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## Contents

## ABOUT COVER

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*MINIREVIEWS*

# Transitioning of renal transplant pathology from allograft to xenograft and tissue engineering pathology: Are we prepared?

Muhammed Mubarak

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## Abstract

Currently, the most feasible and widely practiced option for patients with end-stage organ failure is the transplantation of part of or whole organs, either from deceased or living donors. However, organ shortage has posed and is still posing a big challenge in this field. Newer options being explored are xenografts and engineered/bioengineered tissues/organs. Already small steps have been taken in this direction and sooner or later, these will become a norm in this field. However, these developments will pose different challenges for the diagnosis and management of problems as compared with traditional allografts. The approach to pathologic diagnosis of dysfunction in these settings will likely be significantly different. Thus, there is a need to increase awareness and prepare transplant diagnosticians to meet this future challenge in the field of xenotransplantation/regenerative medicine. This review will focus on the current status of transplant pathology and how it will be changed in the future with the emerging scenario of routine xenotransplantation.

**Key Words:** Xenotransplantation; Bioengineered tissues; Pathology; Allograft; Xenograft

**Core Tip:** End-stage organ failure is a significant public health problem worldwide. Currently, treatment options are limited and organ shortage for allotransplantation is one of the biggest challenges. Alternative options being explored are xenografts and engineered/bioengineered tissues/organs. These developments will pose different challenges for the diagnosis and management of transplant pathologies as compared with traditional allografts. The approach to pathologic diagnosis of dysfunction in these settings will likely be significantly different. Thus, there is a need to increase awareness and prepare transplant pathologists to meet this imminent challenge in the field of xenotransplantation/regenerative medicine.

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## INTRODUCTION

End-stage organ failure of vital organs is one of the leading causes of morbidity and mortality worldwide. Currently, the only treatment or in the case of some organs, the treatment of choice for these patients is the transplantation of those organs. In the United States of America alone, more than 1.2 million people need transplantation for end-stage organ failure; the vast majority of these await kidney transplants. Currently, less than one-third of these succeed in getting transplants with varying rates in different countries, as shown in Figure 1[1-3].

The remarkable success in the progress of solid-organ transplantation in the last few decades of the preceding century is rightly considered a milestone in the history of modern medicine. However, it has not completely met the goals. Many challenges remain to be surpassed and unmet needs to be fulfilled. Currently, the main limitations to the field of transplantation include complications related to lifelong immunosuppression, chronic rejection, and shortage of organs. Among these, the latter is one of the biggest challenges facing the transplant community worldwide. The rejection of transplanted organs, particularly chronic rejection, represents another formidable challenge. Fortunately, the rate of acute rejection has been reduced drastically over the past few decades due chiefly to the development of more potent immunosuppressive agents. However, chronic rejection still poses a big challenge and it remains the leading cause of graft failure worldwide and the dominant indication for second or third transplants. Efforts to prevent or treat it effectively have not been successful till date[4,5].

Against the backdrop of the above facts and challenges, the scientific community has been striving hard to find alternative solutions. The use of animal cells or tissues (xenogeneic) is one of such solutions that could easily reduce the ever-increasing gap between the demand of organs and their supply[6-10]. Although non-human primates are closely related to humans phylogenetically, it is the pig that has been identified as the optimum donor species for xenotransplantation (XenoTx) into humans. The pig kidneys are suited for transplantation in humans on both anatomical and physiological grounds[11,12]. Pig breeding is easy and does not pose major ethical issues, and considerable progress has been made in genetically modifying the animal to improve the acceptance of swine organs. Xenografts from genetically modified pigs have become one of the most promising solutions to the shortage of human organs available for transplantation. The use of organs from such modified pigs together with different methods of inducing tolerance has paved the way to prolonged survival of xenografts, removing the early obstacles that cause hyperacute rejection (HAR) and immediate graft loss[13-17]. Transgenic modifications including knockout of carbohydrate epitopes and additions of the complement cascade and coagulation cascade regulatory proteins have extended the xenograft survival in pig-to-non-human primate transplants of kidneys, hearts, and livers. In addition, improvements in immunosuppressive drugs such as the introduction of mammalian target of rapamycin inhibitors and blockers of co-stimulatory pathways have resulted in better outcomes. However, delayed antibody-mediated rejection and thrombotic microangiopathy (TMA) continue to be the major challenges in the field and need further focused research[18-20].

Regenerative medicine/tissue engineering is another promising field to bridge the existing gaps in transplantation. It can be used to lengthen the lifespan and improve the function of suboptimal donor organs, thereby greatly augmenting the existing donor organ pool, and has the capability to save the remaining vast majority of patients waiting for transplants, by generating or repairing organs. The discipline of regenerative science is older than that of organ transplantation. The first textbook on regenerative medicine was written in 1901. Similarly, a major regenerative science conference was held in 1988, while first Banff renal transplant pathology meeting was held three years later[21]. Contrary to this, the subject of regenerative medicine/tissue engineering pathology (TEP) never received much attention till the recent past[21-25]. In the near future, the discipline of transplantation will expand manifold, through a combination of tissue engineering with the prevalent approaches to decrease the

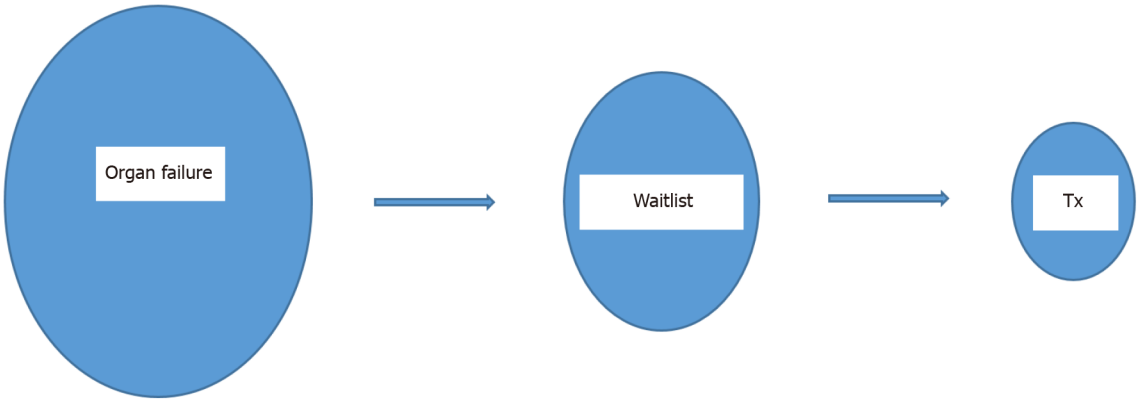


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**Figure 1 Schematic illustration of global estimates of patients with terminal organ failure, waitlist patients, and patients undergoing transplantation.** This is only an approximation and not actual numbers. More patients are waiting for kidneys than any other organ.

organ shortage. These new options will bring along with them new challenges in related fields such as pathology and diagnostic fields.

The role of transplant pathology in the diagnosis and management of graft rejection, particularly in the allotransplant setting, cannot be overemphasized. Although it is easy to conceptualize and classify rejection in theory, its diagnosis and classification are not always easy or straightforward in practice. The Banff process was initiated more than 30 years back to standardize the criteria for diagnosing and classifying the rejection process in human allotransplants. Considerable refinements and improvements in the classification system have been made over the last three decades and there are still many unmet needs to be fulfilled[21-25]. However, the Banff group is not oblivious to the developments or progress in the related fields of regenerative medicine/tissue engineering and XenoTx. In fact, the Banff researchers have proposed to create a new Banff classification, tentatively named TEP, to address issues related to the success or dysfunction of engineered tissues/organs[25]. It is also hoped that the Banff group will also address the issues related to standardization of the diagnostic criteria and classification of XenoTx pathology.

Herein, we review the pathobiology of the rejection process in the XenoTx setting and the current status of histopathology in clinical xenoTx, and focus on current results in the pig-to-primate model, as this is thought as the most relevant to human xenoTx. We will briefly review the histological findings in the three recently performed pig-to-human kidney transplants. We will also explore the future challenges and prospects of XenoTx pathology in light of advancements in human transplant pathology. We will not discuss further the topic of TEP, as it is beyond the scope of the present review.

## PATHOGENETIC BASIS OF REJECTION IN XENOTX

The major barriers to successful solid organ XenoTx are natural antibodies to carbohydrate antigens, present in pigs but absent in humans and non-human primates, mainly galactose-α-1,3-galactose (Gal), which is produced by the enzyme α-1,3-galactosyltransferase (GT). Bi-allelic GT knockout (GTKO) pigs were used in early protocols of solid organ XenoTx[26-29]. The lifespan of grafts improved, and immediate and accelerated injury to xenografts was overcome. GTKO pigs with further genetic modifications in the form of knockout of two other xenospecific antigens, expressed in pigs but not in humans, termed triple knockout (TKO) pigs, were also used in these experiments. Further multiple transgenic modifications including the addition of human complement regulatory proteins, such as CD46 and CD55, and regulatory molecules of human coagulation cascade have further lengthened the lifespans of xenografts. Suggestions to introduce other transgenes to provide multi-dimensional lines of safety for the xenograft have been put forward, with the aim of countering rejection, coagulopathy, or additional mechanisms of immediate or early xenograft injury[30,31].

Three recent cases of pig-to-human kidney xenoTx utilizing 10 gene modifications in genetically engineered (GE) pigs, termed 10-GE pigs, demonstrate the feasibility of the procedure with no HAR. These were performed in brain-dead human recipients and were terminated at 2 to 3 d post-transplant [32,33]. These have attracted considerable public interest and re-kindled the interest of the transplant scientific community. The pigs used as organ donors in both procedures were produced and supplied by Revivicor, Inc., United States. Revivicor (https://www.revivicor.com/), a subsidiary of United Therapeutics Corporation, uses precise gene editing tools to delete or insert genes in the pig genome. Gene editing is carried out *in vitro* in pig cells cultured in Petri dishes. The cells are screened and analyzed to make sure that the gene editing is accurate. Somatic cell nuclear transfer (SCNT) is then

used to produce pigs from the gene-edited cells. SCNT involves the transfer of a nucleus from a gene-edited pig cell into an enucleated pig egg from which the nucleus has been extruded. The eggs are then transferred to surrogate sows where they develop and grow until natural birth (Figure 2). Revivicor raises the organ donor pigs in a designated pathogen-free facility to eliminate infectious agents that could transmit disease to human transplant recipients. Revivicor received approval from the Food and Drug Administration in 2020 for use of the GalSafe™ pig as a source of food for human consumption, and as a source of human therapeutics (https://www.revivicor.com/).

## PATHOLOGIC EVALUATION OF XENOGRAFT AND CURRENT STATUS OF HISTOPATHOLOGY

Histopathology is presently considered the gold standard in the diagnosis of solid organ graft rejection. However, this status of histopathology is subject to certain conditions which must be fulfilled, such as adequacy of sampling and the experience of pathologists. Banff schema of transplant pathology represents a significant scientific effort in the recent past in the field of transplantation diagnostics. Substantial progress has been made in improving the diagnostic criteria and rationalizing the classification of rejection processes in human allografts. This was made possible with continued and concerted efforts by the Banff team and researchers in the transplant field worldwide over the past three decades. However, there are still many unmet needs and challenges in the field, and the Banff process is poised to tackle these in near future[21-24].

The status of histopathology in XenoTx is less developed as compared to its status in allotransplantation (alloTx), principally because there is a lack of extensive literature on this topic. In contrast to alloTx, during the rejection process of a xenograft, the host is likely to use almost the entire armamentarium of its immune mechanisms, encompassing all elements of innate immunity, such as naturally occurring xenoreactive antibodies. In addition, xenograft damage may be caused by mechanisms such as TMA initiated by molecular incongruities in the processes of homeostasis at the surface of the endothelium of blood vessels. Thus, the pathology of xenoTx rejection represents a more complex process and presents a wide variety of histologic features than allograft rejection[32-39]. With multiple transgenic modifications and combinations leading to potentially heterogeneous data in the XenoTx field, systematic study of the xenograft at gross and microscopic levels is crucial. At present, the Banff classification for renal allograft rejection is used by some researchers in some experimental studies [39]. Although the utility of the Banff classification in alloTx is now well-established throughout the world, the pathogenic processes involved in XenoTx and hence the pathologic patterns may be dissimilar. Presently, xenograft pathology classification relates to the diagnostic aspects of rejection, and also reflects, to some extent, the pathomechanisms of rejection. On the other hand, the pathologic evaluation of alloTx rejection provides information not only limited to diagnosis but also on the prognosis and the reversibility of the rejection process with treatment. However, it should be noted that similar to alloTx, histopathology can not be practiced in isolation, but represents a supportive component in the multidisciplinary evaluation of a xenograft[22].

The main focus in earlier Banff classifications of human allograft pathology was on the cellular part of rejection, with the role of alloantibodies relegated to the now obsolete category of HAR[21]. In contrast, in XenoTx, humoral rejection is considered the most important. Accordingly, the classifications have differed in construct and weightage to different categories. More recently, the focus of human allograft pathology has also shifted to humoral rejection beyond immediate and early posttransplant periods[22].

## HISTOPATHOLOGIC CLASSIFICATION IN XENOTX

There are very limited studies that report specific morphological features of xenoTx rejection. Since most work on XenoTx has been done with pig-to-non-human primate models, the study of pathologic features has been reported in this setting[34-39]. Most researchers working with these XenoTx models have implemented a simple and mechanistic classification of rejection processes. The current xenograft pathology classification originated at Imutran, Cambridge, UK, in 2002 and has subsequently been used by other researchers as well[40]. Basically, three main diagnostic categories are distinguished: (1) Hyperacute rejection; (2) acute humoral xenograft rejection (AHXR); and (3) acute cellular xenograft rejection (ACXR), as shown in Table 1. As with Banff classification of human allograft pathology, this classification relies not only conventional morphology, but also needs immunofluorescence for immunoglobulin and complement proteins including C4d, and correlation with clinical information, including status of graft function for optimal evaluation and potential significance of pathological lesions[39,40].

Although the xenograft rejection pathology classification primarily relates to pathogenetic rejection mechanisms, each type of rejection incorporates a wide range of cellular and humoral elements of the specific immune system and innate inflammatory mechanisms. It is likely that different histopathologic

**Table 1 Classification and diagnostic criteria used in pig-to-primate solid organ transplantation pathology**

| Hyperacute rejection | Acute humoral xenograft rejection | Acute cellular xenograft rejection |
|---|---|---|
| **Time period** | | |
| Immediately after reperfusion of the graft (typically within 24 h) | Later after reperfusion (after 24 h) | After 3 d |
| **Immediate graft function** | | |
| No (no urine since reperfusion) | Yes, urine formation initially | Yes, urine formation initially |
| **Histopathologic features** | | |
| Massive hemorrhage; Immunoglobulin and fibrin deposition; Complement (C5b-9) deposition; Presence of neutrophils; Thrombosis, ± | Hemorrhage present; Immunoglobulin and fibrin deposition; Complement (C5b-9) deposition; Presence of neutrophils; Lymphocytes may be present; Necrosis and transmural infiltration by neutrophils in blood vessels can be present; Apoptosis may be present; Thrombosis present | No hemorrhage; Immunoglobulin and fibrin deposition, rare; Complement, ±; Presence of mononuclear (lymphoid) cells associated with tissue destruction (*e.g.*, tubulitis); No thrombosis |

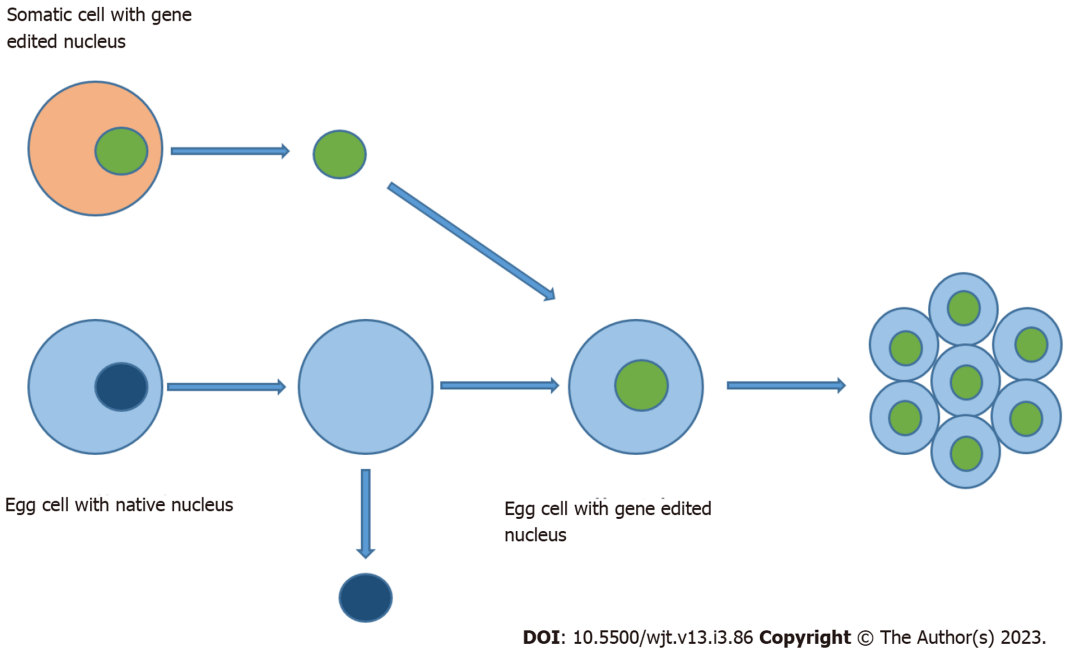


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**Figure 2 Schematic diagram showing steps involved in somatic cell nuclear transfer.** The process involves both *in vitro* and *in vivo* procedures under strict quality control mechanisms.

entities will emerge and the classification will evolve when additional genetically altered animal organs and novel immunosuppressive agents that subdue the immune system are made available. In expectation of this, the proponents of the above classification have kept the nomenclature of the classification schema relatively simple at this stage, leaving room for expansion or modification of the classification in the future, as more data accumulates on this subject[33].

## HYPERACUTE REJECTION

HAR entails immediate destruction of the microvasculature and subsequently, the graft parenchyma following reperfusion, resulting in intravascular thrombosis and diffuse interstitial hemorrhage. HAR has, fortunately, become exceedingly rare in human alloTx. In the pig-to-primate model, HAR is mainly caused by the binding of naturally occurring xenospecific antibodies to Gal epitopes exposed on cell-surface glycoproteins and glycolipids of pig organs, followed by activation of the complement cascade. It can be prevented by the removal of naturally occurring anti-Gal antibodies or by chemical inhibition of complement activation. The removal of naturally occurring antibodies can be accomplished by extracorporeal immunoadsorption or these can be neutralized by the intravenous administration of soluble glycoconjugates. Many such conjugates have been tried with variable success rates. Similarly, many agents have been developed and tried that inhibit complement activation. Alternative options include the use of knockout or transgenic animal organs to circumvent this problem. Multi-transgenic

pigs by inserting multiple complement regulatory proteins have been developed in an attempt to augment complement inhibition. More recently, 10-GE pig kidneys have been used in clinical-grade xenotransplants in brain-dead human recipients with promising results[32,33]. However, there were some caveats in these trials, which need to be addressed in the future. Nevertheless, the reports have attracted public attention towards xenotransplantation which, if properly harnessed, should be of significant benefit to future progress. Although the above strategies have successfully overcome the barrier of HAR, a similar but less fulminant type of rejection still develops later and is called AHXR.

## AHXR

AHXR, also known as ''acute vascular rejection'' or ''delayed xenograft rejection'', is an important form of antibody (Ab)-mediated rejection in xenoTx setting. The use of the AHXR term was adopted as it more closely represents the potential mechanism of Ab-induced rejection, mediated by the activation of complement pathway and/or infiltration by polymorphonuclear leucocytes. The other two terms either reflect the morphological aspect of rejection or the clinical aspect (delayed rejection) and hence, are better avoided.

The antibodies that cause AHXR can be both naturally occurring xenospecific antibodies, *e.g.*, anti-Gal antibodies in the pig-to-primate and pig-to-human situations, or they may be formed *de novo*. In the former case, AHXR is best considered a delayed form of HAR. In the latter situation, AHXR is due to induced antibodies after sensitization by the graft. The *de novo* antibodies may be directed against Gal or non-Gal antigens.

AHXR is the only type of humoral rejection to occur after Tx of organs between the concordant species, in which setting, HAR characteristically does not develop. The hamster-to-rat solid organ Tx model is the most commonly used such animal model, particularly in studies on immunosuppression or induction of immune tolerance in the presence of the development of sensitization. Among discordant species combinations, discrimination between the roles of pre-formed and *de novo* antibodies in the rejection process is vital for the development of plans to preclude or control the rejection. A few researchers have tried to address this topic but none has been completely successful in discriminating between the roles and significance of natural *vs de novo* antibodies in AHXR.

## ACXR

ACXR has been studied to a much less extent than AHXR and hence, detailed accounts of this rejection type are scarcely reported[41]. This is mainly due to the fact these xenografts are usually lost as a result of HAR, which occurs before cellular rejection. In general, ACXR is more or less similar to that observed in human alloTx rejection. Cell-mediated rejection, in isolation, is a comparatively rare occurrence in pig-to-primate solid organ grafts. More commonly, it occurs in combination with AHXR but usually is of lesser intensity than AHXR. Morphologically, the entire range of mononuclear cells, including T lymphocytes (both CD4+ and CD8+ cells), B lymphocytes, and natural killer cells and macrophages, may be present in the interstitium and inside the tubules (tubulitis). Endothelialitis, and in severe forms, transmural vasculitis can be present in cellular rejection.

## CHRONIC REJECTION

Chronic rejection is defined on theoretical grounds as the progressive and unremitting destruction of a transplant over months to many years. As one of the major causes of delayed graft failure from human donors, chronic rejection is currently considered as the major obstacle to the long-term success of alloTx. Chronic rejection has not been widely observed in xenoTx because of the generally short survival of xenotransplants and thus remains understudied. With recent developments resulting in prolonged graft survival in xenoTx, the challenge of chronic rejection is more likely to become prominent in the future as the initial barriers are surmounted and graft survival is extended. Currently, the literature on chronic vasculopathies and other chronic xenograft changes is scarce. In a GTKO porcine to baboon discordant xenoTx of hearts, morphological changes of chronic xenograft rejection were reported 78-179 d post-transplantation[42]. As is well described in the literature, in cardiac transplants, chronic rejection is mainly expressed by vascular changes. Hisashi *et al*[42] described four types of chronic xenograft vasculopathy in GTKO pig hearts transplanted into baboons, as shown in Table 2. Among these, fully developed and chronic antibody-mediated rejection-associated vasculopathy types were predominant in graftectomy specimens removed from the gradually weakened group between 78 and 179 d after transplantation. They hypothesized that fully developed vasculopathy was the end-result of combined chronic humoral and cell-mediated rejection-associated mechanisms, as the intimal fibrosis usually followed the infiltration of cells and deposition of fibrinoid material in the arterial intima. On the other

**Table 2 Types of chronic xenograft vasculopathy**

| Type of vasculopathy | Histopathological features |
|---|---|
| Chronic humoral rejection-associated vasculopathy | Arterial intimal thickening; Presence of TUNEL+ cells; Deposits of fibrin, immunoglobulins (IgG and IgM), and complement components (C3, C4d, and C5b-9) |
| Chronic cellular rejection-associated vasculopathy | Mononuclear cell infiltration in the neointima; Active endothelialitis; TUNEL+ cells |
| Combined chronic humoral and cellular rejection-associated vasculopathy | Fibrinoid material deposition and cellular infiltration in the arterial neointima with immunoglobulin and complement deposition and infiltration of T cells, macrophages, and polymorphonuclear leukocytes |
| Fully developed vasculopathy | Narrowing of arteries with a fibrotic neointima, but without fibrinoid material, or cellular infiltration |

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

hand, evidence of chronic cell-mediated rejection-associated vasculopathy or a combination of both chronic humoral and cell-mediated rejection-associated vasculopathy was less frequent. Nevertheless, the precise mechanism by which chronic rejection and vasculopathy are mediated is still incompletely understood.

The relative roles of cellular and humoral immune components along with the roles of soluble serum inflammatory cytokines, such as tumor necrosis factor-alpha, interleukin-6, and interleukin-17, and their antagonists are yet to be fully explored in human recipients of xenografts[43-49]. Contemporary studies have focused on prolonging the graft survival and preventing the causes of early graft loss but future studies are likely to focus on detailed pathologic assessment of prolonged surviving grafts and protocol biopsies for elucidating the pathomechanisms of chronic rejection.

## FUTURE PROSPECTS

Although extensive pig genome editing and recent pig-to-human kidney transplants in brain-dead recipients have opened the doors of clinical xenoTx in humans, much remains to be done before this becomes a routine activity. The potential advantages of kidney XenoTx are enormous if the immunological barriers are surmounted. Immunological tolerance to the graft may be easier to induce in models of xenoTx than in alloTx. It is hoped that eventually, XenoTx will compete favorably with alloTx. The recent cases of pig-to-human kidney transplants, albeit in brain-dead recipients, have also provided an opportunity to educate healthcare professionals and the public about xenoTx's potential, as well as its public health risks and logistic and economic implications. Increased public awareness and full transparency during clinical trial planning and execution will be required to generate support for xenoTx trials. Kidney xenoTx pathology will also evolve as the activity in clinical xenoTx increases and the xenograft survival is prolonged. There is abundant opportunity to learn from the evolution of the Banff process of human kidney allograft pathology.

## CONCLUSION

Overall, XenoTx pathology is a comparatively new and evolving field of transplant diagnostics. The currently used classification is simple, mechanistic-cum-time based, and flexible. In general, humoral components of the specific immune system and innate immunity play important roles in the immediate and early post-transplant period. These are relatively well reported in the literature and now well controlled, resulting in improved xenograft survival. The transplant obstacles related to the regulation of complement and coagulation cascades due chiefly to species incompatibility and chronic rejection are yet to be investigated and understood. Obviously, with the prolongation of graft survival to years, there is more need of understanding the mechanisms of chronic rejection and to enrich the diagnostic armamentarium for pathologic evaluation. There is a need to increase awareness and train transplant pathologists in this emerging field of transplant diagnostics.

## FOOTNOTES

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