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Long-term safety from transmission of porcine endogenous retrovirus after pig-to-nonhuman primate corneal transplantation

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Abstract

Background—The risk of xenozoonosis mainly by porcine endogenous retrovirus (PERV) has been considered as one of the main hurdles in xenotransplantation and therefore should be elucidated prior to the clinical use of porcine corneal grafts. Accordingly, an investigation was performed to analyze the infectivity of PERVs from porcine keratocytes to human cells, and the long-term risk of transmission of PERVs was determined using pig-to-nonhuman primate (NHP) corneal transplantation models.

Methods—The infectivity of PERVs from the SNU miniature pig keratocytes was investigated by co-culture with a human embryonic kidney cell line. Twenty-two rhesus macaques underwent xenocorneal transplantation as follows: 1) group 1 (n=4): anterior lamellar keratoplasty (LKP) with freshly preserved porcine corneas, 2) group 2 (n=5): anterior LKP with decellularized porcine corneas followed by penetrating keratoplasty (PKP) with allografts, 3) group 3 (n=3): PKP under steroid-based immunosuppression, 4) group 4 (n=4): PKP under anti-CD154 antibody-based immunosuppression, 5) group 5 (n=4): deep anterior lamellar keratoplasty with freshly preserved

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J.Y.K. and H.J.L. primate care and collection of specimens.

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porcine corneas under anti-CD40 antibody-based immunosuppression, and 6) group 6 (n=2): PKP under anti-CD40 antibody-based immunosuppression. Post-operative blood samples were serially collected, and tissue samples were obtained from thirteen different organs at the end of each experiment. The existence of PERV DNA and RNA was investigated using PCR and RT-PCR.

Results—Using two independent *in vitro* infectivity tests, neither PERV *pol* nor pig mitochondrial cytochrome oxidase II was detected after 41 and 92 days of co-culture, respectively. After xenocorneal transplantation, a total of 257 serial peripheral blood mononuclear cell samples, 34 serial plasma samples and 282 tissue samples were obtained from the NHP recipients up to 1176 days post-transplantation. No PERV transmission was evident in any samples.

Conclusions—Within the limits of this study, there is no evidence to support any risk of PERV transmission from porcine corneal tissues to NHP recipients, despite the existence of PERV-expressing cells in porcine corneas.

Keywords

porcine endogenous retrovirus; xenocorneal transplantation; xenozoonosis

Introduction

Xenotransplantation using porcine corneas has been suggested as a possible alternative to overcome an emerging shortage of human donor corneas. The physical properties of the porcine cornea are comparable to those of the human cornea, which means humans suffering from corneal blindness will see better after xenocorneal transplantation, and their use is ethically acceptable [1]. The main hurdle, huge antigenic differences between species, has been overcome in clinically applicable pig-to-nonhuman primate (NHP) corneal transplantation models using different immunosuppressive strategies according to the type of transplantation. Long-term survivals of decellularized porcine corneal lamellar grafts were achieved in an anterior lamellar keratoplasty (LKP) model using only steroid-based immunosuppression [2]. Even full-thickness porcine corneal grafts survived more than 6 months under an anti-CD154 antibody-based potent immunosuppressive regimen [3]. Through these promising results, clinical trials of xenocorneal transplantation are becoming a reality.

However, another obstacle to overcome is the cross-species transmission of porcine pathogens. Recently, public concerns about xenozoonosis have increased, as this happens not infrequently. The latest outbreak of Middle East respiratory syndrome in South Korea is a good example of such concerns [4]. Although no exogenous retroviruses such as human immunodeficiency virus-1 or human T-cell lymphotropic virus-1 have been found in pigs, the potential risk of infection by porcine endogenous retroviruses (PERVs), which are integrated into the genome of all pig strains [5], has been a key safety concern since PERVs were reported to be capable of infecting human cells *in vitro* [6]. Unapparent infection with these viruses may cause altered gene regulation, oncogenesis, or DNA recombination in the recipient.

Xenocorneal transplantation recipients are also considered to be at risk from this xenozoonosis, even though the total number of transplanted cells is much smaller than that of other solid organs. Moreover, the longer the recipients survive with functioning xenocorneal grafts or the longer the duration of potent immunosuppression lasts, the risk of xenozoonotic infections might become greater. Therefore, to be clinically relevant, evidence on the long-term safety from PERV infection should be provided through clinically applicable xenocorneal transplantation models as well as *in vitro* infection experimental models.

In this study, we evaluated the infection potential of PERVs in keratocytes from the SNU miniature pigs using *in vitro* infectivity tests and investigated long-term evidence of transmission of PERVs using a clinically relevant pig-to-NHP corneal transplantation model.

Methods

All procedures used in this study conformed with the ARVO Statement regarding the Use of Animals in Ophthalmic and Vision Research. In addition, all animal experiments were performed after receiving approval of the Institutional Animal Care and Use Committee (IACUC: 12-0374-C2A2) of the Seoul National University Hospital Biomedical Research Institute AAALAC accredited facility and according to the National Institutes of Health guidelines.

In vitro infection model

To culture keratocytes from the SNU miniature pigs, the epithelium of the cornea was removed using a surgical blade, and the Descemet's membrane was peeled off. The remaining stroma was treated with 1.2 U/mL dispase I (Roche, Basel, Switzerland) at 37°C for 2 hours, to which 5 mL of type I collagenase was added (Worthington, Lakewood, NJ, USA). The tissues were shaken 3 times every 30 minutes. The harvested keratocytes were centrifuged at 1200 rpm for 5 minutes and, the cell pellets were inoculated into the culture dishes containing DMEM:F12 (1:1; Cambrex, East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA). The primary keratocytes were cultured at 37°C in a CO_2 incubator. The human embryonic kidney cell line (HEK-293, ATCC CRL-1573) was cultured in DMEM containing 10% FBS at 37°C in CO_2 incubator.

The PERV infectivity test of porcine keratocytes was performed using a co-culture method as described elsewhere [7]. Briefly, 1×10^6 keratocytes/mL were plated onto 9×10^5/mL monolayer-cultured HEK-293 cells. After co-culture for 24 hours, the culture media was removed and replaced with fresh medium. Then, the infected cells were sub-cultured every 3 days and grown in the culture medium.

In vivo Pig-to-NHP corneal transplantation model

Porcine corneas were obtained from designated pathogen-free SNU miniature pigs, which were kindly donated by Yoon Berm Kim and had been bred at the Center for Animal Resource Development, Seoul National University College of Medicine. A hypertonic saline-based decellularization procedure was performed as described previously [2, 8, 9].

Each donor cornea was preserved in Optisol (Chiron Ophthalmics, Irvine, CA, USA) for up to 9 days before transplantation.

Twenty-two Chinese rhesus macaques (Macaca mulatta), which had been bred at the Seoul National University Hospital Non-human Primate Center, were divided randomly into each experimental group and underwent xenocorneal transplantation as follows: 1) group 1 (n=4): anterior LKP with freshly preserved porcine corneas, 2) group 2 (n=5): anterior LKP with decellularized porcine corneas followed by penetrating keratoplasty (PKP) with allograft, 3) group 3 (n=3): PKP with freshly preserved porcine corneas using steroid-based immunosuppression, 4) group 4 (n=4): PKP with freshly preserved porcine corneas using anti-CD154 antibody-based immunosuppression, 5) group 5 (n=4): deep anterior lamellar keratoplasty (DALK) with freshly preserved porcine corneas using anti-CD40 antibody-based immunosuppression, and 6) group 6 (n=2): PKP with freshly preserved porcine corneas using anti-CD40 antibody-based immunosuppression. LKP and PKP procedures were performed as described previously [2, 3, 9], and the DALK procedure was performed using the big-bubble technique. In group 2, subsequent PKPs were performed using 8.5-mm-sized rhesus allocorneal grafts after previously transplanted decellularized porcine grafts were completely removed. In group 4, one recipient (R062) underwent a subsequent PKP using an 8.5-mm-sized porcine corneal graft with the same immunosuppressive regimen after total rejection of the first xenocorneal graft.

After transplantation, all recipients received levofloxacin 0.5% (Cravit®, Santen Pharmaceutical, Osaka, Japan) and prednisolone acetate 1% (Pred forte®, Allergan, Irvine, CA, USA) topically to the cornea once a day. Dexamethasone (JW Pharmaceutical, Seoul, Republic of Korea) was injected weekly subconjunctivally for up to 6 months as a local adjuvant. The systemic immunosuppressive regimen used in this study was intramuscular injection of methylprednisolone (Solu-medrol®, Pfizer, New York, NY, USA) for 5 weeks. In addition, recipients in group 4 received intravenous immunoglobulin (IVIG) and recombinant anti-CD154 antibodies [3], and recipients in group 5 and 6 received IVIG and mouse-rhesus chimeric monoclonal anti-CD40 antibodies (2C10R4, NIH Nonhuman Primate Reagent Resource) [10] according to the program schedule. In group 2, after subsequent PKP, subconjunctival dexamethasone and intramuscular methylprednisolone were administered for up to 3 months and 3 weeks, respectively.

Peripheral blood samples were serially collected from the rhesus recipients, and peripheral blood mononuclear cells (PBMCs) were obtained at the interface after the centrifugation on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) at 400 g for 30 minutes. At the end of each experiment, tissue samples from 13 different organs were obtained from the sacrificed recipients: brain, heart, kidney, large bowel, liver, lung, mesenteric lymph node, muscle, skin, small bowel, spleen, stomach, urinary bladder. All tissue samples were frozen at −80°C until required.

Detection of PERV DNA and RNA

DNA of cells and tissues was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For detection of PERV RNA expression in plasmas and tissues, total RNA was extracted using a QIAamp Viral RNA

Mini Kit and RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol, respectively. To avoid any DNA contamination in samples, RNA extracts were treated with DNase I (Thermo Fisher Scientific, Waltham, MA USA). Complementary DNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manuals.

Primary PCR was carried out as described previously [11], using AccuPower PCR premix (Bioneer, Seoul, Republic of Korea) containing 10 pmol of primary forward and reverse primers for PERV *pol* or pig cytochrome oxidase II (COII) and 10 pg of prepared DNA in a total volume of 20 μL. Amplification was performed with 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in *pol* amplification, and with 30 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min in COII amplification. Nested PCR was performed with the same conditions using 1/20 of the amplified products instead of the prepared DNA. The products were then separated on 2% agarose gels (SeaKem, Rockland, ME, USA). In this nested PCR, PERV *pol* and COII could be detected up to 0.1 pg and 1×10^{-3} pg per reaction, respectively.

Results

PERV expression in the SNU miniature pig cornea

Both PCR and RT-PCR reactions using the SNU miniature pig corneas showed positive signals for both PERV *pol* and COII, which implies the presence of PERV expressing cells in the porcine corneal tissues (Fig. 1).

PERV detection in the in vitro infectivity test

Two independent experiments detected both PERV *pol* and COII in all early co-culture samples, which suggested the presence of porcine keratocytes. However, both PERV *pol* and COII were not detected in any sample after 41 and 92 days of co-culture, respectively (Supplemental Table 1).

PERV detection in the recipients after xenocorneal transplantation

Three different types of porcine corneas (freshly preserved partial thickness, decellularized partial thickness and freshly preserved full-thickness) were transplanted into NHP recipients. These porcine corneal grafts survived from 21 to 986 days in the recipients' corneal beds. Total exposure time to PERVs, calculated from when porcine grafts were transplanted until when the grafts were substituted for allografts (group 2) or when the recipients were sacrificed (other groups), ranged from 28 to 1176 days. Final blood and tissue samples were obtained from 28 to 1176 days post-transplantation (Table 1).

After xenocorneal transplantation, 257 PBMC samples from 22 rhesus recipients were serially collected and investigated for the presence of PERV DNA. All the PBMC samples, except one, were negative for PERV *pol* as well as COII. One collected from the recipient R034 at 4 weeks post-transplantation was positive for both PERV *pol* and COII, which implied the presence of porcine cells in the sample. However, all blood samples thereafter until euthanasia (546 days after the first exposure to a porcine graft) were negative for both

PERV *pol* and COII. Therefore, productive viral replication did not seem to be possible in this recipient (Table 1, Supplemental Table 2). Regarding PERV RNA expression in the recipients' plasma, 34 samples from 12 rhesus recipients showed neither PERV *pol* nor COII (Table 1, Supplemental Table 3).

In total, 282 tissue samples were obtained at the end of each experiment from thirteen organs in 22 rhesus recipients, which were investigated for the existence of PERV DNA. Neither PERV *pol* nor COII was evident in any tissue sample (Table 1, Supplemental Table 4).

Discussion

In this study, HEK-293 cells co-cultured with keratocytes from the SNU miniature pigs showed no detectable PERV DNA after 41 or 92 days co-culture, respectively. Employing clinically relevant xenocorneal transplantation models using various types of porcine corneas, NHPs were exposed to PERV-containing xenoantigens for up to 1176 days and demonstrated no detectable PERV DNA and RNA in any long-term (up to post-operative day 1176) follow-up samples, including PBMCs, plasmas and tissues from thirteen different organs.

It has been well documented that PERV expression is different depending on the pig strain, the individual pig of a given strain, and different organs [12, 13], although to date, there has been no report on PERV expression in the porcine corneal tissue. In this study, the authors confirmed the existence of PERV DNA and RNA in the porcine corneal tissue, which would suggest the cornea is not exempt from the concerns of xenozoonosis. In addition, it is advised that the source pig herd should be screened for PERV expression in the porcine cells and/or for *in vitro* transmission of PERV to human target cells [14–16]. Especially, pig strains carrying PERV-C, with the potential to generate PERV-A/C recombinant viruses, are advised to be avoided as source pigs [17]. The SNU miniature pigs were known to carry PERV types A, B, and C in the genome [18], however, previous *in vitro* infectivity tests suggested that PERVs from PBMCs and islet cells of the SNU miniature pigs cannot replicate productively in HEK-293 cell lines [7], and genetic analysis showed almost all of the PERV type C have defective *env* genes in these pigs [19]. Similarly, PERVs in keratocytes from these pigs did not show productive replications in HEK-293 cell lines in this study. These results suggest that the infection potential of PERVs from the SNU miniature pigs is low, despite the presence of PERV-C, and is supportive of their use as source pigs in future clinical trials.

There is no evidence for PERV transmission to NHP recipients of various transplanted porcine tissues including aortic endothelial cells, acellular vessels, skin, heart, kidney, thymic lobe, and encapsulated islets [20–28]. However, it is difficult to apply these results directly to the human clinical setting because the actual exposure durations of PERV-containing porcine products were relatively short, ranging from a few days to a few months in most cases, and long-term follow-up samples could not be tested due to short-term survival of each porcine graft. This study also found no PERV transmission to NHP recipients after various types of xenocorneal transplantations. These results are noteworthy, as the recipients were continuously exposed to xenoantigens for an extended period of time

(> 6 months in 17 of 22 (77.3%) recipients, and > 1 year in six recipients); thus, long-term follow-up samples were collected up to post-operative day 1176. Moreover, this study expands upon the previous studies mentioned above, as PERV DNA was investigated not only in blood samples but also in thirteen different organs.

An extensive literature review did not find any evidence for long-term PERV transmission from porcine products into human subjects. Although exposure time to porcine materials was very short, extracorporeal spleen/liver/kidney perfusion [29–31] and *ex vivo* treatment using a bioartificial liver support system [32–35] did not show PERV transmission for up to 8.7 years [35]. No PERV infection was reported for up to 8 years [36], even after *in vivo* xenotransplantation using porcine neural tissues [37], islet cells [30, 36, 38], or encapsulated islets [39–41]. A recent study in burn patients treated with live porcine skin grafts showed a negative result for up to 408 months, the longest term analyzed to date [42]. As more evidence accumulates, the risk of PERV transmission to humans seems to be low. Our results support this general consensus. Above all, our results were derived from a clinically applicable pig-to-NHP xenocorneal transplantation model, where all procedures were very similar to those used in the human clinical setting. Moreover, long-term survivals of xenocorneal grafts (> 6 months in 16 of 22 (72.7%) grafts, > 1 year in five grafts, and the longest survival of 986 days) were achieved under a clinically acceptable immunosuppressive regimen. In fact, Group 2, 4 and 5 almost met the minimum requirement for preclinical efficacy to justify initiating a clinical trial by the International Xenotransplantation Association - survival of porcine corneal graft for more than 6 months in five of eight consecutive NHPs, and ideally for 12 months in one or two successful cases [16].

There are some limitations in this study. First, the SNU miniature pigs possessed PERV-C in their genome. Although a series of studies have verified the low transmission potential of PERVs from these pigs, recently introduced genome-wide inactivation of PERVs using precise genome-editing tools would be a promising strategy to eliminate the concerns associated with xenozoonosis [43]. Second, it is not clear whether NHPs used in this study represent a suitable animal model to assess clinical PERV risk, although cells from NHPs have been shown to carry the PERV receptors [44]. In fact, PERV-A receptor 1 (the main receptor for PERV entry) was found to be genetically deficient in rhesus macaque [45], and NHP cells are not permissive to productive replication by PERV [46]. Nonetheless, our results are meaningful, as this is the first report of no evidence of PERV infection in the field of xenocorneal transplantation. In particular, NHP recipients were exposed to PERV-containing functional grafts for up to 1176 days, and the presence of PERV DNA and RNA was determined in long-term follow-up samples (up to post-operative day 1176), not only in blood samples but also in tissues from thirteen different organs. Furthermore, our results were drawn from a clinically applicable pig-to-NHP corneal transplantation model, which showed long-term survival of xenocorneal grafts under a tolerable immunosuppressive regimen.

In summary, no detectable PERV transmission is evident through long-term follow-up of pig-to-NHP corneal transplantations, despite the existence of PERV-expressing cells in porcine corneas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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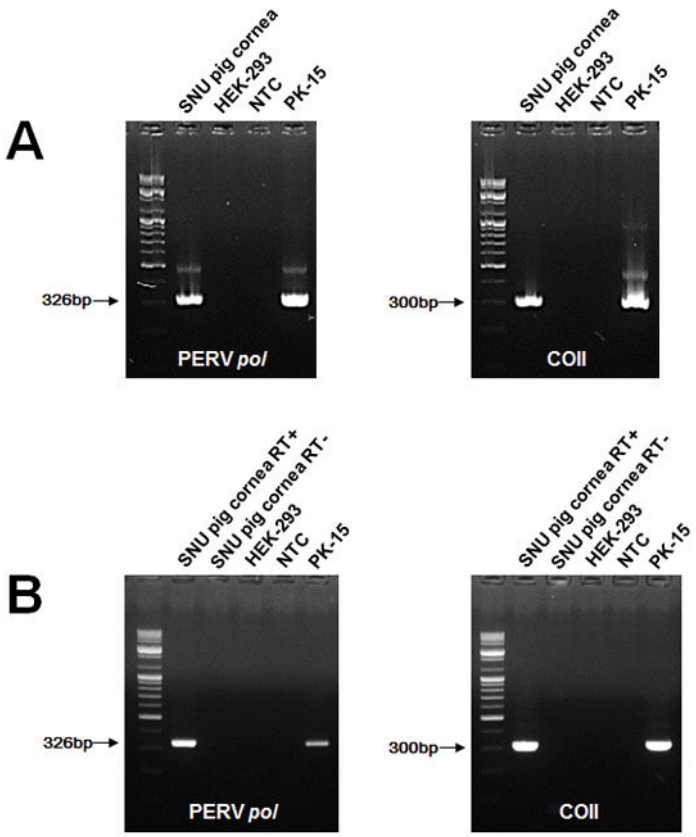

Figure 1. Expression of PERV DNA and RNA in the SNU miniature pig cornea

(A) DNA assay, (B) RNA assay. The SNU miniature pig cornea showed positive DNA and RNA signals for both PERV *pol* and pig mitochondrial cytochrome oxidase II.

PERV, porcine endogenous retrovirus; HEK-293, human embryonic kidney cell line (negative control); NTC, non-template control; PK-15, porcine kidney cell line (positive control); RT, reverse transcriptase; COII, pig mitochondrial cytochrome oxidase II.

Table 1

Porcine endogenous retrovirus expression in rhesus recipients' samples after xenocorneal transplantation.

Rhesus Recipients				Xenotransplantation procedure		Systemic immunosuppression	Survival of the first xenograft (days)	Exposure time to Xenoantigen (days)*	Time to euthanasia (days)†	PERV DNA (PERV *pol*/COII)		PERV RNA (PERV *pol*/ COII)
Group	**Age (Months)**	**Sex**	**Body Weight (Kg)**	**Method**	**Porcine cornea**					**PBMCs**	**Organs‡**	**PBMCs**
Group 1												
R028	41	M	4.38	LKP	Fresh	Methylprednisolone	> 398	398	398	−/−	−/−	−/−
R025	48	M	5.24				112	132	132	−/−	−/−	ND
R035	49	M	5.48				69	102	102	−/−	−/−	ND
R036	49	M	5.18				> 220	220	220	−/−	−/−	−/−
Mean ± SD								213.0 ± 133.1	213.0 ± 133.1			
Group 2												
R027	40	F	4.62	LKP	Decellularized	Methylprednisolone	> 392	392	771	−/−	−/−	−/−
R029	44	F	4.88				49	245	328	−/−	−/−	ND
R030	44	F	4.34				> 266	266	687	−/−	−/−	ND
R031	48	M	5.88				> 209	209	251	−/−	−/−	ND
R034	48	M	5.42				> 222	222	546	−/−§	−/−	−/−
Mean ± SD								278.0 ± 79.6	509.3 ± 258.0			
Group 3												
R032	48	M	5.48	PKP	Fresh	Methylprednisolone	21	28	28	−/−	−/−	ND
R058	54	M	4.90				29	36	36	−/−	−/−	−/−
R022	137	F	4.94				28	28	28	−/−	−/−	−/−
Mean ± SD								30.7 ± 4.6	30.7 ± 4.6			
Group 4												
R062	70	F	5.54	PKP	Fresh	Methylprednisolone IVIG Anti-CD154 antibodies	986	1176	1176	−/−	−/−	−/−
R065	65	F	4.94				> 243	243	243	−/−	−/−	ND
R019	114	F	5.64				318	407	407	−/−	−/−	−/−
R023	122	M	9.04				> 192	192	192	−/−	−/−	ND

Rhesus Recipients				Xenotransplantation procedure		Systemic immunosuppression	Survival of the first xenograft (days)	Exposure time to Xenoantigen (days)*	Time to euthanasia (days)†	PERV DNA (PERV *pol*/COII)		PERV RNA (PERV *pol*/ COII)
Group	**Age (Months)**	**Sex**	**Body Weight (Kg)**	**Method**	**Porcine cornea**					**PBMCs**	**Organs‡**	**PBMCs**
Mean ± SD								504.5 ± 457.0	504.5 ± 457.0			
Group 5												
R020	139	F	6.32	DALK	Fresh	Methylprednisolone IVIG Anti-CD40 antibodies	> 389	389	389	−/−	−/−	−/−
R088	74	F	7.12				> 382	382	382	−/−	−/−	−/−
R138	56	F	5.02				> 236	236	236	−/−	−/−	ND
R139	57	F	5.60				> 201	201	201	−/−	−/−	ND
Mean ± SD								302.0 ± 97.5	302.0 ± 97.5			
Group 6												
R141	59	F	4.91	PKP	Fresh	Methylprednisolone IVIG Anti-CD40 antibodies	> 204	204	204	−/−	−/−	−/−
R142	59	F	4.50				> 196	196	196	−/−	−/−	−/−
Mean ± SD								200.0 ± 5.7	200.0 ± 5.7			

PERV, porcine endogenous retrovirus; COII, pig mitochondrial cytochrome oxidase II; PBMCs, peripheral blood mononuclear cells; LKP, lamellar keratoplasty; PKP, penetrating keratoplasty; DALK, deep anterior lamellar keratoplasty; IVIG, intravenous immunoglobulin; ND, not done.

*calculated from when porcine grafts were transplanted until the grafts were substituted to allografts (group 2) or the recipients were sacrificed (other groups).

†calculated from when porcine grafts were transplanted until the recipients were sacrificed.

‡brain, heart, kidney, large bowel, liver, lung, mesenteric lymph node, muscle, skin, small bowel, spleen, stomach, urinary bladder.

§Blood sample at 4 weeks post-transplantation showed positive for both PERV *pol* and COII. However, all blood samples afterward were negative for both PERV *pol* and COII.