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Encapsulated and 'free' pig islet xenotransplantation: recent experience and clinical progress*

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Abstract

Genetically-engineered pigs offer a possible alternative to deceased human donors as a source of isolated islets for transplantation into patients with life-threatening diabetes. We here consider the advantages and disadvantages of 'free' pig islet transplantation into immunosuppressed recipients vs. 'immunoisolated' pig islet transplantation into non-immunosuppressed recipients. Although hurdles to successful free pig islet transplantation remain, e.g., the instant blood-mediated reaction (IBMIR) and the immune response, we are optimistic that, as new genetically-engineered pigs become available, the remaining barriers may be overcome. In contrast, we have several concerns with regard to the ultimate success of immunoisolation. Without exogenous immunosuppressive therapy, we suggest that immune injury will occur. If immunosuppressive therapy is required, the primary advantage of encapsulation is lost. A key point is that the lack of adequate nutrition and oxygen to the encapsulated islets has not yet been overcome. Furthermore, an optimal site for the placement of the islets has also not been determined. We also very briefly review several other points of importance to islet xenotransplantation, namely (i) Human leukocyte antigens/Swine leucocyte antigens sensitization, (ii) physiological aspects of pig islet xenotransplantation, (iii) the safety of islet xenotransplantation, and (iv) what will be required to initiate a clinical trial.

Abbreviations

HLA = human leukocyte antigens, IBMIR = instant blood-mediated inflammatory reaction, NHP = nonhuman primate, PERV = porcine endogenous retrovirus, SLA = swine leukocyte antigens, WT = wild-type.

Introduction

The incidence of diabetes is increasing worldwide. In the USA alone, there are an estimated 1-2 million people with Type 1 diabetes and perhaps 30 million with Type 2 diabetes. Islet allotransplantation might be curative but, with less than 2,000 pancreatic organs from deceased human donors becoming available each year, there will never be sufficient human islets to resolve this problem.

Genetically-engineered pigs offer a possible alternative as a source of isolated islets for transplantation into patients with life-threatening diabetes[1]. During the past several years, there have been very considerable advances in xenotransplantation research, with non-human primates (NHPs) with heterotopic (non-life supporting) pig heart transplants surviving for more than 2 years, and life-supporting pig kidney transplants for more than a year (Figure 1)[2-8]. After pig islet transplantation, NHPs have remained normoglycemic for periods longer than two years.

One of the major reasons why both islet allotransplantation and islet xenotransplantation have not expanded as quickly as might be thought possible has been because the patient requires lifelong immuno-

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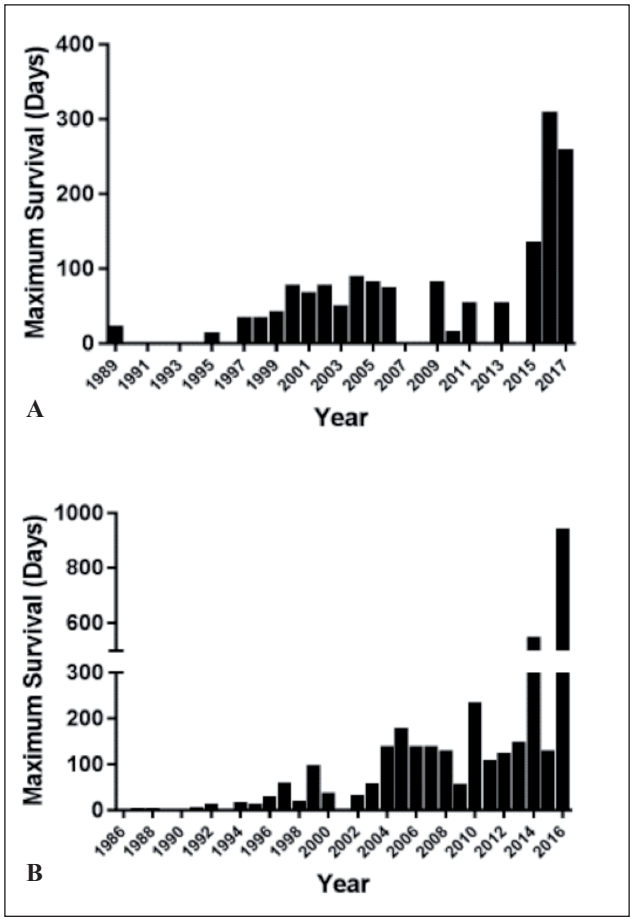

Figure 1. Survival after pig organ transplantation in nonhuman primates, 1986-2017. *A*, After life-supporting pig kidney xenotransplantation, maximum survival has improved from 23 days in 1989 to >1 year. *B*, After heterotopic (non-life-supporting) pig heart xenotransplantation in nonhuman primates, maximum survival has improved from <8 hours in 1986 to 945 days. (Reproduced with permission from Wang et al[8]).

suppressive therapy, which is associated with significant complications, such as infection and malignant change. Because of this, many physicians believe that one debilitating illness, namely diabetes, is being exchanged for another, namely immunodeficiency. In an effort to resolve this problem, experiments have been performed over a number of years in which attempts have been made to physically "isolate" the islets from the human immune response, e.g., by encapsulation, thus abrogating the need for exogenous immunosuppressive therapy.

We here consider the advantages and disadvantages of each approach, i.e., 'free' pig islet transplantation into immunosuppressed recipients *vs.* 'immunoisolated' pig islet transplantation into non-immunosuppressed recipients.

'Free' pig islet transplantation in diabetic non-human primates

The preparation of pig islets is technically more difficult than that of human islets, but the technique has now been refined over a number of years, and a large number of pig islets can be successfully obtained from either adult or neonatal pigs (Figure 2). Although transplanted adult islets begin producing insulin immediately (whereas neonatal islets may not be mature enough to do so), there are several logistic and other advantages of using neonatal pigs as sources of islets that have been discussed elsewhere[9,10]. In order to prevent an inflammatory response, both free and encapsulated pig islets have been cotransplanted with regulatory cells, such as mesenchymal stromal cells or Sertoli cells, but without complete success. Most investigations to date have transplanted the islets into the recipient portal vein, using methods identical to those used in human islet allotransplantation. Their injection into the blood, however, is associated with a major complication known as the instant blood-mediated inflammatory reaction (IBMIR)[11,12]. This response also occurs after islet allotransplantation into the portal vein, but there are *in vitro* data indicating that the loss of islets is much greater when the islets are from pigs (Figure 3)[13,14]. Perhaps as many as 75% of islets are lost within the first few hours, if not minutes. If the number of islets surviving is

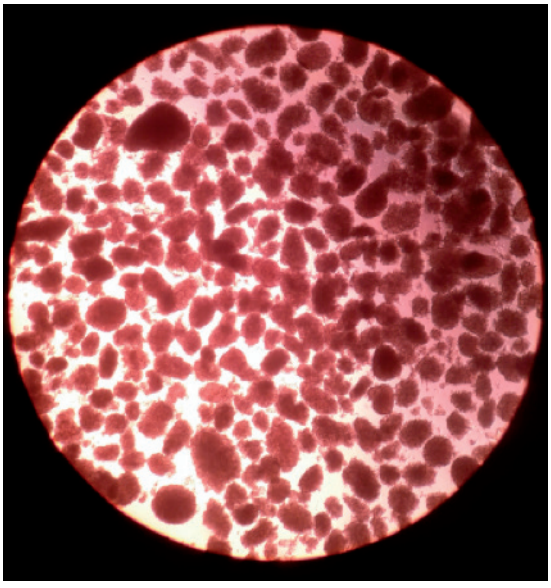

Figure 2. Adult pig islets after isolation. Adult pig islets stained in red with dithizone after isolation and purification (Magnification 40x).

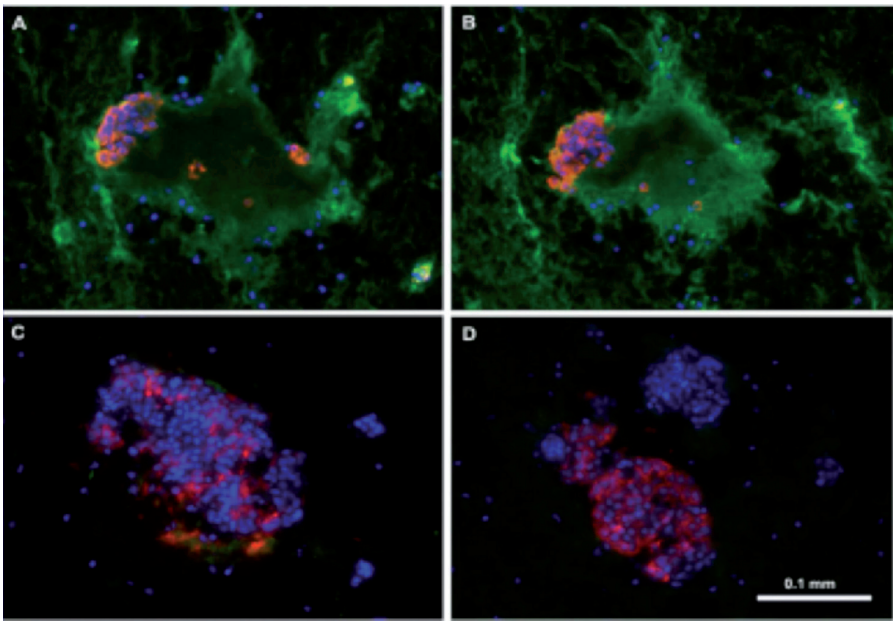

Figure 3. Binding of human IgM and IgG antibody to pig islets (xenogeneic) *(A-B)* and to human islets (allogeneic) *(C-D)*. IgM (green, *A, C*), IgG (green, *B, D*), insulin (red), nucleus (DAPI/blue). Yellow indicates colocalization of insulin and IgM/IgG. The greatly increased binding of human IgM and IgG to pig islets (compared to human islets) is obvious. (Reproduced with permission from van der Windt et al[13]).

sufficient, then long-term normoglycemia can be obtained in NHPs receiving immunosuppressive therapy, particularly if that is with agents that block the CD154-CD40 T cell costimulation pathway.

Many efforts have been made to avoid or reduce IBMIR. One approach has been to genetically engineer the pigs so that the islets are to some extent protected against the inflammatory response that occurs immediately when they are introduced into the blood. Genetic engineering of the pigs has involved two major approaches[15-17]. (i) Deletion of expression of one or more of the three known carbohydrate antigens expressed in pigs against which humans have natural, pre-formed anti-pig antibodies. These three antigens are galactose-α1,3-galactose (Gal), N-glycolylneuraminic acid (Neu5Gc), and Sda (a product of the enzyme, β-1,4N-acetylgalactosaminyltransferase [β4GalNT2]). (ii) Transgenic expression of one or more human complement-regulatory proteins (e.g., CD46, CD55, CD59) and human coagulation-regulatory proteins (e.g., thrombomodulin, endothelial protein C receptor [EPCR], tissue factor pathway inhibitor [TFPI], CD39). Combinations of these genetic manipulations in the pig are associated with reduced early graft loss from IBMIR, although there remains a significant loss[18-20]**.** This may be reduced further when triple knock-out (TKO) pigs expressing human complement- and coagulation-regulatory proteins become readily available[21].

Although methods have been developed to genetically-engineer the pig to reduce or control the adaptive immune (T cell) response[22-25], in all pig-to-NHP studies to date, exogenous immunosuppressive therapy has been administered, usually in the form of agents that block costimulation, e.g., anti-CD154 monoclonal antibodies or anti-CD40 monoclonal antibodies[26,27]. The combination of genetically-engineered pig islets and costimulation blockade has resulted in significant success with pig islet xenotransplantation, resulting in normoglycemia for periods of more than a year in streptozotocin-induced diabetic NHPs (Figure 4)[18,19].

When a potent costimulation blockade agent is administered, even islets from wild-type (i.e., genetically-*un*modified) pigs have functioned adequately for periods in excess of two years [Figure 5][28,29]. There is significant evidence that, the greater the extent of genetic manipulation, the less immunosuppressive therapy will be required. Nevertheless, unless very intensive immunosuppressive therapy is delivered, these excellent results cannot be obtained consistently.

Pigs are now available in which all three known carbohydrate xenoantigens have been deleted (TKO pigs), and in which six human transgenes have been introduced to provide protection against human complement and coagulation activation, and against the human inflammatory response (by the introduction of human hemeoxygenase-1 [HO-1])[21]. In addition, the expression of CD47 is known to reduce macrophage activation and may have some suppressive effect on the T cell response. Although

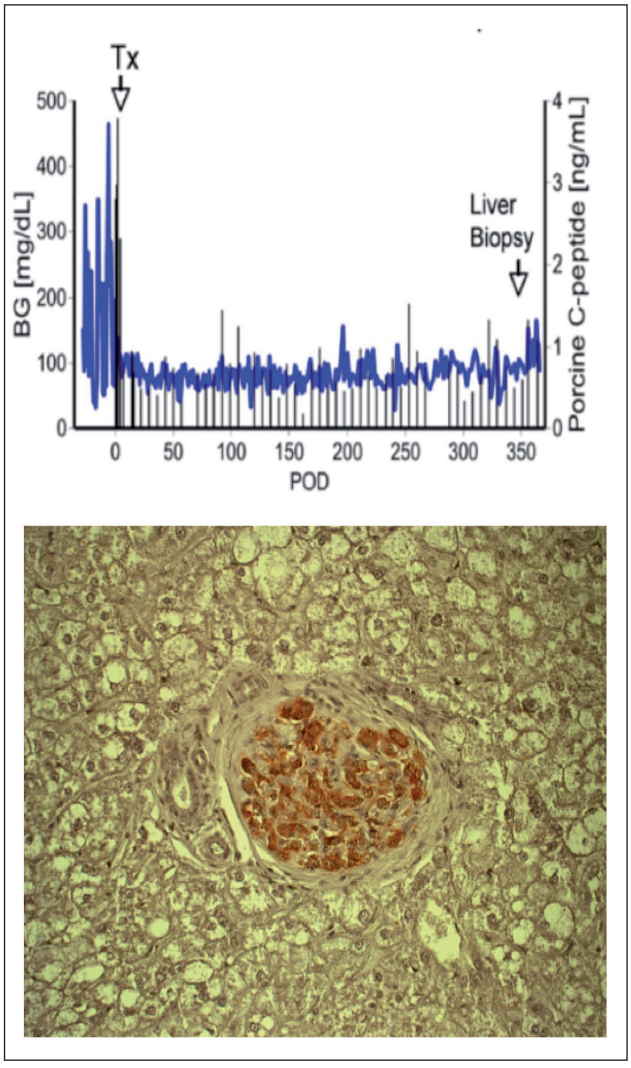

Figure 4. *A*, Blood glucose and pig C-peptide levels in a streptozotocin-induced diabetic cynomolgus monkey before and after intraportal transplantation of islets from a pig expressing the human complement-regulatory protein, CD46. No exogenous insulin was administered after the transplant. The normoglycemic monkey was electively euthanized after 12 months. Day 0 = day of islet transplantation. *B*, Insulin immunostaining (in red) of a liver section in a monkey recipient of islets from a pig transgenic for human CD46, showing a healthy pig islet 12 months after transplantation. (Magnification x200). (Reproduced with permission from van der Windt et al[18]).

not yet tested in NHP models, when these pigs are used as sources of islets, it is likely that both IBMIR and the T cell response will be reduced. However, alternative approaches are being explored.

It is becoming increasingly clear that introducing the pig islets into the blood (e.g., the portal vein) is less than optimal, and so several other sites have been explored[30]. If a novel yet clinically feasible site for the islets can be identified in which IBMIR is no longer problematic, then the number of islets that would require transplantation could be significantly reduced. In our own experience, the gastric submucosal space is one such site that warrants further exploration (Figure 6)[31-33].

The fact that islets can be transplanted successfully under the kidney capsule in rodents and in pigs asks the question as to why this site has not been explored further in primates, including humans. There is evidence that if autologous islets are placed under the kidney capsule of a pig, and even baboon, then, in the absence of any immune or inflammatory response, they are revascularized rapidly, enabling the composite islet kidney graft to be transplanted into a recipient successfully[34,35].

Immunoisolation of pig islets

The concept of protecting the islets by isolating them within a device or through encapsulation is not new, and efforts to develop these devices or capsules have been undertaken for more than 50 years, but have not yet been proven entirely successful. The islets can be placed within an intravascular device or in the form of macroencapsulation or microencapsulation[10,36-39]. Microencapsulation is perhaps the approach that has been investigated most intensively. Whatever the approach, there are significant considerations[37].

1) The encapsulation material must be fully biocompatible so that it does not induce an inflammatory response itself.
2) Protection of the islets from the recipient's immune cells, antibodies, and cytokines/chemokines need to be complete, and yet the capsules must allow insulin to be released from the islets into the surrounding tissues. This has proved a major biomechanical engineering problem. If the islets undergo loss of viability, then antigen can leak through the pores in the capsule, and thus sensitize the recipient to pig antigens. Theoretically, if the immunoisolation technique were fully successful, there would be no need to provide exogenous immunosuppressive therapy. As to date this has not proved to be the case, thought has been given to providing a low level of immunosuppressive therapy to patients with encapsulated islets. However, if immunosuppression is necessary, then the advantages of immunoisolation over free islet transplantation are greatly minimized. Consideration has also

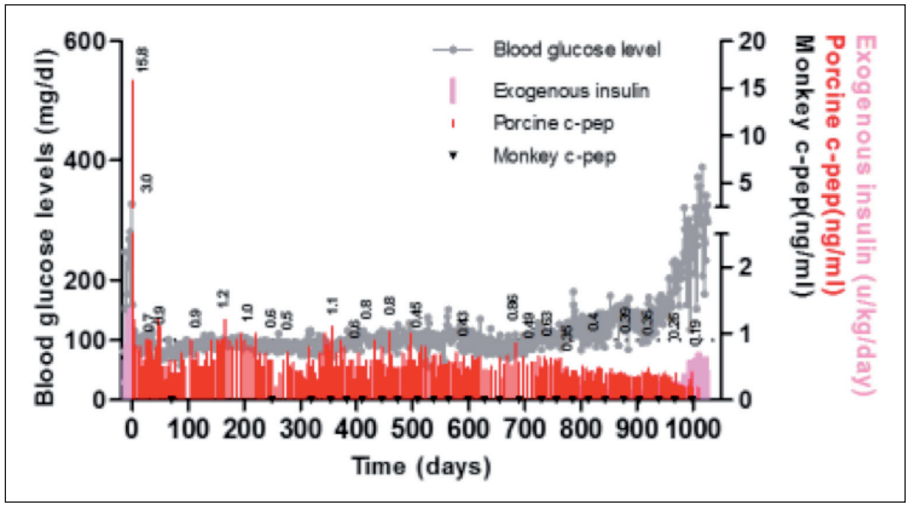

Figure 5. Blood glucose, porcine and monkey C-peptide levels, and exogenous insulin requirements in a monkey following adult wild-type pig islet transplantation (with graft function for >2 years). (Courtesy Dr. Chung-Gyu Park, Seoul National University.)

been given to employ islets from genetically-engineered pigs within the capsules to protect against the immune response (which again should not be needed if the capsules completely protected the islets from the immune response).

3) Perhaps the major problem has been providing sufficient nutrition and oxygen to the islets within the capsules. The provision of nutrition and oxygenation is complicated by a number of related factors, including the rate of diffusion, the distance that has to be traveled by the nutrients and oxygen to reach the islets, and the size of the capsules. Even if these are optimized, there is always an altered insulin response from encapsulated islets than from free islets, thus reducing their efficiency in maintaining normoglycemia. Thoughts have been given to revascularize the islets within the capsules, but if this is achieved, it negates the benefit of the immunoisolation as it enables immune cells, antibodies, complement, etc., to reach the islets and damage them.

There have been a number of small clinical trials of immunoisolated islets using encapsulated intraperitoneal adult wild-type pig islets with no immunosuppressive therapy[40-42]. Two of the more important and well-regulated trials have been carried out

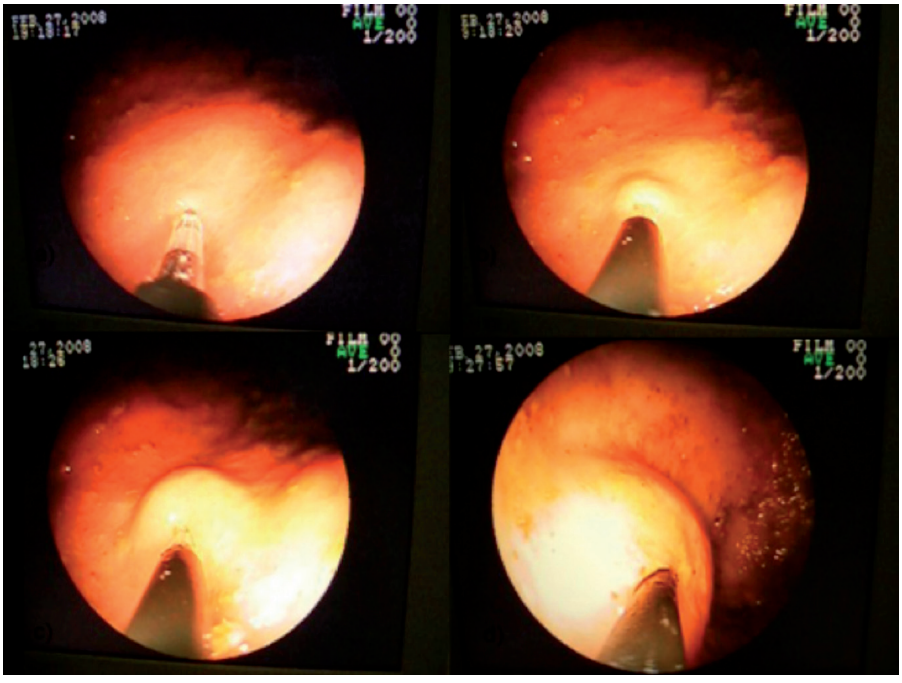

Figure 6. Sequence of adult pig islets being injected into the gastric submucosal space by endoscopy. (Reproduced with permission from Echeverri et al[31]).

in New Zealand and Argentina under national or local regulatory authority supervision. Although neither trial has been fully reported, it is believed that graft survival was not perfect, although there may have been some reduction in hemoglobin A1c levels. Nevertheless, there were no significant infectious or other complications from these trials, indicating that the concept is a safe one.

A recent study throws considerable light onto the factors limiting the success of microencapsulation. Saffley et al[43] carried out the transplantation of microencapsulated adult islets into the peritoneal cavity of diabetic NHPs that received immunosuppressive therapy in the form of costimulation blockade. Glycemia was controlled for 20-70 days (with pig C-peptide being measured for between 7-125 days). There was no clinical or histopathological evidence of rejection. These data strongly suggested that failure of the grafts was not immune-related, but was associated with a lack of nutrition and/or oxygenation of the islets during the first two to three months' post-transplantation. If immunosuppressive therapy had not been administered, however, it would have been interesting to see whether the islets failed at an earlier time-point from immune injury.

The case for 'free' pig islet xenotransplantation

In summary, therefore, there are several current concerns with regard to immunoisolation. Without exogenous immunosuppressive therapy, we suggest that immune injury will occur. If immunosuppressive therapy is required, there is no advantage over free islet transplantation. A key point is that the lack of adequate nutrition and oxygen to the islets has not yet been overcome. Furthermore, an optimal site for the placement of the capsules containing islets has also not been determined. Most encapsulated islets have been implanted into the peritoneal cavity, but whether this is an ideal site is uncertain. Within the peritoneal cavity, the islets tend to cluster together caudally (i.e., on the floor of the cavity) and may not be well-distributed, perhaps increasing the likelihood that nutrients and oxygen fail to reach them.

Other considerations

Sensitization

The current evidence is that sensitization to a human graft (i.e., to human leukocyte antigens [HLA]) may or may not result in sensitization to a pig graft[44-49]. When there is cross-reactivity between HLA and swine leukocyte antigens (SLA), it should be possible in the not-too-distant future to overcome this barrier by genetic engineering, e.g., (i) by deletion of expression of SLA class I[25] and/or downregulation of SLA class II[24], or (ii) by deleting a specific SLA amino acid and replacing it with a 'non-offending' SLA amino acid[50].

Sensitization to a pig graft (i.e., to SLA) does not appear to result in sensitization to a human graft[47]. Therefore, failure of pig islet transplantation would not necessarily preclude the patient from receiving a subsequent human islet allograft.

Physiological aspects of pig islet xenotransplantation

There are significant differences in glucose metabolism between human, NHP, and pig islets[51-53]. For example, the normal C-peptide level in monkeys is 0.47-3.14 nmol/l, whereas in humans, it is only 0.17-0.66, and in pigs it is 0.11-0.32. Therefore, the transplantation of pig islets (that normally maintain only a low C-peptide level) into a monkey (where a much higher C-peptide level is required) is a major hurdle. The difference in response to a glucose tolerance test is considerable between monkey islets and pig islets. It is perhaps surprising that any pig islet transplants in NHPs have proved so successful, and we speculate that pig islets may function more efficiently in humans.

There are, however, genetic-engineering techniques that might help improve pig islet production of insulin. Gianello and his colleagues have carried out some innovative studies in which they have increased insulin production in pigs by introducing genes for glucagon-like peptide-1 (GLP-1) and Type 3 muscarinic receptor (M3R), resulting in the pig islets producing higher levels of insulin[54]. However, with this approach there is a possibility that exhaustion of the pig islets might be caused, resulting in early graft failure independent of any immune response.

Safety of islet xenotransplantation

Concern regarding potential transfer of infectious microorganisms from the pig islets to the recipient and, even more, to the personal contacts of the recipient, e.g. family, friends, medical and nursing staff, and members of the public, has been raised. Nevertheless, pigs bred and housed under the conditions that will be required by the regulatory authorities

('designated pathogen-free pigs') will exclude most pathogenic microorganisms and viruses[55,56]. There remains some concern about the transfer of porcine endogenous retroviruses (PERVs), that are viruses and virus particles present in the genome of every cell in the pig[39,57]. There is no evidence that these are related to any health problems in the pig, nor is there evidence that the human equivalent (HERVs) are associated with any health problems in humans. Although it remains unknown what will happen when PERVs are transplanted (with islets or an organ) into an immunosuppressed human recipient, the current evidence is that the risk of infection or malignant change will be small[58,59].

In addition, if deemed essential, there are genetic engineering techniques to prevent activation of PERV[60-62] or delete them from the pig genome[63].

Concern has also been raised about the potential of hitherto unknown microorganisms being transferred with the islets, but this can also be a problem with the transplantation of human islets. Furthermore, if we conclude that no advances should be made because of the potential risk of an 'unknown', then there would be no progress made in many aspects of medicine.

Initial clinical trial

Experimentally, islet graft viability and function for at least six months, and possibly a year, will be required after pig islet transplantation in NHPs in a significant number of experiments, e.g., in 4 of 6, or 6 of 8 consecutive experiments, indicating a realistic possibility of relatively long-term benefit to a patient who has life-threatening diabetes[64-66]. However, there are other requirements that need to be met.

(i) The preparation of 'clean' islets will almost certainly be possible from pigs
(ii) bred and housed under the 'designated pathogen-free', biosecure conditions mentioned above.
(iii) A clinically acceptable immunosuppressive regimen would need to have been demonstrated in NHPs to effectively prevent the adaptive immune response.

Pigs have been used by humans as food for many centuries. For example, more than 100 million pigs are slaughtered in the USA each year for food. In China, the number must be very significantly more and, in addition, it is estimated that 500 million pigs are slaughtered each year to produce the anticoagulant heparin. Worldwide, approximately 250,000 pig heart valves are implanted to replace defective heart valves in human patients. Therefore, using pig islets as an approach to therapy in patients with life-threatening diabetes will not be very different from use of pigs for other purposes.

Conclusions

In the light of the present evidence available to us, we would suggest that (i) free islets have more potential than immunoisolated islets; (ii) the portal vein is not ideal for the transplantation of free islets; (iii) the islets will need to be genetically-engineered to protect against IBMIR, inflammation, and antibody-mediated and cellular rejection; and, ideally, (iv) minimal or no exogenous immunosuppressive therapy should be required (and may well be achieved by further genetic manipulation of the source pigs)[15,21]. We are optimistic that, as new pigs become available, protection may be provided against both IBMIR and the immune response.

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Conflict of Interest:

No author declares a conflict of interest.

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