Peer Review File
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Reviewer A

I commend the authors on a timely and good discussion. Please see my thoughts.

The authors convey their ideas under the assumption that a xenograft will offer similar outcomes as an allotransplant. Please add caveats and/risks to your positive views on xenotransplantation such as xenozoonosis, multiple surgeries and possible prolonged hospital length of stay due to infection possibility.

Response:
We have added passages on pages 8 (xenozoonoses), 11 (costs), 11 (hospital stay), and 14 (multiple surgeries) to address these points.

Although children may have some immunologic advantage to xenotransplantation compared to adults it is important to consider that its use as a bridge may expose the patient to additional surgeries and procedures that could also increase a patients PRA, like blood transfusions and then make them a worse candidate for allotransplantation even though there is low fear of raised anti-pig antibodies. Please note limitations within the manuscript about multiple/additional surgery/procedures exposure.

Response:
We understand the reviewer's concern but suggest that the risk of allosensitization from xenotransplantation is probably less than if mechanical circulatory support is provided to the patient. We have not commented on this point in the text.

Line 205 paragraph where costs are discussed please consider discussing similar points along the lines above about surgeries for implanting and removing the xenograft as this can also be costly. Surgical and hospitalization costs related to xenograft implantation/removal may exceed that of 69 thousand noted. A cost benefit analysis would need to be performed to weigh the implementation of xenotransplantation.

Response:
We accept this comment but, if the patient is able to be discharged home, the costs will be significantly less than if mechanical circulatory support is necessary.

Line 65 add the word "human" to "primary transplant" until clinical trials are performed

you cannot compare the outcomes of a pig organ to that of an allotransplant even if superior to SV palliation.

Response:

The word 'human' has been added (page 15).

Line 67 please avoid extreme statements such as "but the milieu and technology have collided to offer the best opportunity up to this point in history". Extreme statements can be biased, subjective and contested to be untrue. Either reword or remove.

Response:

This sentence (at the end of the Abstract) has been reworded, as follows: "There are many remaining issues to be resolved before cardiac xenotransplantation enters regular pediatric clinical use, but experience in this field is progressing rapidly."

If the patient displays signs of Xenozoonosis risk this would ultimately also carry long hospital stays. It is unknown how long a patient will be monitored or remains in the hospital after a xenograft. Clinical management and algorithms are not in place for such scenarios yet. The cardiac xenotransplantation patient in Maryland was never able to be discharged. The possible recipients you discuss will be sick patients that even if they receive a xenograft may still not be able to go home to wait. Please add this caveat or expand discussion to where you discuss that the patient would simply be discharged home around line 215.

Response:

Based on our own experimental experience and that of others, we anticipate that the pig heart will function well for several weeks or months, and so we fully expect that the patient will be able to be discharged home as soon as a patient with an allograft. The adult patient in Maryland was an extreme case because he was extremely debilitated before the transplant, having been confined to bed on ECMO for 7 weeks. We have not commented on this in the text.

Reviewer B

Summary:

The recent clinical trial of pig cardiac xenograft (life-supporting, orthotopically) in the human could lead to a significant step forward in the application of xenotransplantation for future medicine. Although the use of pig heart xenografts in adult humans as an alternative to allotransplantation is easy to understand, the application in pediatric care remains obscure.

Heart transplantation in neonates is associated with the best results of any organ transplantation, but the lack of deceased human donor hearts in this age group limits its application. Therefore, cardiac xenotransplantation as a short-term bridge to allotransplant would be a significant advantage in infants with complex congenital heart disease.

In the present manuscript, the authors have described the indication of heart xenograft in pediatric patients (e.g., first-stage single ventricle palliation patients with end-stage heart failure).

The topic is timely and interesting. It might be important to discuss the application of cardiac xenotransplantation in pediatric patients for future clinical trials. Therefore, the reviewer believes that this review should contribute significantly to the field of xenotransplant research as well as clinical application, as xenotransplantation appears to be approaching the clinical trial phase.

However, the reviewer has two questions that the authors need to consider to answer if this manuscript will be published.

Minor comment:
Q1. What is the first milestone the authors would like to achieve regarding xenograft survival (e.g., 6 months?) as a bridge for allotransplantation?

Response:

In the laboratory, we are aiming to demonstrate consistent good function of an orthotopically-transplanted pig heart for 4-6 months, followed by its replacement by a baboon allograft that functions well for 2-4 months. If this can be achieved, we would propose a clinical trial in which we would anticipate that the pig graft supports the patient until a deceased human donor heart becomes available, which on average would take 4-6 months. We have now added statements to this effect in the text (pages 12-13), as follows:

"As a research group we are actively evaluating orthotopic cardiac transplants from GEPs into juvenile baboons (4-6 kg in weight). Our goal is to demonstrate consistent 4-6 months survival of orthotopic (life sustaining) cardiac xenotransplants with no evidence of cross-reactivity of anti-pig antibodies with human antigens that would preclude subsequent allotransplantation. To ensure clinical feasibility, at 4-6 months we will excise the pig heart and replace it with a baboon allograft, which we will monitor for a further 2-4 months. If these milestones are achieved, we believe a clinical trial in infants failing traditional single ventricle palliation is warranted."

Q2. If unfortunately, the xenograft would be rejected before allotransplantation, what

approach do the authors consider as an alternative therapy (the native heart has been removed)?
Please address them in the text.

Response:

There is clearly a risk of the procedure as no alternative therapy is likely to be available. However, we would not proceed to a clinical trial unless our laboratory studies in the pig-to-nonhuman primate indicated that a clinical trial would have a realistic chance of success. As nonhuman primates are more likely to have preformed antibodies to TKO pig cells whereas our in vitro data indicate that *no* human infants have anti-pig antibodies (and our current immunosuppressive regimen successfully prevents the production of de novo antibodies) we believe that rejection is unlikely to be problematic. We have added a statement to this effect (page 13), as follows:

"There is clearly a risk in this approach because, in the event of graft failure, there is likely to be no alternative therapy available. However, without a xenograft, the risk of death on MCS is also high. However, we would not proceed to a clinical trial unless our laboratory studies in the pig-to-nonhuman primate model indicated that a clinical trial would have a realistic chance of success. As nonhuman primates are more likely to have preformed antibodies to TKO pig cells whereas our in vitro data indicate that *no* human infants have anti-pig antibodies (Figure 1) (and our current immunosuppressive regimen successfully prevents the production of de novo antibodies) we believe that rejection is unlikely to be problematic."

Reviewer C

Cleveland et al present an interesting article in discussion of cardiac xenotransplantation as a management strategy for infants with complex congenital heart disease, especially those with single ventricle disease. The article provides a refreshing overview of the current state of proceedings as things in this field are rapidly evolving.

They provide an interesting insight to the limitation of donor hearts and the current status of pushing boundaries to increase pool of donors. Uniquely, CHD patients may be limited in outcomes with MCSD, warranting the need for xenotransplantation. The group gives a detailed argument about why infants with CHD are uniquely poised for xenotransplantation both immunologically and limited applicability of MCSDs in these patients. Additionally, there is a good amount of comparison to current three-stage palliation and xenotransplantation as a unique solution.

Article should discuss more of limitations to clinical application as it currently stands with xenotransplantation, what more is needed to get to clinical trials?

Response:

We have included more discussion about potential limitations (pages 8, 11, and 14). We have also added a comment on page 12. However, we suggest that (i) there are few surgical hurdles that are not already faced with cardiac allotransplantation, (ii) there are now encouraging experimental data that the currently-available immunosuppressive regimens prevent rejection at least for several months, and (iii) the concerns regarding xenozoonoses are now greatly reduced, particularly as pigs are now available in which PERVs have been inactivated.

Reviewer D

I had the privilege of reviewing your manuscript entitled The Potential of Cardiac Xenotransplantation for the Treatment of Infants with Complex Congenital Heart Defects.

This is a very important and topical issue. The manuscript is well written and easy to understand. I enjoyed reading the manuscript. What we already know and what the authors have shown in their review:

- Page 4-5: Due to the lack of donors, we have longer waiting times on the list worldwide. 20% to 30% of patients die on the list.
- Page 5-6: Mechanical circulatory support is often necessary when patients are very ill and require MCS to stay alive until a suitable organ is obtained. Outcomes in infants and newborns with MCS are inferior to results in infants without MCS.
- Page 6-7: Modern technology and experience in early molecular biology techniques, in addition to the lack of identified xeno-antigen targets, have shown that rapid and typical clinical implementation is precluded. Scientists identified the three major carbohydrate antigens expressed on porcine endothelium against which humans have natural antibodies. Genetically engineered triple knockout (TKO) pigs that do not express either of these antigens are now available.
- Page 8: ABO-incompatible heart transplantation has shortened the waiting time in some cases. However, serious organ shortages in general persist. This type of transplantation has theorized that because human infants lack humoral responsiveness to stimulation by carbohydrate antigens and have low levels of antibodies to non-self A and B group antigens; they tolerated transplants from ABO-incompatible donors. That is already known about donor-specific B-cell tolerance and a similarly blunt and responsive humoral response to a xenograft.
- Page 9: High costs for pediatric patients with MCS under the age of 12 years as they remain hospitalized until they receive a suitable organ, which increases the costs enormously.

Pediatric application of xenotransplantation: This point is the most important part of this work. In my opinion, as pediatric cardiac surgeons, we should consider two important standpoint in performing of pediatric xenotransplantation. First we have high experimental competence with little clinical experience in xenotransplantation. However, we are accelerating the clinical implementation of pediatric

xenotransplantation. In this case, if this first step in pediatric xenotransplantation leads to serious complications, then there will be a heavy price to pay in terms of moral and ethical consequences worldwide.
On the other hand, further clinical developments in the field of xenotransplantation in adult patients could be expected and then with more greater clinical experience, pediatric xenotransplantation could be considered with greater certainty.
In any way, performing of pediatric xenotransplantation in critically ill pediatric patients with parental consent, treatment protocol (pre- and post-transplant), and ethically informed consent would have a very serious impact on the management of congenital heart disease in severe end-stage heart failure.

Response:

We thank the reviewer for his/her clear understanding of the points we have raised.

We have included the following paragraph on pig heart transplantation (page 14):

We believe there are significant advantages of our proposal to employ a pig xenograft as a bridge to allotransplantation. No NHP has yet survived beyond 9 months after orthotopic transplantation of a pig heart, and therefore the prospects for destination therapy are presently limited. Bridging of infants for 4-6 months would be much more feasible. Bridging does not commit an infant to a lifetime dependent on a pig heart which, in view of our limited knowledge of the field at present, must be an advantage.

Reviewer E

1. the Authors failed to discuss the importance of the pre-natal diagnosis, currently available at least in 90-95% of cases. This, in particular in the presence of complex congenital heart defects such as single ventricle, hypoplastic left heart syndrome, would allow sufficient time for counseling the parents about the potential availability of xenotransplantation among the treatment options, and in the case of their interest, of organizing the required prenatal clinical preparation.

Response:

We thank the reviewer for raising this important point. We have now included two paragraphs addressing this point (page 15), as follows:

"Over the past two decades there have been significant improvements in fetal diagnosis. In the present era, there is an expectation of accurate and detailed diagnosis of complex CHD in fetuses referred to pediatric cardiologists for diagnosis. Diagnosis before birth would allow sufficient time for counseling of the parents about the potential availability of xenotransplantation among other treatment options. If they wished to explore xenotransplantation, the time

before birth would allow prenatal clinical preparation.

The ability to accurately diagnose CHD in the fetus also opens up the possibility of modifying the neonatal immune response to xenoantigens and thereby improving the outcome of cardiac xenotransplantation. In fact, this ability could potentially result in cardiac xenotransplantation utilized as destination therapy for a group of neonates that have been documented to have poor prognosis."

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2. The availability of xenotransplantation could allow, in grown up children with heart failure and increased pulmonary vascular resistance, the possibility of assisted heterotopic assisted heart transplantation, with a second left ventricle providing the pulmonary blood flow
https://pubmed.ncbi.nlm.nih.gov/3290401/

Response:

The reviewer is correct although growth of the heterotopic thoracic donor pig heart can interfere with the function of both hearts by compressing the native heart (Baur A., et al. Xenotransplantation 2010;17:243-249). We have not discussed this point in the text.