

Reducing porcine corneal graft rejection, with an emphasis on porcine endogenous retrovirus transmission safety: a review

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Abstract

• Donor cornea shortage is a primary hurdle in the development of corneal transplantation. Of all species, porcine corneas are the ideal transplantation material for humans. However, the xenoimmune rejection induced by porcine corneal xenotransplantation compromises surgical efficacy. Although the binding of IgM/IgG in human serum to a genetically modified porcine cornea is significantly weaker than that of the wild type (WT), genetically modified porcine corneas do not display a prolonged graft survival time *in vivo*. Conversely, costimulatory blockade drugs, such as anti-CD40 antibodies, can reduce the xenoimmune response and prolong graft survival time in animal experiments. Moreover, porcine endothelial grafts can survive for more than 6mo with only the subconjunctival injection of a steroid-based immunosuppressants regime; therefore, they show great value for treating corneal endothelial disease. In addition, zoonotic transmission is a primary concern of xenotransplantation. Porcine endogenous retrovirus (PERV) is the most significant virus assessed by ophthalmologists. PERV integrates into the porcine genome and infects human cells *in vitro*. Fortunately, no evidence from *in vivo* studies has yet shown that PERV can be transmitted to hosts.

• **KEYWORDS:** corneal xenotransplantation; genetically modified pigs; wild type pigs; costimulatory blockade drugs; porcine endothelial grafts; porcine endogenous retrovirus safety

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INTRODUCTION

Corneal disease is one of the most common blindness-related eye diseases in the world, second only to cataracts. An estimated 100-150 million people are affected worldwide^[1]. Corneal disease severely compromises patients' visual acuity. The most effective treatment for corneal blindness is corneal transplantation. Corneal transplantation has been performed for more than 100y, and has been proven to be safe and effective^[2-4]. However, the lack of donors significantly restricts the application of corneal transplant surgery. Thus, some alternatives for human corneas must be explored. In fact, people have attempted to use animal corneas as a substitute for more than 100y^[5]. Currently, pigs are considered suitable animals for the supply of heterologous organs, because their physiological structure is similar to that of humans. Genetic modifications can be easily performed in pigs, and the application of porcine organs avoids many ethical problems^[6]. In addition, pigs are raised widely and accessed easily. Porcine corneas are also very similar to human corneas in terms of anatomy and biomechanical properties^[7-8]. Therefore, some researchers have focused on using porcine corneas for corneal xenotransplantation since the late 20th century^[9]. However, reducing porcine corneal xenotransplantation rejection remains a significant challenge. Briefly, corneal xenografts may trigger the recipients' immune system to synthesize corresponding antibodies and then mediate complement-dependent cytotoxic effects^[10]. Moreover, xenografts rejection is induced by indirect pathways because of the significant difference in major histocompatibility complex (MHC) between donors and recipients^[11]. Like allograft rejection, it may cause an inflammatory response, corneal opacity, edema and neovascularization, which damage corneal tissues and reduce graft survival time^[12-13]. Xenograft rejection shows more serious signs than allograft rejection. It is crucial to decrease xenoantigens or adopt immune tolerance in recipients' bodies. We will discuss this topic in the following sections.

In addition, the safety of porcine organ xenotransplantation is also a major concern. After all, zoonotic transmission is intolerable regardless of whether the surgical outcomes are satisfactory in human clinical practice. Fortunately, designated pathogen-free (DPF)-pigs, which are obtained through cesarean section from special pathogen-free (SPF) species, have been designed as medical donors^[14] because they are free of most bacteria, fungi, viruses and parasites. However, porcine endogenous retrovirus (PERV) remains a concern because the DPF technique can not eliminate this pathogen^[15]. This article reviews recent progress aiming to safely reduce porcine corneal graft rejection and zoonotic transmission, particularly for PERV.

STUDIES ON REDUCING PORCINE CORNEAL XENOTRANSPLANTATION REJECTION

Pathogenesis of Corneal Xenotransplantation Rejection

Corneal xenotransplantation rejection pathogenesis does not differ so much different from allograft rejection. As we know, unlike other solid organs that include blood vessels, immune privilege shields the cornea and prevents it from developing hyperacute rejection. Because the central area of the cornea is verified as vascular-free, the aqueous humor maintains the anterior chamber-associated immune deviation (ACAID) due to the presence of free Fas ligand and negative immune-regulatory molecules such as IL-10 and TGF- β ; moreover, tight junctions between corneal endothelial cells prevent the cells and proteins in the aqueous humor from entering the stroma. Corneal immune privilege is a prerequisite for performing xenografts transplantation. However, immune privilege may be defective or impaired in some cases, such as ocular inflammation, corneal dystrophy, neovascularization, trauma, topical allergies, *etc.* and in such cases, the cornea would yield to rejection^[16]. Antibody-dependent or complement-mediated innate humoral immune responses induce hyperacute rejection, so comparatively, CD4⁺ T cells play a major role in corneal xenotransplantation rejection^[16]. In addition, Reichart *et al*^[17] have confirmed that the humoral and cellular immune responses both contribute to corneal xenotransplantation rejection. They also concluded that rejections are primarily correlated with corneal immune privilege, the extent of genetic homology between donors and recipients, and the dose of the immunosuppressants regime^[17].

There are three major xenogeneic antigens, including galactose α 1,3-galactose (α Gal), N-glycolylneuraminic acid (NeuGc) and β 1,4 N-acetylgalactosaminyl transferase (B4GALNT2) that are expressed in pigs and may bind to the antibodies of humans or nonhuman primates (NHPs) to induce a humoral immune response^[18]. Complement also damages porcine corneal grafts *via* antibody-dependent and -independent pathways because porcine corneal endothelial cells are highly

sensitized to the cytotoxicity of complement binding to antibodies. In addition to the humoral immune response, the cellular immune response, particularly for the cytotoxic effect of CD4⁺ T cells on corneal endothelial cells, is also a main cause of corneal xenotransplantation rejection. If there are not sufficient CD4⁺ T cells, CD8⁺ T cells and dendritic cells will replace them.

Unlike corneal allograft rejection, dendritic cells in the corneal xenograft can directly bind to the recipient's T lymphocytes for antigen presentation and then cause a cellular immune response. In addition, porcine corneal endothelial cells continuously express costimulatory molecules (CD80 and CD86), which allows them to stimulate human T cells completely to induce an immune response by direct pathways^[19].

Corneal Xenotransplantation Using Genetically Modified Pigs

Genome editing techniques on pigs In recent years, there has been significant improvement in porcine genetically modified techniques aiming to eliminate xenoantigens. The first generation of nuclease-mediated technique-zinc finger nucleases (ZFNs) was created, and zinc finger proteins were applied to recognize and bind specific DNA sequences in 2005. Combined with endonuclease Fok I, double-stranded DNA would then break^[20]. The second generation of nuclease-mediated technique transcription activator-like effector nucleases (TALENs) was successfully applied to edit eukaryotic cells in 2012^[21]. The TALEN technique identified DNA sequence specifically with TALE, and hydrolyzed DNA with the help of endonuclease Fok I^[22]. Currently, clustered regularly interspaced short palindromic repeating associated nuclease 9 (CRISPR/Cas9) is an acquired immune system existing in bacteria and archaea that is one of the most advanced genome editing techniques. Under the guidance of guide RNA (gRNA), CRISPR/Cas9 can make nuclease Cas9 recognize and degrade exogenous DNA^[23]. This technique is much simpler, more effective and more economical than ZFNs and TALENs, and it has been widely used to knock out the targeted genes of encoding porcine xenoantigens such as GGTA1/CMAH/B4GalNT2^[24-26]. It is predicted that these genetically modified pigs for xenotransplantation could reduce xenoantigens-antibodies reactions to some extent.

In vitro studies on genetically modified porcine cornea

α Gal is a carbohydrate antigen mainly expressed in the porcine vascular endothelium; it is catalyzed and synthesized by α 1, 3-galactosyltransferase^[27-28]. α Gal can be recognized by anti-pig antibodies in primates and then causes hyperacute rejection in the recipient's body^[29]. According to some reports, α Gal is mildly expressed in the anterior stroma of the wild type (WT) porcine cornea, but it is not expressed in the epithelium or

endothelium^[30]. α 1,3-galactosyltransferase knockout (GTKO) pigs were successfully cultivated in 2002^[31]. Hara *et al*^[32] confirmed that the GTKO porcine cornea did not express α Gal, and indicated that the immune response from the recipient to the GTKO porcine cornea was weaker than that to the WT after corneal xenotransplantation.

A primate's complement system is activated to form membrane attack complexes and different types of multiple active fragments after pig-primate xenotransplantation. The complement system would damage graft cells and mediate the inflammatory response^[33]. The human complement-regulatory protein can interact with different complement components, enabling complement to mediate activation and suppression balanced reactions. Therefore, the expression of the human complements regulatory gene in donor pigs can effectively reduce the recipient's complement response^[34]. Some researchers have successfully cultivated the transgenic pig expressing human complement-regulatory protein-CD46^[35]. Even transgenic GTKO/CD46 pigs expressing GTKO and CD46 simultaneously were also successfully bred and have become one of the major strains used for xenotransplantation research^[36]. Hara *et al*^[32] reported that human CD4⁺ T cells displayed a significantly weaker immune response against GTKO/CD46 porcine corneal endothelial cells than that of the WT *in vitro*. Moreover, CD55 and CD59 were also synthesized in some genetically modified pigs as transfection proteins. However, there were no reports showing that GTKO/CD55 and GTKO/CD59 species were used for corneal xenotransplantation.

Pigs also express some non- α Gal antigens. Thus, there are still antibodies from the recipient that bind to non- α Gal antigens in the porcine cornea even though α Gal is knocked out through gene knockout techniques, which would cause immune rejection. A non- α Gal antigen called NeuGc was discovered in 1990^[37]. Subsequent studies confirmed that NeuGc was expressed in pigs, orangutans and rhesus monkeys, but not in humans^[38]. Moreover, the anti-NeuGc antibody was expressed in humans. Given that NeuGc is expressed in some NHPs, research on investigating this antigen cannot draw support from pig-NHP models. Researchers had to perform some experiments on NeuGc *in vitro*. Cohen *et al*^[30] demonstrated that NeuGc was strongly expressed in all layers of the corneas regardless of whether WT or GTKO strains were used, and IgM/IgG in human serum was positively expressed in the epithelium, anterior stroma and limbus of porcine corneas from WT and GTKO after binding to porcine xenoantigens. They also hypothesized that anti-NeuGc antibodies in human serum would bind to NeuGc in GTKO porcine corneas. Subsequently, some researchers attempted to cultivate a newly genetically engineered pig named NeuGcKO to eliminate NeuGc expression.

As we learned from articles reported by Gao *et al*^[39], Wang *et al*^[40] and other researchers in recent years, GTKO/NeuGcKO pigs have been successfully cultivated. It has been confirmed that the complement response in human serum binding to GTKO/NeuGcKO pig cells was weaker than that of GTKO *in vitro*. Moreover, Lee *et al*^[41] indicated that there was no significant difference between IgM/IgG in human serum binding to xenoantigens in GTKO/NeuGcKO and GTKO porcine corneal endothelial cells. However, in another article published in the same year, they reported that the binding of IgM/IgG in human serum to GTKO/CD46/NeuGcKO porcine corneal tissue was weaker than that of GTKO/CD46^[42]. The discordance between these two studies may attribute to the contents of xenoantigen in porcine corneal endothelial cells, which were minor and not sufficient to cause significant differences in the xenoimmune response between the GTKO/NeuGcKO and GTKO groups.

Byrne *et al*^[43] characterized the porcine B4GALNT2 gene sequence, genomic organization and expression, which encoded another non- α Gal antigen. Then Zhang *et al*^[44] cultivated GTKO/NeuGcKO/ B4GALNT2KO pigs. Wang *et al*^[45] investigated the xenoantigenicity of tissues and organs derived from GTKO/NeuGcKO/B4GALNT2KO pigs and found that compared to WT pigs, IgM/IgG in human serum binding to the cornea was significantly weaker in GTKO/NeuGcKO/ B4GALNT2KO pigs. However, the difference between human serum IgM/IgG binding to GTKO/NeuGcKO/B4GALNT2KO and GTKO (or GTKO/NeuGcKO) strains has not been reported.

Corneal xenotransplantation using genetically modified pigs Porcine corneas have been applied to donors in corneal xenotransplantation for the past 10y. NHPs are considered highly homologous with human beings. Therefore, NHPs, particularly Macacus, which is classified as an old world monkey, were frequently used as recipients. Zhiqiang *et al*^[46] first performed pig-NHP corneal xenotransplantation. Their results showed that WT porcine corneal grafts could survive for 4mo by injecting betamethasone subconjunctivally after penetrating keratoplasty, and lamellar corneal xenografts could maintain transparency for 3mo without betamethasone. Dong *et al*^[47] first attempt to perform penetrating keratoplasty with GTKO/CD46 and WT porcine corneal grafts in 2017. In their studies, the mean corneal graft survival time in GTKO/CD46 group was 100.8 \pm 59.11d (47-171d), whereas that in the WT group was 77.5 \pm 60.45d (28-157d). Unfortunately, compared to the WT porcine corneas, the GTKO/CD46 porcine corneas showed no correlation with prolonged corneal graft survival time or reduced immune response from the recipients' antibodies to the porcine xenoantigens. Based on the analysis of their report, we attributed this phenomenon to the complications that emerged in some xenografts such as anterior synechiae and retrocorneal

membrane, which disrupt the postoperative efficacy. Lee *et al*^[48] pointed out that the origin of retrocorneal membrane was the donor porcine corneal stroma, and young GTKO miniature porcine corneas should still be recommended for operation because their corneal thickness and biomechanical properties are closer to those of primates. Above all, GTKO/CD46 techniques alone may not improve porcine corneal survival status *in vivo*. Secondary postoperative complications and significant differences in corneal thickness between pig and rhesus monkey compromised corneal grafts survival.

Conversely, some researchers investigated to reduce the cellular immune response. There is a major costimulatory molecule named B7/CD28. B7-1 (CD80) and B7-2 (CD86) are expressed on the surface of antigen presenting cells (APCs) and bind to CD28 expressed on the surface of T cells. They then induce CD28 to transmit signals into T cells to activate a response. B cells also express B7 when they recognize antigens. Another receptor of the B7 group named cytotoxic lymphocyte associated antigen-4 (CTLA-4) was discovered later. Both CTLA-4 and CD28 are expressed on the transmembrane receptors of the surface of T cells, and they are similar in structure, particularly in the intracellular region. Therefore, CTLA-4 binding to B7 can naturally inhibit the combination of B7 and CD28. CTLA-4/B7 interactions can transmit inhibitory signals into activated T cells, therefore reducing T cells-mediated immune responses. Vabres *et al*^[49] used porcine corneas from transgenic pigs that secreted human CTLA4Ig (hCTLA4Ig), GTKO and WT pigs, respectively, for corneal xenotransplantation in rhesus monkeys. Before transplantation, hCTLA4Ig was mainly expressed in the porcine corneal stroma and moderately expressed in the epithelium and endothelium. After surgery, hCTLA4Ig was still expressed in the porcine corneal grafts. Although the corneal grafts were eventually rejected in the hCTLA4Ig group, the quantity of inflammatory, T and B cells in porcine corneal grafts was less than that in the WT group. The longest final rejection was 120d in the hCTLA4Ig group whereas there was no significant difference in mean final rejection days between the hCTLA4Ig and WT groups ($P=0.12$).

Corneal Xenotransplantation Using Wild Type Pigs Given that there were no advantages in prolonging corneal grafts survival days with genetically modified pigs, researchers tend to utilize WT pigs to reduce experimental costs. One feasible method in current research that involves blocking the costimulation pathway has gradually gained more attention than before; this method is intended to enhanced recipients' immunological tolerance to WT porcine corneal grafts^[6,50]. Briefly, naive T cells activation requires two different extracellular signals, which both worked well. The first signal is derived from the major MHC-antigen peptide complex on

the surface of APCs, this type of APC can interact and bind with T cell receptors (TCRs). The second signal is derived from the costimulatory molecule, which is the innate immune component that responds to the microorganism. As we know, the second signaling molecules and their ligands include B7/CD28, ICAM-1/FFA-1, LFA-3/CD2 and CD40/CD40L. Normal tissues and residual APCs may or may not express costimulatory molecules at low levels. The second signal deficiency can place autoreactive T cells in an anergic state, which is conducive to maintaining autoimmune tolerance. Therefore, the blocking activity of costimulatory molecules is expected to reduce rejection and prolong corneal graft survival time.

Some researchers have blocked CD40/CD40L pathways in animal experiments. The interaction between CD40L on the surface of T cells and CD40 on the surface of APCs can enhance the T cells response. The reaction mechanism involves the activation of APCs, which transmit intracellular signals that promote B7 expression and increase IL-12. CD40 is also expressed on the surface of B cells and binds to CD40L expressed on the surface of activated CD4Th⁺ cells. The CD40/CD40L complex stimulates quiescent B cells into the proliferation cycle and is the strongest costimulatory molecule that enables the activation of B cells. As early as 2001, some researchers attempted to block CD40/CD40L to prolong the corneal graft survival time after allograft transplantation. Subsequently, Choi *et al*^[51] performed pig-NHP corneal xenotransplantation on 4 rhesus monkeys and administered intravenous immunoglobulin (IVIG) and anti-CD40L antibodies within 6mo after surgery. Compared to the control group, the corneal xenograft survival time in recipients who received anti-CD40L antibodies was significantly longer (192-933d), and administrating anti-CD40L antibodies significantly reduced the deposition of inflammatory cells in corneal tissues. Conversely, some researchers have attempted to use anti-CD40 antibodies to reduce the T cells immune response. Kim *et al*^[52] performed deep lamellar corneal xenotransplantation on 5 rhesus monkeys. After administrating IVIG and anti-CD40 antibodies within 6mo after surgery, the xenografts survival time in recipients who received anti-CD40 antibodies was significantly longer than that of the control group (61d-12mo). The local expansion and activation of central and effector memory T cells were also effectively suppressed. Kim *et al*^[53] further compared anti-CD40 antibody efficacy with a commercially available immunosuppressants regime (anti-CD20/basiliximab/tacrolimus). They performed full-thickness corneal xenotransplantation on 13 rhesus monkeys. In the anti-CD40 antibodies group, graft survival time ranged from 41 to 511d, whereas that in the anti-CD20 antibodies group ranged from 97 to 470d. There was no significant difference

in the concentration of T cells in the recipients' blood, anti- α Gal antibodies in the recipients' plasma and α Gal expression in porcine corneal grafts. Unfortunately, 3 rhesus monkeys in the anti-CD20 antibodies group suffered from adverse events (anorexia/pneumonitis/shigellosis) after receiving an immunosuppressants regime. Combined with Choi and Kim's studies, we can conclude that costimulatory blockade drugs may significantly improve the corneal xenograft survival time. However, some reports found that anti-CD40L antibodies may cause thrombosis when treating immune-related diseases. Consequently, anti-CD40L antibodies are not recommended to patients in clinical practice. We believe that it is also necessary to pay close attention to the safety of anti-CD40 antibodies.

Some researchers chose not to administer a systemic immunosuppressants regime in corneal xenotransplantation to avoid side effects. This expectation might result in endothelial keratoplasty. Currently, corneal endothelial keratoplasty has been widely developed. It is considered to be an effective therapy for bullous keratopathy, Fuchs corneal endothelial dystrophy and corneal endothelial dysfunction. With the rapid development of phacoemulsification in recent years, the incidence of corneal endothelial dysfunction is expected to increase significantly^[54]. Rejection risks after pig-NHP endothelial keratoplasty are estimated to be significantly lower than those after penetrating keratoplasty because surgeons make minor incisions without sutures, xenoantigens in the porcine corneal endothelium are scarce^[41] and porcine endothelial grafts are fully protected by ACAID. Liu *et al*^[55] first studied the application of porcine corneal endothelial grafts. They successfully prepared porcine corneal endothelial grafts for endothelial keratoplasty through mechanical stripping and liquid bubble techniques, respectively. There was no significant difference in the success rate between these two methods. The liquid bubble technique was less time-consuming than mechanical stripping, but the former method led to significantly higher endothelial cell loss.

Subsequently, Liu *et al*^[56] performed Descemet's stripping automated endothelial keratoplasty (DSAEK) on 7 rhesus monkeys. They adopt a mechanical stripping technique for the preparation of porcine endothelial grafts. Their results showed that 5 of 7 corneal grafts became transparent within 30d postoperatively, and 4 corneal grafts survived more than 180d (180-270d) with only injecting betamethasone subconjunctivally. In addition, immunohistochemistry showed that there was no obvious inflammatory cell infiltration in the survival grafts. Immunofluorescence showed that α Gal expression was very scarce, and both anti-pig IgG and complement C3 were not found in the surviving endothelial grafts. Above all, the long-term survival of porcine corneal endothelial grafts may not depend on the systemic

immunosuppressants regime. However, we could not find other studies about the utilization of porcine endothelial grafts, so more rigorous animal studies are necessary to strengthen Liu's conclusion.

We summarized the characteristics of pig-NHP corneal xenotransplantation mentioned above in Table 1.

Safety Issues with Porcine Endogenous Retrovirus The microorganism safety of xenotransplantation is also an important concern. Donor animals should be examined before xenotransplantation to determine whether they carry pathogenic microorganisms. Like other animals, many microorganisms cling to the porcine alimentary canal and skin. Tissues and organs for transplantation, including porcine corneas, should be obtained under aseptic conditions^[57]. Some reviews on microorganism safety of xenotransplantation reported that many microorganisms could infect humans through transplantation and result in zoonotic diseases. However, it was unclear which types of microorganisms should be monitored before operation. Currently, hepatitis E virus (HEV), porcine cytomegalovirus (PCMV), porcine circovirus (PCV), porcine lymphotropic herpesviruses (PLHV) and PERV are considered the primary contagious viruses^[58]. Some strategies for eliminating these high-risk pathogens have been described in previous detailed reports (except for PERV)^[59-60]. Briefly, screening for zoonotic virus carriers in all cultivated pigs and administering caesarean sections, antiviral treatment or vaccines to the qualified pigs (or those that may be carriers but with values lower than the baseline) help identify virus-free pigs that can be bred and further used for xenotransplantation.

We reviewed published articles to date and found that ophthalmologists mainly focused on the risk of PERV transmission in corneal xenotransplantation^[61-63]. Therefore, we will emphasize the relevant contents as follows. PERV is characterized as a retrovirus that transmitted across species and integrated into the porcine genome. It is classified as a C-type retrovirus, γ 1 subgroup and RNA virus^[64]. PERV exists as a proviral formation that is integrated and exists in the host genome. Its genetic monomeric length is approximately 7-9 kb, which is composed of 5'LTR, 3'LTR and a middle region encoding the gag, pol, and env genes. Gag and pol display high homology even when obtained from different sources of PERV; however, env varies significantly^[64]. Because of env gene differences and cellular tropism *in vitro*, PERV can be divided into three different subtypes: PERV-A, PERV-B and PERV-C. PERV-A and B are expressed in all porcine genomes and possess different copy levels, whereas PERV-C cannot be integrated into all porcine genomes. PERV-A and B display polytropism and infect humans' and other species' cells; PERV-C only infects porcine cells^[65]. Since it is difficult to remove PERV from porcine genomes, effective strategies are

Table 1 Characteristics of pig-NHP corneal xenotransplantation

Study	Surgical technique	Donor type	Survival days	Topical immunosuppressants regime	Systemic immunosuppressants regime
Dong (2017)	PKP	WT	157, 28, 92, 33	Tobramycin-dexamethasone eyedrop; Subconjunctival betamethasone	None
		GTKO/CD46	128, 57, 47, 171		
Vabres (2014)	LKP	WT	9, 70, 70, 21	Dexamethasone ointment	Intravenous methylprednisolone
		GTKO/hCD39/hCD55/hCD59	9, 34		
		hCTLA4Ig	21, 50, 90, 120		
Zhiqiang (2007)	PKP	WT	12, 16, 16, 16, 12, 18	None	None
	LKP		129, 276, 182, 144	Subconjunctival betamethasone	
Choi (2015)	PKP	WT	21, 28, 29	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone	None
			>933, >243, 318, >192	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone	
Kim (2017)	LKP	WT	>389, >382, >236, >201	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone	IVIG; Intravenous anti-CD40 Ab
			>61, >192, >192	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone	None
			>192	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone; Intramuscular tacrolimus	IVIG
Kim (2018)	PKP	WT	>203, >196, >511, 41, >422, >273	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone	IVIG; Intravenous anti-CD40 Ab
			>260, 134, >97, >470, 297, >210, >184	Topical prednisolone; Subconjunctival dexamethasone; Intramuscular methylprednisolone; Intramuscular tacrolimus	IVIG; Intravenous anti-CD20 Ab; Intravenous basiliximab
Liu (2018)	EKP	WT	>180, >180, >298, >270, 30, 30	Subconjunctival betamethasone	None

PKP: Penetrating keratoplasty; LKP: Lamellar keratoplasty; EKP: Endothelial keratoplasty; WT: Wild type; GTKO: α 1,3-galactosyltransferase knockout; hCTLA4Ig: Human cytotoxic lymphocyte associated antigen-4 immunoglobulin; IVIG: Intravenous immunoglobulin; Ab: Antibody.

necessary overcome the infection. First, pigs with low copy numbers and low PERV expression should be preferentially assigned. Second, to avoid PERV-A/C recombination, PERV-C negative pigs should be utilized as much as possible. Third, genetically modified pigs that express low or even negative PERV should be cultivated to reduce the possibility of PERV transmission. Finally, vaccines should be used to block PERV transmission^[66-67].

In fact, some researchers have suppressed or eliminated the PERV gene with aforementioned gene editing technology. In brief, Yang *et al*^[66] at Harvard University adopted CRISPR/Cas9 technology to knock out all 62 copies of the PERV pol (polymerase) gene in the porcine kidney cell line (PK15), which could reduce endogenous viral transmission risks in pig-recipient by 1/1000. Niu *et al*^[68] analyzed the porcine fetal fibroblast (PFF) genome sequence. They then found 25 copies of the PERV gene and inactivated them by utilizing CRISPR/Cas9. In addition, they successfully cloned first endogenous retrovirus-inactivated pigs in the world with somatic cell nuclear transfer (SCNT) technology. Moreover, aforementioned reports^[15] showed that the integrase inhibitors might be the most efficient inhibitors of PERV. Of course, their potency against PERV infection in the recipient body should continue to be analyzed.

PERV can infect a variety of human cell lines, such as stem cells, bone marrow cells, NK cells and kidney cell lines *in vitro*^[69]. However, Meije *et al*^[70] demonstrated that it was difficult for “endogenous ‘retroviral restriction factors’” such as intracellular proteins and components of the innate immune system to take effect *in vitro* environment. In some *in vivo* studies, Kim *et al*^[71] found that no transmission of PERV was detected in the process of PERV-producing porcine cells transplanted into mice unless the murine cells were pretreated with PERV. Denner^[72] confirmed this finding that although recombinations between the human tropic PERV-A and the ecotropic PERV-C in pigs showed high replication activity, recombinant PERV-A/C failed to infect pigs.

Some ophthalmologists also investigated the possibility of PERV transmission. Choi *et al*^[62] cultured porcine keratocytes with the human embryonic kidney cell line-HEK-293 to explore PERV infectivity *in vitro*. They performed pig-NHP corneal xenotransplantation on 13 monkeys. Some monkeys were administered a steroid-based immunosuppressants regime ($n=3$) or anti-CD40 antibodies ($n=4$) postoperatively. Their results showed that neither PERV pol nor pig mitochondrial cytochrome oxidase II were detected in cocultured cell lines after 41 and 92d, respectively. Moreover, 257 rhesus monkeys' peripheral blood mononuclear cell (PBMC) samples, 34

serial plasma samples and 282 tissue samples were detected by polymerase chain reaction (PCR) and real time-PCR, respectively. As a result, no positive signal of PERV DNA and RNA were found. Li *et al*^[61] cultured porcine aortic endothelial cells (PAECs) with monkey vein endothelial cells (MVECs) and performed pig-NHP corneal xenotransplantation on 10 rhesus monkeys. Their results showed that PERV was transmitted from PAEC to MVEC *in vitro*. PCR and RT-PCR showed that positive signals of PERV-A and B were present in porcine corneas. Fortunately, no evidence showed that PERV was transmitted into the recipients *in vivo*.

CONCLUSION

The global severe shortage of donor corneas will not be improved for a long time. Thus, porcine corneas as a replacement for humans is a foreseeable future possibility. Although genetically modified pigs have been successfully cultivated and raised, their corneal grafts did not survive longer than those of the WT *in vivo*. Some newly genetic engineered pigs, such as GTKO/NeuGcKO and GTKO/NeuGcKO/ B4GALNT2KO strains, have not yet been applied to animal experiments. Costimulatory blockade drugs can reduce the immune response that targets WT porcine corneal grafts and prolong their survival time. Aoyagi *et al*^[73] studied the pharmacodynamic effects of a novel anti-CD40 antibody-4D11, which could mitigate some side effects including thrombosis. Of course, more preclinical experiments should be performed to ensure that a reasonable dose is used. WT porcine endothelial grafts could survive for months without a systemic immunosuppressants regime due to their low level of xenoreactivity and moderate complications. More comprehensive studies must be performed to verify their efficacy and safety to progress into clinical trials.

Obviously, there are no cases on PERV transmission during pig-NHP corneal xenotransplantation. However, it is indicated that there is no receptor for PERV-C in NHPs^[74]; therefore, non-crossover into humans cannot be 100% assured even though pig-NHP studies showed no PERV infection. Thus, effective PERV detection must be performed in porcine corneal grafts before implanting them into human eyes in the future. It is better to use low level or negative PERV pigs as donors.

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