the method including or comprising the steps of:

(I) harvesting the pancreas of piglets at or near full term gestation, and

(ii) extracting pancreatic β islet cells from the harvested pancreas

wherein the islets (at least at some stage in the performance of the method) are exposed to nicotinamide.

Preferably the method includes or comprises the steps of:

(I) harvesting the pancreas of piglets at or near full term gestation, and

(ii) preparing a culture of the pancreatic β islet cells

(iii) simultaneously with step (ii) and/or after step (ii) extracting pancreatic β islet cells from the culture of the harvested pancreas

Preferably said piglets from which the pancreatic β islet cells are extracted are at from -20 to +10 days full term gestation.

Preferably said piglets are at from -7 to +10 days full term gestation.

Preferably the extraction is performed using a suitable collagenase

Preferably the collagenase is selected from human Liberase® or porcine Liberase®.

Preferably said collagenase is human Liberase®.

Preferably the culture includes harvested pancreas in a supportive mammalian albumin substantially free of non-human microbiological agents.

Preferably the mammalian albumin is human serum albumin (HSA).

Preferably the islets are treated with nicotinamide after their extraction from the pancreas.

Preferably the method includes the further step of treating the islets with IGF-1 or the N-terminal tripeptide of IGF-1 (GPE).

Preferably the exposure to IGF-1 or to GPE is greater for those cells from piglets furthest from full term gestation, more preferably there is exposure to IGF-1 for all cells extracted irrespective of their relationship to full term gestation.

Preferably the pancreas and/or islets are subject to a trauma protecting agent selected from suitable anaesthetic agents.

Preferably the trauma protecting agent is lignocaine.

Preferably step (iii) of the method includes mechanically reducing the harvested pancreas in the presence of the islet trauma protecting agent.

Preferably an antibiotic is associated with the islet cells.

Preferably said antibiotic is ciproxin.

In another aspect the invention consists in **a method of preparing a xenotransplantable porcine islet preparation capable upon xenotransplantation of producing porcine insulin in an appropriate recipient mammal,** said method including or comprising the steps of:

(I) harvesting the pancreas of piglets at or near full term gestation, and

(ii) preparing a culture of the pancreatic β islet cells

(iii) simultaneously with step (ii) and/or after step (ii) extracting pancreatic β

islet cells from the culture of the harvested pancreas

and

(iv) encapsulating the islet cells with a biocompatible xenotransplantable material, said material **in vivo** being both glucose and insulin porous,

wherein nicotinamide is introduced to the islets or islet cells prior to encapsulation at any one or more stages of the procedure.

Preferably said piglets at or near full term gestation from which the pancreatic β islet cells are extracted are at from -20 to +10 days full term gestation.

Preferably said piglets are at from -7 to +10 days full term gestation.

Preferably the extraction is performed using a suitable collagenase.

Preferably the collagenase is selected from human Liberase® or porcine Liberase®.

Preferably said collagenase is human Liberase®.

Preferably the culture includes harvested pancreas in a supportive mammalian albumin substantially free of non-human microbiological agents.

Preferably the mammalian albumin is human serum albumin (HSA).

Preferably the islets are treated with nicotinamide after their extraction from the pancreas.

Preferably the method includes the further step of treating the islets with IGF-1 or the N-terminal tripeptide of IGF-1 (GPE).

Preferably the exposure to IGF-1 or to GPE is greater for those cells from piglets furthest from full term gestation but preferably there is exposure to IGF-1 for all cells extracted irrespective of their relationship to full term gestation.

Preferably the pancreas and/or islets are subject to a trauma protecting agent selected from suitable anaesthetic agents.

Preferably the trauma protecting agent is lignocaine.

Preferably step (iii) of the method includes mechanically reducing the harvested pancreas in the presence of the islet trauma protecting agent.

Preferably an antibiotic is associated with the islet cells.

Preferably said antibiotic is ciproxin.

Preferably said biocompatible material is a suitable alginate.

Preferably the alginate is in ultra pure form.

Preferably each islet or grouping of islets is entrapped in an *in vivo* insulin and glucose porous biocompatible alginate or alginate-like surround.

Preferably the encapsulation provides a surround which prevents, once implanted, direct tissue contact with the islets.

Preferably each encapsulation involves presenting islets and a suitable alginate solution into a source of compatible cations thereby to entrap the islets in a cation - alginate gel.

Preferably said cation alginate gel is calcium-alginate gel.

Preferably said alginate used in the solution is sodium alginate, and the islet and sodium alginate solution is presented as a droplet into a bath of suitable cations.

Preferably the islet and sodium alginate solution is of 1.6% w/w.

Preferably the islet and sodium alginate solution is presented as a droplet through a droplet generating needle.

Preferably the suitable cations are calcium chloride.

Preferably the gel encased islets are coated with a positively charged material and thereafter are provided with an outer coat of a suitable alginate.

Preferably the positive charging material is poly-L-ornithine.

Preferably the gel entrapping the islets within the outer coating is then liquified.

Preferably the liquification involves or comes about by the addition of sodium citrate.

Preferably the encapsulation produces capsules.

Preferably the capsules contain a plurality of islet cells.

Preferably the capsules contain substantially three islet cells.

Preferably the capsules have a diameter of substantially from about 300 to 400 microns.

Preferably following liquification of the alginate entrapping the islets there are the further steps of:

- washing the capsules
- further coating the capsules with alginate to neutralize any residual change on the poly-L-ornithine coating and prevents direct contact of the poly-L-ornithine with tissues when the entire capsule is transplanted.

Preferably the alginate has been produced via a process involving the steps of:

Seaweed harvest→Washing→Alginate extraction→Filtration →Precipitation→Drying.

In another aspect the invention is **a xenotransplantable capsule** prepared according to the above method.

In another aspect the present invention is **a xenotransplantable preparation** being or including viable porcine islets prepared according to a method of the present invention.

In still a further aspect the present invention consist in a **xenotransplantable capsule** of at least one porcine pancreatic β islet cell comprising at least one viable porcine pancreatic β islet cell enclosed in an *in vivo* glucose porous and insulin porous biocompatible material.

In another aspect the invention consists in **a method for treatment of a mammalian patient** suffering from diabetes which comprises:

(a) extracting pancreatic β islet cells from piglets at or near full term gestation;

(b) Simultaneously with, and/or after a), treating said islets with nicotinamide,

(c) encapsulating said islets in a biocompatible material which will allow *in vivo* glucose movement to and insulin movement from the islets, and

(d) injecting or otherwise implanting the encapsulated islet cells of step (c) so as to transplant into said mammalian patient an effective amount of viable piglet islet cells capable of producing insulin in the patient,

Preferably the method further includes the step of administering nicotinamide to the mammalian patient at least subsequent to transplantation.

Preferably the method further includes the step of prescribing to the patient, prior to or after the implantation step, a casein-free diet (as herein described).

Preferably the method further includes the step of exposure of the pancreatic β islet cells at some stage after extraction from the piglets and prior to encapsulation to IGF-1 or to GPE.

Preferably the harvesting of the islets at least during any substantial confrontation (eg; mincing and/or enzymatic challenge) is in the present of a trauma protecting agent.

Preferably the trauma protecting agent is used during the isolation and/or preparation thereof for encapsulation.

Preferably the agent is a trauma protecting agent is selected from suitable anaesthetic agents.

Preferably the trauma protecting agent is lignocaine.

Preferably the patient prior to, during or after the step (d) has been subjected to a cholesterol lowering drug regime.

Preferably the drug is of the "statin" family.

Preferably the drug is pravastatin.

Preferably the yield of viable porcine islets obtained from the extraction of step a) is enhanced by the use of a suitable collagenase.

Preferably the collagenase is selected from human Liberase® or porcine Liberase®.

Preferably said collagenase is human Liberase®.

Preferably the extraction of step a) includes mechanical treatment of the islets.

Preferably the mechanical treatment follows application of a suitable anaesthetic to the pancreatic tissue.

Preferably the anaesthetic is lignocaine.

Preferably said piglets from which the pancreatic β islet cells are extracted are at from -20 to +10 days full term gestation.

Preferably said piglets are at from -7 to +10 days full term gestation.

Preferably said biocompatible material is a suitable alginate.

Preferably the alginate is in ultra pure form.

Preferably each islet or grouping of islets is entrapped in an *in vivo* insulin and glucose porous biocompatible alginate or alginate-like surround.

Preferably the encapsulation provides a surround which prevents, once implanted, direct tissue contact with the islets.

Preferably each encapsulation involves presenting islets and a suitable alginate solution into a source of compatible cations thereby to entrap the islets in a cation - alginate gel.

Preferably said cation alginate gel is calcium-alginate gel.

Preferably said alginate used in the solution is sodium alginate, and the islet and sodium alginate solution is presented as a droplet into a bath of suitable cations.

Preferably the islet and sodium alginate solution is of 1.6% w/w.

Preferably the islet and sodium alginate solution is presented as a droplet through a droplet generating needle.

Preferably the suitable cations are calcium chloride.

Preferably the gel encased islets are coated with a positively charged material and thereafter are provided with an outer coat of a suitable alginate.

Preferably the positive charging material is poly-L-ornithine.

Preferably the gel entrapping the islets within the outer coating is then liquified.

Preferably the liquification involves or comes about by the addition of sodium citrate.

Preferably the encapsulation produces capsules.

Preferably the capsules contain a plurality of islet cells.

Preferably the capsules contain substantially three islet cells.

Preferably the capsules have a diameter of substantially from about 300 to 400 microns.

Preferably following liquification of the alginate entrapping the islets there are the further steps of:

- washing the capsules
- further coating the capsules with alginate to neutralize any residual change on the poly-L-ornithine coating and prevents direct contact of the poly-L-ornithine with tissues when the entire capsule is transplanted.

Preferably the alginate has been produced via a process involving the steps of:

Seaweed harvest→Washing→Alginate extraction→Filtration →Precipitation→Drying.

In yet another aspect the invention is **a method** for the treatment of a mammalian patient suffering from or predisposed to diabetes, said method including or comprising the steps of:

(A) (i) harvesting the pancreas of piglets at or near full term gestation,

(ii) culturing the harvested pancreas in Mammalian Albumin substantially free of non-human microbiological agents,

(iii) simultaneously with step (ii) and/or after step (ii), extracting the islets from the harvested pancreas using a suitable Liberase®,

wherein the islets (at least at some stage in the performance of (A)) are exposed to nicotinamide;

(B) (i) encapsulating the islets prepared by (A) with a suitable encapsulation material that allows both glucose and insulin movement therethrough, and

(ii) implanting the encapsulated porcine islets into the recipient mammal.

Preferably the Liberase® is selected from human Liberase® or porcine Liberase®.

Preferably the Liberase is human Liberase®.

Preferably the extraction of step a) includes mechanical treatment of the islets.

Preferably the mechanical treatment follows application of a suitable anaesthetic to the pancreatic tissue.

Preferably the anaesthetic is lignocaine.

Preferably the method further includes the step of administering nicotinamide to the recipient mammal prior to or after the implantation step.

Preferably the method further includes the step of prescribing for the patient, prior to or after the implantation step, a casein-free diet (as described herein).

Preferably the method further includes the step of subjecting the patient prior to or after the implantation step to a cholesterol lower drug regime.

Preferably the cholesterol lowering drug is of the "statin" family

Preferably said cholesterol lowering drug is pravastatin or simvistatin.

In a further aspect the present invention consists in **encapsulated pancreatic islets** of a kind useful in a method aforesaid.

In still a further aspect the present invention consists in **a method of porcine β islet cell production and/or method of xenotransplantation thereof in an encapsulated form** when preformed by a procedure substantially as hereinbefore described and/or substantially as hereinafter described and/or as shown in Figure 1 of the accompanying drawings.

In a further aspect the present invention consists in **any isolated porcine islets or xenotransplantable preparations including viable porcine islets** where the digestion has been in accordance with the method in accordance with the present invention.

In yet another aspect the invention is **a method of treating a mammalian patient predisposed to or suffering from diabetes** which involves the xenotransplantation into such patient at least one capsule of the present invention.

DETAILED DISCUSSION

1. General

The present invention recognises the ability to source appropriate islets from piglets which have similar structural similarities of insulin to humans, and similar physiological glucose levels to humans. The piglets used are at or near full term gestation. The islets are converted into an appropriate xenotransplantable source of islets with viability in a human being by following certain procedures in respect of the harvesting and extraction of the islets, the treatment of the islets prior to xenotransplantation as well as regimes of use of such islets.

The major advantage of porcine islet cell transplantation over human islet cell transplantation is that the islet cell source can be readily expanded, and the biosafety of the cells can be thoroughly explored prior to transplantation. From a practical viewpoint, pancreas removal and islet cell isolation can be performed expeditiously in an ideal environment.

Important considerations relevant to the use of porcine islet cells in transplantation approaches for type 1 diabetes include the following:

- The structural and biological similarities of porcine and human insulin
- The fact that porcine insulin has been used to treat diabetes for several decades (and has only been replaced by human sequence insulin relatively recently); and
- The similarity of physiological glucose levels in pigs and humans. (Weir & Bonner-Weir 1997). This effectively means that pig islet cells can be expected to react similarly to their human counterparts in maintaining equivalent blood glucose concentrations.

2. The Nature of the Disease causing Diabetes

Successful long-term allotransplantation of human islets can be achieved in over 80% of patients when the disease is caused by non-immune processes. In contrast, even islets obtained from a non-diabetic twin cannot reverse autoimmune diabetes long-term in the diabetic twin member. This emphasises the critical role of autoimmunity in the failure of islet transplantation. This observation has been validated in allotransplantation of rodents with diabetes caused by autoimmunity as compared with diabetes due to pancreatectomy or chemical β cell destruction. No large animal model of autoimmune diabetes exists. It is possible that the use of islets from different species (xenotransplantation) could avoid autoimmune destruction of transplanted islets, as the immune process of xenotransplant rejection is different to that of allotransplant rejection, but this is entirely hypothetical in humans.

3. Isolation and Preparation of Porcine Islet Cells for Xenotransplantation

3a. Animal Source and Transportation

All animals intended as a source of pancreatic tissue for xenotransplantation are obtained

from a specific pathogen-free (SPF) pig breeding facility which is maintained in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC). The facility maintains a high-health status colony with excellent standards of husbandry, and operates a record system that is readily accessible and archived indefinitely. Donor sows and sires are selected with the underlying objective of producing strong heterosis in donor litters.

3b. Isolation and Purification of Islet Cells

Following surgical removal, the donor pancreases are transferred to a cleanroom facility for further processing in a cold plastic container in 50ml tubes containing cold Hanks' Balanced Salt Solution (HBSS) with 0.2% human serum albumin (HSA) added. Blood samples from each donor are sent for virology testing and toxoplasma serology. Samples from each organ are kept in a freezer at –80°C for future testing if necessary.

3c. Digestion

The islet cells are isolated by standard collagenase digestion of the minced pancreas via the procedure documented by Ricordi et al. (1990), though with some modifications. Using aseptic technique, the glands are distended with Liberase ® (1.5mg/ml), trimmed of excess fat, blood vessels and connective tissue, minced, and digested at 37°C in a shaking water bath for 15 minutes at 120 rpm. The digestion is achieved using lignocaine mixed with the Liberase ® solution to avoid cell damage during digestion. Following the digestion process, the cells are passed through a sterile 400mm mesh into a sterile beaker. A second digestion process is used for any undigested tissue.

We have determined that much greater yields per neonatal pig pancreas can be obtained using either pig or human Liberase™ (eg; sourced in New Zealand from Roche) rather than collagenase. Whilst there is disclosure in "*Improved Pig Islet Yield and Post-Culture Recovery Using Liberase P1 Purified Enzyme Blend*", T J Cavanagh et al. Transplantation Proceedings 30, 367 (1998) and in "*Significant Progress In Porcine Islets Mass Isolation Utilizing Liberase ® HI For Enzymatic Low-Temperature Pancreas Digestion*", H. Brandhorst et al. Transplantation Vol 68, 355-361 No. 3, August 15, 1999 the yields therefore therein are low compared to those we have discovered. If, for example, in following the procedure of Brandhorst et al. there is a yield increase of islets over

collagenase of from 400 to say 800 with the procedure using human Liberase ® (ie; Liberase ® HI) as in the Brandhorst et al. procedure but confined to neonatal porcine islets such as those as 7 days post delivery extra ordinarily larger yields are possible, namely, the equivalent to from 400 which would be the case with crude collagenase to 30000 which as can be seen as very much greater than that to be expected from following the procedure of Brandhorst et al. with pigs.

3d. Washing and Culture

The digested tissue is washed three times, and seeded into cell culture media RPMI 1640 to which is added 2% human serum albumin (HSA), 10 mmol/L nicotinamide, and antibiotic (Ciproxin).

3e. Quality Control Procedures

To exclude any contamination of the tissue, quality control procedures are undertaken on cell culture samples after isolation and before encapsulation. Three days after isolation, the cell culture is tested for microbiological contamination by accredited laboratories. Testing for porcine endogenous retrovirus (PERV) is undertaken at the Virology Laboratory, Auckland Hospital.

The *islet yield* is determined via dithizone (DTZ) staining of the cells. Dithizone is a zinc-chelating agent and a supravital stain that selectively stains zinc in the islets of Langherhans, producing a distinctive red appearance.

The *viability* of the islet cells is determined using acridin orange and propidium iodide. Acridin orange is a fluorescent stain that readily passes through all cell membranes to stain the cytoplasm and nucleus. Bright green fluorescence in both the nucleus and cytoplasm on exposure to ultraviolet (UV) light denotes intact live cells. Conversely, propidium iodide is a fluorescent stain that cannot pass through an intact membrane. It emits a bright red fluorescence when exposed to UV light, and the presence of propidium iodide in a cell nucleus indicates severe damage or a dead cell.

3f. Determination of *in vitro* Insulin Secretory Capacity

Static glucose stimulation (SGS) is used to assess *in vitro* function of the porcine islets by

exposing them to low and high concentrations of glucose and theophylline. Determination of the *in vitro* insulin secretory capacity is undertaken on both free islets (after 3 days in culture) and after their subsequent encapsulation.

4. Xenotransplantation

4a. The viability of the islets for xenotransplantation

The processes by which islets are purified prior to transplantation are traumatic to these highly specialised tissues. Such trauma can induce necrosis or apoptosis – the latter can be quite delayed.

Further trauma may result from encapsulation. Processes used by us in both the preparation of islets and their encapsulation have been optimised to ensure minimal damage to the islets. Such procedures have ensured zero warm ischaemia (compared with hours with most human islet preparations), have involved the use of nicotinamide to enhance successful *in vitro* explantation, have involved minimal incubation time with collagenase or Liberase, have involved swift non-traumatic encapsulation technology, have involved the use of IgF-1 (or the GPE tripeptide thereof), the use of an anaesthetic such as lignocaine, and the use of an antibiotic such as ciproproxin etc.

Our preferred preparation preferably uses neonatal (7-day old) islets which is crucial in both limiting islet trauma during purification, and assuring sufficient maturation of the islets for stimulated insulin production.

The IgF-1 (Human Insulin-like Growth Factor I) is used in order to induce unmatured porcine islets to mature to their insulin-producing form. IgF-1 is a potent mitogenic growth factor that mediates the growth promoting activities of growth hormone postnatally. Both IgF-1 and IgF-2 are expressed in many cell types and may have endocrine, autocrine and paracrine functions. The preferred form of IgF-1 we have found to be the amino-terminal tripeptide glycine-proline-glutamate of IgF-1 (GPE).

4b. Alginate Encapsulation Procedure

Sodium alginate used for this procedure is extracted from raw material sources (seaweed) and prepared in a powdered ultrapure form. The sterile sodium alginate solution (1.6%) is

then utilised at the Diatranz Islet Transplant Centre to manufacture encapsulated islets.

Generally each encapsulation involves presenting islets and a suitable alginate solution (usually sodium alginate) into a source of compatible cations thereby to entrap the islets in a cation - alginate gel (usually calcium-alginate gel).

The encapsulation procedure involves extruding a mixture of islets and sodium alginate solution (1.6%w/w) through a droplet generating needle into a bath of gelling cations (calcium chloride). The islets entrapped in the calcium-alginate gel are then coated with positively charged poly-L-ornithine followed by an outer coat of alginate (0.05%). The central core of alginate is then liquefied by the addition of sodium citrate. Most capsules contain 3 islets and have a diameter of 300 to 400μm.

After liquification of the alginate entrapping the islets, the "capsules" are washed, and again coated with alginate which neutralizes any residual change on the poly-L-ornithine coating and prevents direct contact of the poly-L-ornithine with tissues when the entire capsule is transplanted.

The encapsulated islets are kept in cell culture, and then checked for contamination, insulin release and viability before transplantation. They are only released for transplantation if all quality control tests are negative.

Ideally the alginate production process has involved the following steps:

Seaweed harvest→Washing→Alginate extraction→Filtration (preferably a 0.2 μm filter→Precipitation→Drying.

The ultrapure alginate used is ideally Kelco LV produced by Monsanto-Kelco, US and has the following specifications:

1. Viscosity: 2% - 100-300 cps (Brookfield 25°C, speed 3,60 rpm)
2. pH: 6.4-8.0
3. Protein content <0.5%
4. Filtration: through 0.2 μm
5. Chemical analysis:

Ca: <100 ppm	Mg <40 ppm	Mn: <10 ppm
Cu: <40 ppm	Zn: <40 ppm	Sr: <40 ppm
Fe: <60 ppm	Pb: <50 ppm	As: <100ppb
Hg: <40 ppb	Si: <10 ppm	

6. Endotoxin level - measured by LAL test (at University of Perugia): 39 EU/g
   [NB. Any level below 100 EU/g in this test is considered endotoxin-free].
7. Molecular weight: 120,000 - 190,000 kD
8. Mannuronic acid (M) content: M fraction (F_m) 61%
9. Guluronic acid (G) content: G fraction (F_G) 39%

Ideally the filtration has been with a multiple filtration process employing positively charged filters that remove any lipopolysaccharide content.

4c. Drugs used in the recipient

Transplantation does not require and avoids the need for cytotoxic agents to suppress the immune system. Such agents are able to enter the alginate microcapsule and cause islet toxicity, as well as causing systemic toxicity. Instead, nicotinamide and a special diet are used (for rationale, see section 1.4 below).

The transplantation procedures of our earlier patent specification have the ability over a period prior to rejection of providing porcine insulin. In this respect, we ourselves conducted clinical trials.

Four type 1 diabetic adolescents received 10,000 free islets/kg bodyweight by intraperitoneal injection. The islets were located from term piglets using the standard collagenase digestion, purification and culture techniques described in section 3.2. All four recipients received oral nicotinamide (1.5 g/day) and a casein-free as herein defined diet both pre- and post-transplantation. A prompt reduction in insulin requirements, which was not clearly dose-related, was noted in the first week after transplantation. The reduction in insulin dosage range from 21 to 32%, and the response lasted for up to 14 weeks. However, insulin doses subsequently returned to their previous levels.

The most likely reason for the transplant failure in these patients was chronic rejection.

However, no adverse effects were noted.

We have now shown alginate-encapsulated porcine islet cell transplants in two human diabetic patients, prolonged functioning of the transplants. The islets were transplanted by intraperitoneal injection, one patient receiving 15,000 IEQ/kg (total 1,300,000 islets) and the other 10,000 IEQ/kg (total 930,000 islets). Both patients were treated pre- and post-transplantation with oral nicotinamide and a soy-based/casein-free as herein defined diet.

The preferred procedure as shown in Figure 1 was used for the preparation, the encapsulation being as aforesaid. Islet cells of -7 days to +10 days full gestation were used.

DESCRIPTION OF THE DRAWINGS

Preferred forms of the present invention or examples of working will now be described with reference to the accompanying drawings in which:

Figure 1 shows a preferred procedure for harvesting, isolating and preparing islet cells (with either confinement or encapsulation) and the associated treatment regime for a diabetic human patient in order to receive ongoing benefit from the xenotransplantation,

Figure 2 shows the effect of collagenase from various sources on islet yield and function,

Figure 3 shows the stimulation index of Liberase® against Collagenase clearly showing that Liberase® preparations (both human and porcine at suitable concentrations) gave higher yields and function in vitro than an optimised concentration of Collagenase P,

Figure 4 shows the stimulation index of free islets when comparing the use of ciproxin against a penicillin/streptomycin mix and against a control of no antibiotics,

Figure 5 shows the results of exposure of neonatal porcine islets in culture with GPE in comparison with control cells.

5. Examples

5a. Examples of use of IgF-1

*Note: in the following , different experiments used different islet preparations so control values vary.

- porcine islets in culture which were exposed to IgF-1, increased their insulin response to glucose, by up to a 3-fold increase.

	Incubated 24hrs with 0.1ug/ml IgF-1 after isolation	**CONTROL- no IgF-1**
Insulin secretion In response to 19.4mM Glucose +10mM Theophylline After 3 days culture Post isolation	236uU/hr/100IEQ	75.2uU/hr/100IEQ

- A concentration of 0.1ug/ml IgF-1 in culture is sufficient to produce optimal insulin secretion during glucose challenge. No further benefit was achieved by increasing the concentration of IgF-1.

	Incubated 24hrs with 0.1ug/ml IgF-1	**Incubated 24hrs with 1.0 ug/ml IgF-1**
Insulin secretion In response to 19.4mM Glucose +10mM Theophylline After 3 days culture Post isolation	58uU/hr/100IEQ	56.8uU/hr/100IEQ

- Variations on the duration of IgF-1 exposure were tried on the porcine islet cells. However no increased benefit was found on culturing the islets with IgF-1 beyond a 24hrs period, post isolation.

	Incubated 7 days With 0.1ug/ml IgF-1	Incubated 24hrs with 1.0 ug/ml IgF-1
Insulin secretion In response to 19.4mM Glucose +10mM Theophylline 7days post isolation	58uU/hr/100IEQ	57.5uU/hr/100IEQ

- This increased insulin production persisted to 14 days post IgF-1 exposure. Longer durations are yet to be investigated.

	14 days post IGF-1 Exposure	3 days post IGF-1 Exposure
Insulin secretion In response to 19.4mM Glucose + 10mM Theophylline	1.3-fold increase Compared to control	1.5-fold increase Compared to control

- Withdrawal of Nicotinamide from the culture media eliminated the benefit of IGF-1 on islet insulin production.

	Incubated 3 days With 0.1ug/ml IGF-1 Without Nicotinamide	Incubated 3 days With culture Media Without Nicotinamide
Insulin secretion In response to 19.4mM Glucose +10mM Theophylline After 3 days culture Post isolation	47.6uU/hr/100IEQ	55.9uU/hr/100IEQ

- A concentration of 0.1ug/ml IGF-2 during culturing appeared to increase insulin production of porcine islet cells, after an initial exposure of 24 hrs. However, this increase was transient to 3 days post exposure.

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	Control
Insulin secretion In response to 19.4mM Glucose +10mM TheophylineAfter 3 days culture Post isolation	105.8/100IEQ	75.2r/100IEQ

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	**Control**
Insulin secretion In response to 19.4mM Glucose +10mM TheophylineAfter 3 days culture Post isolation	32uU/hr/100IEQ	39.8 uU/hr/100IEQ

- Prolonged exposure to IGF-2 beyond 24hrs, failed to increase the insulin production of the islet cells in response to glucose.

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	**Control**
Insulin secretion In response to 19.4mM Glucose +10mM TheophylineAfter 3 days culture Post isolation	105.8/100IEQ	75.2r/100IEQ

	Incubated 7 days With 0.1ug/ml IGF-2	**Control**
Insulin secretion In response to 19.4mM Glucose +10mM TheophylineAfter 7 days culture Post isolation	38.4uU/hr/100IEQ	39.8uU/hr/100IEQ

5b. Effect of N-Terminal Tripeptide (GPE) of Insulin like growth factor (IGF-1) on the function of neonatal porcine islet cells.

GPE is a tripeptide (gly-pro-glu) derived from IGF-1. It is a novel neuroactive peptide with a potent effect on acetylcholine and dopamine release in cortical slices. Previous studies done using GPE support the concept that the proteolytic products of the IGF-1 precursor play a role in the regulation of brain functions.

The aim of this example was to present the effect of GPE on the function of isolated porcine islets in vitro.

Method

- Islet cell isolation with 2 pancreases;

- Isolation following the previously discussed protocol;
- RPMI media added with Ciproxin, nicotinamide, Human serum albumin
- GPE, IGF1 (1-3), Bachem AG, Lot No.0538925, stock solution of 100 ug/ml (in water): dilute further in RPMI medium to the final concentrations: 1ug/ml (1:100), 0.1 ug/ml (1: 1000) and 0.01 ug/ml (1: 10 000)

1. GPE 0.01ug/ml
2. GPE 0.1ug/ml
3. GPE 1.0ug/ml

Keep the cells 3 days in culture before Static Glucose Stimulation (SGS). SGS involves exposure of the cells to low and high concentration of glucose to check insulin production. Using 0.1 ug/ml concentration add GPE to two plates 24 hours before SGS (day 2 after isolation)

Results of Example 5b

Exposure of neonatal porcine islets in culture to GPE increased the insulin response to glucose up to 11.5 compared with the control cells.(Stimulation Index control 13.3 compared to 24.8 when GPE was used) Viability of the cells was >85% DTZ, AO/PI staining)

A concentration of 0.01ug/ml of GPE in culture is sufficient to produce optimal response during glucose challenge. No further benefit was achieved by increasing the concentration of GPE in culture. See Figure 5 below.

The results suggest that GPE could be used during porcine islet cell culture to improve the quality and function of the cells before transplantation. Furthermore GPE is a novel neuroactive peptide found in human brain.

5c. Examples of the effect of lignocaine when used during porcine pancreatic digestion, on islet yield and viability.

Lignocaine is a membrane stabiliser and phospholipase A2 inhibitor. When used at a 1mM

concentration during Collagenase digestion of 7d old porcine pancreas, a 2-fold increase in islet yield is produced.

Islet endocrine function was assessed after 3 days in culture via static glucose stimulation. Islets isolated with Lignocaine during digestion produced a 3-fold increase in insulin secretion in response to glucose challenge.

	Collagenase alone	**Collagenase + 1mM Lignocaine**
Average islet yield	40,960 IEQ/g	88,183 IEQ/g

	Collagenase alone	**Collagenase + 1mM Lignocaine**
Insulin secretion in response to 19.4mM Glucose +10mM Theophyline After 3 days culture Post isolation	46.4 uU/hr/100IEQ	163.8 uU/hr/100IEQ

Conclusion: The use of Lignocaine during pancreatic digestion increases the insulin production/g of pancreas by 6-fold.

5d. Examples of the effects of Ciproxin on Islet function as assessed by static glucose stimulation.

Freshly prepared neonatal pig islets were prepared by standard isolation procedure and cultured for two days in RPMI medium with standard additions.

Streptomycin (100mcg/ml)and Penicillin (100U/ml) were included in one flask and Ciproxin (3 mcg/ml) in another.

The islets were harvested and an aliquot subjected to stimulation with theophylline and high glucose.

The comparative insulin release from the islets---a measure of viability is shown in Figure 4.

5e. Examples of the effects of collagenase from various sources on islet yield and function

Pancreases of neonatal piglets aged 7 days were obtained as above and islets extracted by the same process, varying only the source and amount of collagenase. The yield/gram of pancreas is shown in the Figure.

Islets extracted using these variations in collagenase source and amount were assessed for viability using propidium iodide and dithizone for insulin content.

DTZ staining >85%

AO/PI >85%

The islets were then assessed for functionality by static glucose stimulation as above. The results are shown in the Figure below.

It is apparent that the Liberase® preparations at suitable concentrations gave higher yields and function in vitro than the previously optimised concentration of Collagenase P.

5f. Examples of the comparative effectiveness of islets prepared with Liberase P or H in vivo

Islets prepared with the best concentration of Liberase® P and H in this way were injected intraperitoneally into CD1 mice made diabetic by intravenous streptozotocin. The dose used was 10 islets/g body weight of mouse. Ten days after such treatment the number of mice no longer diabetic was assessed.

1/7 of the mice treated with the islets isolated with Liberase® P and 4/7 of those isolated with Liberase H were non diabetic.

Similar experiments were performed using spontaneously diabetic NOD mice. Of the surviving mice at 10 days after transplantation 3/7 of the Liberase P treated islets and 3/3 of the Liberase H islets were no longer diabetic

5g. Example of Islet Encapsulation Procedure

The novel medium size microcapsules (300-400μ MSM) are prepared by atomizing the islet-alginate suspension through a special microdroplet generator.

Sodium alginate used for this procedure is extracted form raw material sources (seaweed) and prepared in powdered ultrapure form (Keltone LVCR).

The encapsulation procedure involves extruding a mixture of islets and sodium alginate solution (1.6%) through a droplet generating needle into a bath of gelling cations (calcium chloride). The islets entrapped in the calcium-alginate gel are then coated with positively charged poly-L-ornithine followed by an outer coast of alginate (0.05%). The central core of alginate is then liquified by the addition of sodium citrate. Most capsules contain 3 islets and have a diameter of 300 to 400μ m.

The encapsulated islets are kept in cell culture, and then checked for contamination, insulin release and viability before transplantation.

DEFINITIONS

As used herein:

- "*Administering*" includes self-administering;
- "*Casein-free*" when referring to milk as used herein refers to milk which does not contain a diabetogenic factor, particularly to milk containing no variant of β-casein which stimulates diabetogenic activity in humans. With reference to International PCT Application WO 96/14577, a non-diabetogenic variant for example, may be the A2 variant of β-casein. The full contents of PCT/NZ95/00114 (WO 96/14577) and PCT/NZ96/00039 (WO 96/36239) are here included by way of reference.
- "*Casein-free*" as used herein in respect of dietary considerations means at least a substantial avoidance (preferably total avoidance) of such milk containing or derived diabetogenic factors.
- *IGF-1* is Human Insulin-like Growth Factor I and is a potent mitogenic growth factor that mediates the growth promoting activities of growth hormone postnatally. Both IGF-1 and IGF-2 are expressed in many cell types and may have endocrine, autocrine and paracrine functions.
- The *N-terminal tripeptide of IGF-1* or "*GPE*" is the amino-terminal tripeptide glycine-proline-glutamate of IGF-1.
- "*mammalian albumin*" as used herein means serum albumin from mammals,

preferably human serum albumin (HSA).

- "*appropriate collagenase*" means preferably Liberase ®, ideally human or porcine, ideally Liberase H ®.
- "*mechanically reduced*" as used herein includes any process where pancreatic tissue is increased in surface area eg, mechanical or water jet shredding, grinding, mincing, etc...

CLAIMS:

1. **A method of preparing a xenotransplantable porcine islet preparation capable upon xenotransplantation of producing porcine insulin in an appropriate recipient mammal**, the method including or comprising the steps of:

   (i) harvesting the pancreas of piglets at or near full term gestation, and

   (ii) preparing a culture of the pancreatic β islet cells

   (iii) simultaneously with step (ii) and/or after step (ii) extracting pancreatic β islet cells from the culture of the harvested pancreas

   wherein, and the islets (at least at some stage in the performance of the method) are exposed to nicotinamide, and wherein the piglets are at from -7 to +10 days full term gestation, and wherein the extraction is performed using a suitable collagenase selected from purified human collagenase or purified porcine collagenase, and wherein the culture includes harvested pancreas in a supportive mammalian albumin substantially free of non-human microbiological agents.

2. A method as claimed in claim 1 wherein the collagenase is purified human collagenase.

3. A method as claimed in claim 2 wherein the mammalian albumin is human serum albumin (HSA).

4. A method as claimed in claim 3 wherein the islets are treated with nicotinamide after their extraction from the pancreas.

5. A method as claimed in any one of the preceding claims wherein the method includes the further step of treating the islets with IgF-1 or the N-terminal tripeptide of IgF-1 (GPE).

6. A method as claimed in claim 5 wherein the exposure to IgF_1 or to GPE is greater for those cells from piglets furthest from full term gestation.

7. A method as claimed in claim 5 or 6 wherein there is exposure to IgF_1 for all cells extracted irrespective of their relationship to full term gestation.

8. A method as claimed in any one of the preceding claims wherein the pancreas and/or

islets are subject to a trauma protecting agent selected from suitable anaesthetic agents.

9. A method as claimed in claim 8 wherein the trauma protecting agent is lignocaine.

10. A method as claimed in any one of the preceding claims wherein step (iii) of the method includes mechanically reducing the harvested pancreas in the presence of the islet trauma protecting agent.

11. A method as claimed in any one of the preceding claims wherein an antibiotic is associated with the islet cells.

12. A method as claimed in claim 11 wherein said antibiotic is ciprofloxacin.

13. A **method of preparing a xenotransplantable porcine islet preparation capable upon xenotransplantation of producing porcine insulin in an appropriate recipient mammal,** said method including or comprising the steps of:

(i) harvesting the pancreas of piglets at or near full term gestation, and

(ii) preparing a culture of the pancreatic β islet cells

(iii) simultaneously with step (ii) and/or after step (ii) extracting pancreatic β islet cells from the culture of the harvested pancreas

and

(iv) encapsulating the islet cells with a biocompatible xenotransplantable material, said material **in vivo** being both glucose and insulin porous,

wherein nicotinamide is introduced to the islets or islet cells prior to encapsulation at any one or more stages of the procedure, and wherein the piglets at or near full term gestation from which the pancreatic β islet cells are extracted are at from -20 to +10 days full term gestation.

14. A method as claimed in claim 14 wherein the piglets are at from -7 to +10 days full term gestation.

15. A method as claimed in any one of claims 13 to 14 wherein the extraction is performed using a suitable collagenase.

16. A method as claimed in claim 15 wherein the collagenase is selected from human purified human collagenase or purified porcine collagenase.

17. A method as claimed in claim 16 wherein the collagenase is purified human collagenase.

18. A method as claimed in any one of claims 13 to 17 wherein the culture includes harvested pancreas in a supportive mammalian albumin substantially free of non-human microbiological agents.

19. A method as claimed in claim 18 wherein the mammalian albumin is human serum albumin (HSA).

20. A method as claimed in any one of claims 13 to19 wherein the islets are treated with nicotinamide after their extraction from the pancreas.

21. A method as claimed in any one of claims 13 to 20 wherein the method includes the further step of treating the islets with IgF-1 or the N-terminal tripeptide of IgF-1 (GPE).

22. A method as claimed in claim 21 wherein the exposure to IgF_1 or to GPE is greater for those cells from piglets furthest from full term gestation.

23. A method as claimed in claim 21 or 22 wherein there is exposure to IgF_1 for all cells extracted irrespective of their relationship to full term gestation.

24. A method as claimed in any one of claims 13 to 23 wherein the pancreas and/or islets are subject to a trauma protecting agent selected from suitable anaesthetic agents.

25. A method as claimed in claim 24 wherein the trauma protecting agent is lignocaine.

26. A method as claimed in any one of claims 13 to 25 wherein step (iii) of the method includes mechanically reducing the harvested pancreas in the presence of the islet trauma protecting agent.

27. A method as claimed in any one of claims 13 to 26 wherein an antibiotic is associated with the islet cells.

28. A method as claimed in claim 27 wherein the antibiotic is ciprofloxacin.

29. A method as claimed in any one of claims 13 to 28 wherein the biocompatible material is a suitable alginate.

30. A method as claimed in claim 29 wherein the alginate is in ultra pure form.

31. A method as claimed in any one of claims 13 to 30 wherein each islet or grouping of islets is entrapped in an *in vivo* insulin and glucose porous biocompatible alginate or alginate-like surround.

32. A method as claimed in claim 31 wherein the encapsulation provides a surround which prevents, once implanted, direct tissue contact with the islets.

33. A method as claimed in claim 32 wherein each encapsulation involves presenting islets and a suitable alginate solution into a source of compatible cations thereby to entrap the islets in a cation - alginate gel.

34. A method as claimed in claim 33 wherein the cation alginate gel is calcium-alginate gel.

35. A method as claimed in claim 34 wherein the alginate used in the solution is sodium alginate, and the islet and sodium alginate solution is presented as a droplet into a bath of suitable cations.

36. A method as claimed in claim 35 wherein the islet and sodium alginate solution is of 1.6% w/w.

37. A method as claimed in claim 36 wherein the suitable cations are calcium chloride.

38. A method as claimed in claim 37 wherein the gel encased islets are coated with a positively charged material and thereafter are provided with an outer coat of a suitable alginate.

39. A method as claimed in claim 38 wherein the positive charging material is poly-L-ornithine.

40. A method as claimed in claim 39 wherein the gel entrapping the islets within the outer coating is then liquified.

41. A method as claimed in claim 40 wherein the liquification involves or comes about by the addition of sodium citrate.

42. A method as claimed in any one of claims 13 to 41 wherein the encapsulation produces capsules.

43. A method as claimed in claim 42 wherein the capsules contain a plurality of islet cells.

44. A method as claimed in claim 43 wherein the capsules contain substantially three islet cells.

45. A method as claimed in claim 43 or 44 wherein the capsules have a diameter of substantially from about 300 to 400 microns.

46. A method as claimed in claim 45 wherein following liquification of the alginate entrapping the islets there are the further steps of:

- washing the capsules
- further coating the capsules with alginate

47. **A xenotransplantable capsule** prepared according to the method as claimed in any one of claims 1 to 46.

48. **A xenotransplantable preparation** being or including viable porcine islets prepared according to a method as claimed in any one of claims 1 to 46.

49. **A method of preparing a xenotransplantable porcine islet preparation capable upon xenotransplantation of producing porcine insulin in an appropriate recipient mammal** substantially as herein described with reference to one or more of the Figures and/or Examples.