A clinical trial of xenotransplantation of neonatal pig islets for diabetic patients

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Abstract: **Objective** To ascertain the safety and function of the transplantation of neonatal pig islets (NPIs) for diabetic patients. **Methods** NPIs were injected into the hepatic artery of 22 patients. After the transplantation, the patients were treated with a multiple drug immunosuppressive regimens. The first 14 patients were treated with cyclosporine (CsA), mycophenolate mofetil (MMF) and prednisolon, and porcine C-peptide was not monitored, the following 2 patients were given cyklosporin and MMF only, while the next 6 patients were given a quadruple drug regimen consisting of OKT3, takrolimus, sirolimus and prednisolon. The blood glucose levels, exogenous insulin requirement, HbA1c, porcine endogenous retrovirus (PERV) and liver function were assessed before and after NPI transplantation. The serum porcine C peptide were monitored in last 8 patients. **Results** The first 14 patients required less insulin and the HbA1c dropped after the transplantation. In the 2 subsequent patients, the metabolic parameters remained unchanged and monitor of porcine C-peptide was negative. Insulin requirements were reduced in all 6 patients, and HbA1c was normalized 3 months after the transplantation. Significant levels of porcine C-peptide were detected in the patient serum. Two of the patients were given a second injection of NPIs, and one of them became insulin independent for 7 d. No serious adverse events were noted after the transplantation. There was no evidence of PERV transmission. Six out of the 22 patients were followed up for 4–6 years after the NPIs injection, immunosuppressive treatment was stopped 1 year after the transplantation. The patients started to take insulin at the time of follow up. Four patients restricted the intake of sugar, while the other 2 did not. One patient had ketoacidosis twice and slight diabetic retinopathy, and another patient had ketoacidosis induced by acute gastroenteritis. The remaining 4 patients did not have any complications. Assays for PERV were again negative. **Conclusion** Xenogenic islets can survive and function in the human body. No serious adverse events are noted.

Key words: neonatal pig islet; xenotransplantation; diabetic patient

新生猪胰岛移植治疗糖尿病病人的临床研究

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[摘要] **目的**:评估新生猪胰岛移植治疗1型糖尿病方法的安全性和有效性。**方法**:所有22例病人均接受经肝动脉新生猪胰岛移植治疗,移植后病人接受多种免疫抑制治疗方案;第1组14例病人使用环孢菌素、骁息和甲基强的松龙,没有接受猪C肽检测;第2组2例病人只使用环孢菌素和骁息,第3组6例病人的免疫抑制方案是OKT3、他克莫司、西罗莫司和甲基强的松龙。在移植治疗前和移植后1年,病人进行了血糖、外源性胰岛素用量、糖化血红蛋白、猪内源性反转录病毒(PERV)和肝功能的评估,第2组和第3组的8例病人接受了血清猪C肽检测。6例病人在移植后4~6年进行了复查。**结果**:第1组14例病人移植后胰岛素用量减少和HbA1c水平降低。第2组2例病人在移植后代谢指标没有变化,猪C肽检测阴性。第3组的6例病人在移植3个月以后,胰岛素用量均减少,HbA1c正常,6例病人血清均检测到有意义的猪C肽。其中2例病人接受了第2次新生猪胰岛移植,1例病人短暂脱离胰岛素治疗7 d。所有病人在移植后均未出现严重不良反应,没有PERV感染的证据。6例病人在新生猪胰岛移植4~6年后接受检查,此6例病人均在接受移植治疗1年后停止免疫抑制治疗,复查时6例病人均接受胰岛素治疗。其中4例病人严格限制糖的摄入,2例病人为自由饮食;这2例病人中,有1例出现2次酮症,有轻度糖尿病视网膜病变;1例出现1次由于急性胃肠炎导致的酮症;其余4例均未出现任何并发症。6例病人再次接受PERV检测均为阴性。**结论**:异种胰岛可以在人体内存活并发挥其功能,没有发现严重不良反应。

[关键词] 新生猪胰岛; 异种移植; 糖尿病病人
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Transplantation of islets of Langerhan offer a potential means to treat diabetes, however, the limited supply of human islets would allow for the treatment of only a few of all suitable patients[1].

The use of pig islets would solve this dilemma. Pig and human insulin are structurally similar and the regulation of insulin secretion in pigs resembles that in humans. Also, there is the fact that pig insulin has been previously used to treat diabetic patients[2-4]. As for the immunological barrier, islet grafts are revascularized by vessels of recipient origin, thus, such grafts should not undergo the hyperacute xenorejection, this process being a consequence of the destruction of the xenogenic epithelium. Instead, xenogenic islet grafts undergo cellular rejection[5-7], this process should be amenable to immunosuppressive treatment.

From ethical point of view, the use of pigs as donors seems likely to gain wide acceptance[1]. In the early 1990's porcine fetal islet-like cell clusters (ICC) were transplanted to 10 diabetic kidney transplant patients in Stockholm, Sweden. Four of the recipients excreted small amounts of porcine C-peptide in the urine for several months. In one patient, a biopsy specimen obtained 3 months after the transplantation, revealed an accumulation of cells staining positively for insulin and glucagon. It was concluded that porcine pancreatic endocrine tissue can survive in the human body[2].

In the years 1999–2005, a pilot study was carried out in Changsha, in that 22 diabetic patients had neonatal pig islets (NPIs) injected via the hepatic artery. This clinical pilot trial was approved by the Ethics and Scientific Committees of the Chinese Ministry of Health.

1 MATERIALS AND METHODS

1.1 Preparation of NPIs

Pregnant sows [Xeno-1 pig, weight (2.16 ± 0.39) kg, Hunan Xeno-Life Science Ltd.] were bred in the Animal House of Central South University in Changsha. The animals were negative for fungi (actinamycosis, candidiasis, torulosis, histolasmosis, aspergillosis, mucormycosis), bacterium bugeri, streptococcus, aftosa virus, influenza viruses, cephalitis virus, cytomegalovirus, spirochetes and swine plague. Neonatal pigs were extracted by cesarean section under sterile conditions, the pancreases were removed surgically 3–5 d later. The weight of the pancreases was (1.6 ± 0.4) g. The pancreases were minced, and digested with Liberase PI (Roche) for 12 min. Subsequently, the digest was cultured for 6 d in Ham's F10 medium (Invitrogen) with human serum albumin (HSA) added. On the last day of the incubation, heparin and low-molecular weight dextran were added. Four days after the digested pancreatic tissue had been placed in an incubator, a large number of islets like cell clusters, NPIs, had formed. The number of NPIs per pancreas was $(9 \pm 1.2) \times 10^4$. The number of NPIs was counted, and the purity of the preparation was assessed following staining with dithizone (Sigma,

USA), the viability was assessed after staining with fluorescent diacetate (Sigma, USA). All procedures were performed in a cGMP laboratory.

1.2 Functional assessment of the NPIs in vitro, and after the transplantation to nude mice and dogs

After 6 d in culture, the viability of the NPIs was (85 ± 8.3)%, and the purity was (85.9 ± 4.6)%. The static insulin stimulation test showed a significantly higher insulin concentration in the high-glucose media than that in low-glucose media, indicating that the cultured NPIs were functional (Tab. 1). Samples of the culture media contained no bacteria and no endotoxin (data not shown).

Nude mice were made diabetic (glucose levels > 11.1 mmol/L) by the administration of streptozotoin. Six diabetic mice had approximate 2 000 NPIs placed under the kidney capsule, and blood glucose levels were monitored twice a week by an one touch, blood glucose meter (Johnson & Johnson, USA). Blood sugar levels were normalized (< 10 mmol/L) after 5-6 weeks in all 6 mice (Tab. 2). When the graft bearing kidney was removed, hyperglycemia reoccurred in all animals. Immunohistochemical examination of the subcapsular grafts revealed cells staining positively for insulin, glucagon and somatostatin.

Five dogs, weight 8-10 kg, were made diabetic by the administration of streptozotosin and alloxan. When the hyperglycemia had stabilized, 4×10^5 NPIs were infused through a catheter placed in the hepatic artery. Immunosuppressive treatment was with CsA 8 mg/kg, and MMF 0.5 g/d for 3 months. Following the transplantation, the liver function remained unaffected (Fig. 1). Four of the 5 dogs became temporarily insulin independent, this condition lasted for 5, 9, 16 and 23 d, respectively (Fig. 1). When the animals were sacrificed, well preserved NPIs were identified in the intralobal arteries in 4 of the 5 dogs (Fig. 2). The liver parenchyma was histological intact.

Tab. 1 Insulin releasing test (μU/mL)

Time/d	Low-glucose	High-glucose
2	79	118
4	68	132
6	83	165

Tab. 2 Metabolic parameters from diabetic SCID mice before and after NPI xenotransplantation ($\bar{x} \pm s$)

Days	B-glucose/(mmol/L)	Exog. insulin/U	HbA1c/%
-7	4.1 ± 0.99	40 ± 6.8	12.4 ± 2.9
7	4.7 ± 0.52	54 ± 9.8	—
14	4.8 ± 0.34	46 ± 6.0	—
21	4.9 ± 0.40	31 ± 4.9	—
28	5.0 ± 0.33	25 ± 5.0	—
60	5.0 ± 0.17	23 ± 5.0	—
90	5.4 ± 0.11	23 ± 5.0	7.4 ± 2.0

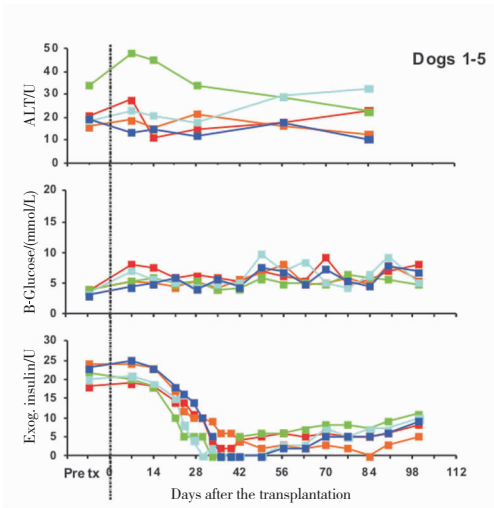

Fig. 1 Metabolic parameters from diabetic dogs before and after NPI xenotransplantation.

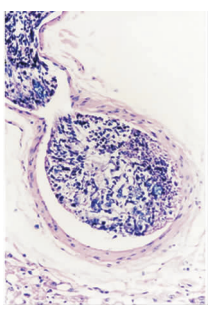

Fig. 2 Histopathology assessment of samples from diabetic canine liver after NPI xenotransplantation (HE staining, ×100). It shows NPIs stay in hepatic artery.

1.3 Transplantation of NPIs in diabetic patients

1.3.1 Patient characteristics

Twenty-two patients with type 1 diabetes (9 females and 13 males) were included in the study. The age of the patients was 18-52 (29.4 ± 10.2)

years. The patients had a history of diabetes for (9 ± 4) years. The patient body weight was (52.7 ± 7.3) kg. Before the transplantation, the basal human C-peptide level was (0.048 ± 0.025) ng/mL, arginine stimulation did not cause any increase (data not shown). All patients had unstable diabetes reflected in hypoglycemic episodes, bouts of coma, or great variations in blood glucose levels. There was no evidence of clinical significant renal, hepatic or cardiac dysfunction in any of the patients. All patients tested negative for cytomegalovirus, tuberculosis, hepatitis A, B, C and HIV. Each patient's medical history was carefully described and submitted for approval to the Ethic Committee of Third Xiangya Hospital. The patients and their spouse and/or parents understood, and signed an authorized, informed consent.

1.3.2 Transplantation procedure

The femoral artery was catheterized using Seldinger's technique. The tip of the catheter was placed in the hepatic artery, the placement was confirmed by injection of a small amount of contrast medium. The number of NPIs injected was (50 600 ± 13 300) IEQ/kg, 30 min were used for the injection. The volume of the tissue injected to hepatic arter was less than 15 mL. Hepatic hemodynamics were monitored by Doppler Ultrasound during the infusion of the NPIs and thereafter.

1.3.3 Treatment with immunosuppressive agents, and other agents

Three different immunosuppressive protocols were applied.

Fourteen patients, treated in 1999–2002, were given methylprednisolone (MP) (500 mg for the first day, 50 mg for the second day, then 10 mg/d for 7 d, Xianju Pharmacy Ltd., Co., China), CsA [8 mg/(kg · d) for 1 year], and MMF (1 g/d for 30 d, Roche, USA).

Two patients, treated in 2003, were given only CsA and MMF (dosing as described above).

Six patients, treated in 2003–2005, were given MP (dosing as described above), OKT3 (5 mg/d for 7 d, Wuhan Biology and Technology Co., China), Tacrolimus (TAC) [0.015 mg/(kg · d) for 1 year, Fujisawa, Japan], sirolimus (SLR) [0.2 mg/kg for the first day, then 0.1 mg/(kg · d) for 1 year].

All recipients were treated with low molecular heparin for 7 d before and 7 d after the transplantation. Aciclovir were given for 1 month after the transplantation; and Ceftriaxone was given for 1 d before and 4 d after the transplantation, Nicotinamide was administered for 1 week before and 1 week after the transplantation.

1.3.4 Measurement of the functional capacity of the NPIs after the transplantation

Early after the transplantation, serum blood glucose was monitored 6 times daily. After patient discharge, such monitoring was performed once a week for 3 months, and then at 6 and 12 months after the transplantation, and the exogenous insulin requirement was noted. HbA1c was assessed before the transplantation, and at 3 and 12 months after the transplantation. Human C peptide was examined by radioimmunoassay (RIA) (RIA kits of 125I C peptide, the Atomic Energy Science Academy of China). Porcine C-peptide was assessed by RIA (HI1.1. Linco Research, USA) in the last 8 patients. Samples were collected during fasting and under an intravenous glucose tolerance test (IVGTT). Porcine C-peptide was assessed in IVGTT.

1.3.5 Analysis of porcine endogenous retrovirus (PERV) by PCR and RT-PCR

In 14 of the patients, peripheral blood mononuclear cells (PBMCs) were isolated at 3 months and 3 years after the transplantation, respectively. RNA and DNA extraction was performed, using genomic RNA and DNA isolation kits (Gentra, Germany). Porcine mitochondrial (mt) DNA cytochrome oxidase subunit II, pol and gag of the *PERV* gene were amplified using previously reported primer pairs[8]. *GAPDH* were used as house keeping gene for analysis of *PERV* mRNA. PCR products were analyzed on 2% agarose gels.

1.3.6 Patient follow up 4–6 years after the transplantation of the NPIs

Following up data from 6 patients were collected 4–6 years after the NPIs injection, it included general condition of the patients, dosage of exogenous insulin, serum porcine C-peptide, and assessment of PERV.

2 RESULTS

2.1 Function of the NPIs

In the 14 patients treated with a CsA based triple drug regimen, a reduction in the exogenous insulin

requirement occurred after the transplantation, the value fell from (40 ± 7) U/d to a nadir of (25 ± 5) U/d, and the HbA1c dropped from 12.4 ± 3 to 7.4 ± 2 (Fig. 3A). In 2 patients given only CsA and MMP, no changes in the metabolic parameters were observed, and monitor of porcine C-peptide was negative (Fig. 3B). In the subsequent 6 patients that were given the quadruple drug regimen, the insulin requirement dropped in all patients after the transplantation, and the HbA1c was normalized (Fig. 3C). After the transplantation, porcine C-peptide appeared in the serum of all 6 patients (Fig. 3C). Two of the patients were given a second injections of NPIs approximately 2 weeks after the first injection. One of these recipients became insulin independent for 7 d (Fig. 3D). Performing an IVGTT in these patients, invariably resulted in an increased level of porcine C-peptide in the serum (Fig. 4).

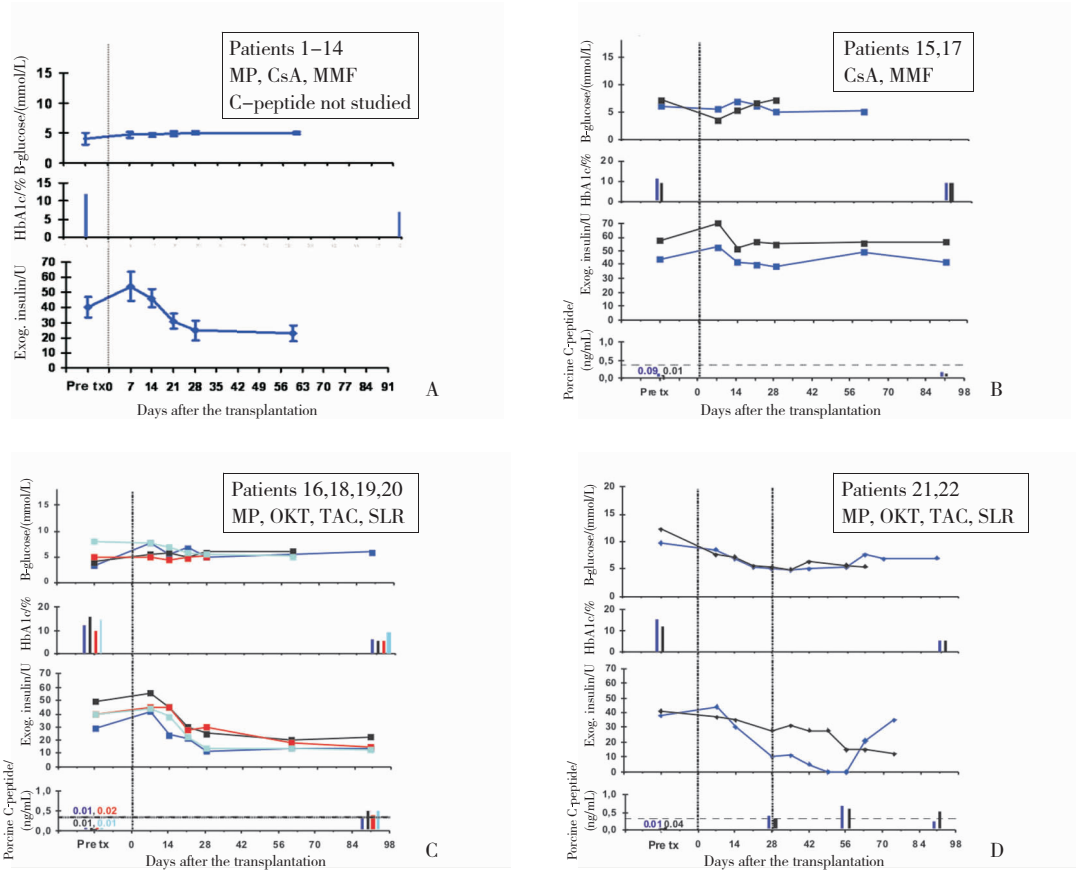

Fig. 3 Metabolic parameters from diabetic patients before and after NPI xenotransplantation.

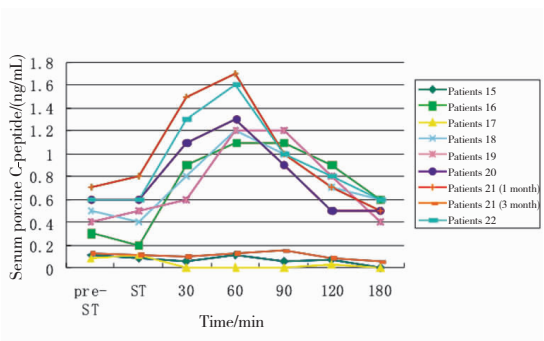

Fig. 4 Porcine C-peptide during IVGTT.

2.2 Studies on PERV

When the PBMC of the transplanted patients were assessed 3 years after the transplantation by RT-PCR analysis of pol, gag, env-A, -B and -C, there was no detectable *PERV* gene expression (data not shown). However, proviral DNA was detected; there were small amounts of mtDNA and pol gene in the peripheral blood of the recipients, whereas no gag gene could be detected. This indicated that there was xenochimerism, but no PERV infection.

2.3 Adverse events during the post transplant course

Two recipients complained of temporary right-

upper abdomen pain during the infusion of the NPIs. Three patients developed fever for 2 d post transplantation, this was followed by a period of leucopenia. One patient developed pneumonia, and the immunosuppressive treatment was discontinued and the patient recovered. Liver function test revealed a slight elevation in alanine aminotransferase (ALT) in 13 out of 22 cases [(65.2 ± 20.7) U, range from 45 to 114 U], the levels had normalized 2 weeks after the transplantation.

2.4 Patient followed up 4–6 years after the transplantation of the NPIs

All the 6 patients stopped immunosuppressive treatment 1 year after the transplantation, and were taking insulin at the time of follow up. Four patients restricted their intake of sugar while the other 2 did not. One patient had ketoacidosis twice and slight diabetic retinopathy, and another patient had ketoacidosis induced by acute gastroenteritis. The remaining 4 patients had not experienced any complications. Assays for PERV were again negative.

3 DISCUSSION

Until now, intraportal injection has been the favoured technique for islet transplantation in patient[9-11]. However, intraportal injection of islets may cause portal hypertension, and in some instances portal thrombosis and liver infarctions have occurred[12]. An alternative approach would be to inject the islets into the hepatic artery. The risk for thrombosis might be less, furthermore the hepatic artery provides only 20% –30% of the total hepatic blood flow, thus a thrombosis in the artery would cause less parenchymal damage. However, when Hirshberg et al.[13] injected allogeneic islets either into the celiac axis or into the portal vein in pancreatectomized rhesus monkeys, all recipients given the islets intraportally became insulin independent, while none of the animals in the arterial injection group had such a favorable outcome. In contrast, we observed function of islets after intrahepatic arterial injection in both dogs and patients. A possible explanation for this discrepancy could be that Hirschberg injected the islets into the celiac axis, which means that only 20% –50% of the islets would pass into the hepatic artery. In our study, the injection of the islets into the hepatic artery ascertained that all islets were delivered to the liver. Also, there was a marked difference in the number of islets transplanted: Hirschberg used approximately 1.5×10^4 islets/kg, while we injected 4×10^4 islets/kg in the dogs, and 5.5×10^4 islets/kg in the patients.

Recently, 3 landmark studies have highlighted the utility of NPIs as a source of insulin producing cells for the treatment of diabetes. Thus, Dr Rajotte and co-workers described large scale isolation of NPIs, and observed subsequent growth and function of the NPIs[14]. Furthermore, the same group reported on the reversal of diabetes in pancretectomized pigs following the transplantation of NPIs[15]. Moreover, these investigators, working in collaboration with the islet transplant group at the Emory University in Atlanta, Georgia, reported that NPIs could restore normoglycemia in diabetic non-human primates[11]. The capacity of the NPIs produced in our laboratory was evaluated in a nude mice model, and also in diabetic dogs with reassuring outcome.

To prevent islet rejection, most investigators have treated the recipients by various immunosuppressive agents. The Swedish patients that underwent transplantation with fetal islets, several years back, were all renal transplant recipients and were thus being treated with conventional immunosuppressive agents, some of the patients were given DSG as an adjunct[2]. Temporary graft survival was observed in some of the patients, however, insulin requirements were not affected. When various conventional immunosuppressive agents were used in rats transplanted with pig islets, Prograf was particularly effective and the rats became normoglycemic for 100 d (Wennberg). In 3 recent studies[11,16-17], reporting extended cure of diabetes in non-human primates transplanted with pig islets, the recipient animals had invariably been given multiple immunosuppressive regimens, including agents that induced costimulatory blockade.

In our study, some of the dogs given cyclosporine, MMF became temporarily insulin independent. The first 14 patients that were given cyclosporine, MMF and prednisolon, experienced a reduction in the insulin requirement, but insulin independence was not achieved. It is noteworthy that 2 subsequent patients that were treated with cyclosporine and MMF but no prednisolon, had no such improvement in glucose control. These 2 patients were the first in our series in whom porcine C-peptide was monitored, however, the findings were negative. The

next 6 patients were treated with tacrolimus, sirolimus, OKT3 and prednisolon. In these patients, there was an improvement in the glucose control, furthermore, there was direct evidence of function of the NPIs in that significant levels of porcine C-peptide were observed in the serum, and during the IVGTT the C-peptide level rose significantly. One of two patients given a second injection of NPIs, became insulin independent for a few days. These findings points to the importance of applying appropriate immunosuppressive drugs, and transplanting a sufficient mass of pig islets.

It was reassuring that the liver function remained in the normal, or near normal range in all patients after the transplantation. However, some of the patients experienced abdominal pain. Similar symptoms were noted in a patient in Stockholm who received a large amount of fetal islets injected into the portal vein. The fever and leucopenia, seen in some of the current patients, probably had the same etiology.

An attempt at bringing the patients back for follow up studies several years after the transplantation was only partially successful. Some of the patients had moved and could not be found, and some patients were not motivated to return for a follow up study. There was no evidence of transmission of PERV to the patients, and a negative findings was observed in the Stockholm cases[2].

This findings in the current pilot study indicated that NPIs is safe and the xenogenic cells can survive and function in the human body for several months.

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