Peer Review File

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Reviewer A

Good paper. Clear and precise

We appreciate the encouraging words from the reviewer.

Reviewer B

Summary:

Bovine- or porcine-derived xenografts are commonly used for surgical reconstruction in congenital heart disease. However, it has been concerned about using xenografts because they are immunogenic for humans. Although aldehyde fixation to crosslink xenoantigens results in masking xenoantigens, aldehyde fixation is a suboptimal solution and degraded over time, which induces immune activation and inflammation, eventually leading to graft failure. Therefore, patients need reintervention to replace the defective bioprosthetic. Decellularisation to remove antigens material has been suggested as an alternative approach; however, incomplete decellularisation cause a substantial immune activation. The authors first reviewed current commercially available xenografts and cryopreserved homografts used in surgical pediatric CHD intervention. Then they discussed alternative strategies for their graft (e.g., using tissue from genetically modified pigs). The manuscript is well written, organized, and a comprehensive review that provides important information and citations. However, several questions (Minor comments) need to be answered if this manuscript is accepted for publication.

We thank the reviewer for this concise summary of our work, we answer the specific questions raised below.

Minor comment:

Q1. Introduction (Page 2). The authors addressed "Two major epitopes present on the -- the biggest hurdles". Currently, three carbohydrates (Gal, Neu5Gc, Sda) are recognized as xenoantigens for humans.

We thank the reviewer for bringing this to our attention. We have amended the text accordingly and included the recognised antigen Sda in your review. Please see line 29 of the introduction, the text is copied below for convenience.

Three major epitopes on transplanted animal tissue present the biggest hurdles: galactose-α1,3-galactose (α-Gal), N-glycolylneuraminic acid (Neu5Gc), and Sda

Q2. The authors primarily focus on the graft, including animal-derived (i.e., xenograft) for surgical reconstruction in congenital heart disease. However, some of the information the authors addressed included heart xenotransplantation. Do the authors want to address the heart xenograft, the tissue graft, or both in this review? The immune

response to aldehyde fixation graft and the strategies to reduce this response differ from those of fresh organ xenograft. For example, severe immune suppressive therapies are definitely necessary for patients after heart xenotransplantation but not in aldehyde fixation graft transplanted patients. The function of the human transgene (e.g., human complement regulatory proteins such as CD55) could be lost after aldehyde fixation and decellularization treatment of the graft. Please address this point in the text.

We agree with the reviewer that some of the information pertains to whole organ transplantation rather than tissue grafts, which is the focus of this review. Currently immunosuppression is not used in cardiac grafting, and we did not wish to mislead the reader. To address this confusion, we have removed reference to whole organ transplantation within the mechanisms of rejection and long-term outcomes section to simplify this narrative. Additionally, we have removed the paragraphs pertaining to whole organ transplant in the pharmacological treatments and emerging strategies of intervention section, copied below:

"A recent study combining decades of preclinical xenotransplantation research achieved up to 195 days of life-supporting xenograft heart function in baboons (140). Until this point, maximum reported survival of a NHP after a life-supporting heart transplant was 57 days (108). Notably, a study which implanted α-Gal KO pig hearts expressing human membrane cofactor protein CD46 and human thrombomodulin concluded that non-ischaemic preservation of the porcine organ and restriction of post-transplantation heart overgrowth by the sirolimus prodrug temsirolimus, an mTOR inhibitor, is critical for prolonged survival. This promising work using a GM pig and immunosuppressive therapy points towards significant progress in the success of heart xenotransplant in NHP and paves the way toward heart xenotransplantation in the clinic.

Though not a paediatric CHD application, a testament to scientific achievement recently made headlines with the first successful pig-to-human xenotransplantation (135, 141). The patient, diagnosed with end-stage heart disease, underwent the experimental surgery as the only chance to save his life. The transplanted heart originated from a GM pig with ten genome edits: knockdown of three immune-rejection genes, insertion of six human genes, and one gene for growth inactivation (135). Short-term postoperative results were positive, with the patient able to move without cardiopulmonary bypass. Though HAR was avoided, an achievement that should not be minimised, the patient's condition deteriorated and he died two months after transplant (135). Despite this, the event is a huge step forward for the use of xenotransplants in a clinical setting."

However, we still believe it is important to highlight that this work is ongoing and thus we have added a sentence to this effect in the pharmacological treatment and emerging strategies of intervention, seen below.

"It is important to note that full xenogenic heart transplantation is not currently a viable

option due to the need for blank immunosuppression, highlighting the need for alternative strategies such as fixatives."

Q3. Ref#21 and 22 (Page 17). The information on the references needs to be completed.
We apologise to the reviewer for this oversight, we have corrected the incomplete references.

Reference 22. Bartoli-Leonard FA-O, Zimmer JA-O, Aikawa EA-O. Innate and adaptive immunity: the understudied driving force of heart valve disease. Cardiovasc Res. 2021;117(13):2506-24.
Reference 23: Farlex Partner Medical Dictionary. 2012. S.v. "Rejection".

Reviewer C

Harris et al. provide a quite comprehensive and well-written review on the role of xenotransplantation and homografts in the use of treating congenital heart disease. They not only have a detailed description of the appropriate preservation method and its unique application to Xenografts but also detail the recent genetic advancements. This review not only is a great perspective of the literature but also gives great clinical insight. The authors should be commended for their work.

We thank the reviewer for the kind words and motivating comments. We hope by publishing such a review we will contribute to furthering scientific discord around novel CHD treatments.

Reviewer D

This manuscript reviews the surgical outcomes of the various patches that have been used in the correction of congenital heart disease procedures. It provides a fairly comprehensive review of the topic, but it needs some significant revisions to render it suitable for publication. It would then be worth publishing. It is an ambitious manuscript because the topic includes widely different techniques and methods. However, the authors are not entirely successful as the paper is a difficult manuscript to read. Many different topics are discussed, and the reader is left rather bewildered.
We are sorry that reviewer D feels bewildered and finds our manuscript difficult to read, we aim to produce an article which appeals to a wide scientific audience and have therefore thoroughly edited the manuscript for clarity and readability. Please see the specific comments to address your individual points below.

I would suggest the authors (i) include more headings and subheadings so that the topics become clearer to the reader, and (ii) break up the text into shorter paragraphs.
We apologise for the lack of clarity regarding the headings within the manuscript. To give further insight into the topics included, we have replaced the title of second section from "Commercial Grafts" with "Utility and Function of Commercially Available Cardiac Grafts".

In line with the editorial instructions given by the journal, subheadings are not allowed in this manuscript and thus have not been included.

Regarding shorter paragraphs, we have broken paragraphs into smaller sections throughout the manuscript for ease or reading. We hope these editorial changes make the manuscript more palatable for the reviewer.

Importantly, after reviewing the literature fairly comprehensively, the authors do not provide us with clear conclusions with regard to which 'patches', etc. to use in reconstructing the heart. Having made such a review, the authors should be in a strong position to come to some conclusions in this respect.

We apologise that the reviewer feels there is no clear conclusions within the manuscript. Unfortunately, the field of implantable cardiac prostheses is still in its infancy and thus strong conclusions cannot be drawn. We hope that this review stimulates discussion around directionality within this continuously evolving and exciting field so more substantiative conclusions can be drawn in the future. In the "Utility and Function of Commercially Available Cardiac Grafts" section, we address the governmental approval of current cardiac tissue repair patches and highlight the lack of definitive solution within the field. Moreover, we have addressed this within the conclusion, please see below for the text.

Utility and Function of Commercially Available Cardiac Grafts

"In the past decade, small intestinal submucosa (SIS) has gained increasing attention and has become one of the bioscaffolds of choice for decelluarisation aimed at different surgical applications. SIS has excellent physical properties, including resistance to deformation, ease of handling and shaping, suture retention, good absorbability, and lack of immunogenicity (81). Commercially available decellularised SIS-ECM scaffold materials are predominantly obtained from pig small intestine, with the most widely used in cardiovascular applications being ProxiCor (previously Cormatrix). ProxiCor has received approval by the FDA and European authorities for cardiac tissue repair and is being used in various cardiovascular surgery applications, from early correction of paediatric CHD to surgical treatment of acquired heart disease (82). Despite huge initial enthusiasm, ProxiCor has not shown any significant advantages over previous biomaterials, providing variable clinical outcomes. A retrospective study observed that ProxiCor is a safer alternative to conventional patches when employed in atrial and ventricular defect closure, however, when used for arterial vessel reconstruction, is associated with higher reintervention rates as opposed to autologous pericardium (83). Additionally, preclinical studies using the ProxiCor fashioned as a trileaflet valved conduit in a pig model of thoracic aorta replacement showed this model failed to remodel in a structural and anatomical manner, leading to early fibrosis and calcification (84). Similar concerns were raised by studies using this SIS-ECM for valve repair in children (85). To conclude, despite being one of the mostly used materials in paediatric cardiac repair, ProxiCor has not proven to be a definitive solution to CHD

corrections."

Conclusion
"There is currently no perfect solution for the reconstruction of defective paediatric hearts, but xenotransplantation for paediatric CHD treatment holds the potential to overcome the imbalance between small-sized homograft availability and demand."

At nearly 6,500 words, the paper is rather long, and an effort should be made to reduce its length.
We are sorry to hear that the reviewer finds the manuscript too lengthy, by including such detail we hoped to present a comprehensive review of the topic. While seeking to maintain the depth of the manuscript and responding to reviewers comments, we have shortened the manuscript and it now reads at 5960 words.

It is not logical to state that "hyperacute rejection can be delayed" or refer to "delayed HAR". Hyperacute rejection is usually defined as rejection occurring within 24 hours, and so it is either 'HAR' or 'acute humoral xenograft rejection' or 'delayed xenograft rejection.'
We apologise if our use of language has confused reviewer D. While hyperacute rejection can be delayed and is used within the field of immunology (Montoliu, et al. hallmark study on kidney transplantation in twins, for example), we agree delayed xenograft rejection is a perfectly suitable synonym and thus have changed the text and Figure 2 accordingly.

"Further evidence of the xenoantigen-mediated delayed HAR is seen in comparisons between homografts and xenografts."
Is now changed to:
"Further evidence of the xenoantigen-mediated delayed xenograft rejection is seen in comparisons between homografts and xenografts."

"Following the delayed HAR through natural xenoantigens, cell-mediated rejection can be seen in xenografts grafts in the months to years following implantation"
Is now changed to:
"Cell-mediated rejection can be seen in xenografts grafts in the months to years following implantation"

"Delayed hyperacute rejection results from reduction of fixation efficacy in the extreme cardiac environment and initiates as immunoincompatible donor antigens become exposed in the days and weeks following implantation."
Is now changed to:
"Delayed xenograft rejection results from reduction of fixation efficacy in the extreme cardiac environment and initiates as immunoincompatible donor antigens become exposed in the days and weeks following implantation."

Reference 93 refers to anti-Neu5Gc antibodies and so is inappropriate where cited . (The pages are not numbered, making it difficult to refer to where changes need to be made.)

We sincerely apologise to the reviewer for this error. Reference 93 has now been replaced with the following references.

Reference 106. Galili U. Anti-Gal: an abundant human natural antibody of multiple pathogeneses and clinical benefits. Immunology. 2013;140(1):1-11.

Reference 107. Cooper DK, Koren E, Oriol R. Oligosaccharides and discordant xenotransplantation. Immunol Rev. 1994;141:31-58.

Reference 108. Helder MRK, Stoyles NJ, Tefft BJ, Hennessy RS, Hennessy RRC, Dyer R, et al. Xenoantigenicity of porcine decellularized valves. J Cardiothorac Surg. 2017;12(1):56.

When the authors discuss the mechanisms in volved in organ xenotransplantation, their relative lack of knowledge is noticeable. The authors refer to "recent research" but it actually relates to discoveries made in the 1990s, possibly reflecting the authors relative lack of knowledge about the field of xenotransplantation. These aspects of the paper should be improved. For example, the work of Cleveland and his colleagues relating to pig heart transplantation in baboons is not discussed.

We are disappointed to read this reviewer does not believe we have the knowledge required to address this topic. Nevertheless, we have undertaken a thorough literature review prior to commencement of this project and believe we are well positioned in the field to publish such a manuscript. The manuscripts we are referring to in our 'recent research' section were published between 2022 and 2021 and are still widely considered up to date in their scientific methodology and findings.

To address the second point, due to the word count and the limited scope of the review we have needed to remove information pertaining to full heart xenotransplantation. However, we thank the reviewer for bringing this seminal piece of work to our attention and agree the Cleveland study is a landmark in the field of xenograft transplantation.

There is some confusion as to what they define as 'bioprosthetic' grafts. These should surely include any graft that includes human or animal-derived tissue.

We apologise to the reviewer for this confusion. Throughout the manuscript we have sought to clarify our nomenclature, by using the word "xeno-" when referring specifically to animal derived grafts, "homo-" when referring specifically to human derived grafts, and the term, "bioprosthetic" when referring to either or both of the aforementioned grafts more generally. These changes can be found highlighted within the manuscript.

The role of the vigorous metabolism of young people is not referred to sufficiently in regard to calcification of bioprostheses.

We appreciate the reviewer bringing this interesting topic to our attention. Within the

manuscript we highlight the fact degeneration of grafts occurs more rapidly in the paediatric population than the elderly. Although we do identify calcification as playing a crucial role in long term graft failure, the in-depth metabolomic process of calcification is outside the scope of this review and we are saddened that we cannot discuss this in more detail. However, we have included the line below to link to metabolism in the context of calcification. However, we cannot find a reference linking "vigorous metabolism" regarding calcification of bioprostheses in the context of cardiac grafting, would it be possible for the reviewer to kindly share this?

"Notably, paediatric patients have a stronger immune system (139) and a higher basal metabolism (140) than the elderly, the two populations which tend to receive xenografts, and that may contribute to the faster graft degeneration seen in the former population".

Many of the references do nor refer to the original work. For example, reference 100 should be replaced by Kuwaki K et al 2005. The paper on the first pig heart transplant by Griffth et al is not referenced. The information provided on this case is very unclear. We apologise for this oversight and thank the reviewer for bringing this to our attention. Reference 100 has been replaced with the Kuwaki K et al paper from 2005:

Reference 147. Kuwaki K, Tseng YL, Dor FJ, Shimizu A, Houser SL, Sanderson TM, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. Nat Med. 2005;11(1):29-31.

Unfortunately, due to the word limit and trimming of the manuscript we have now removed mention of the first pig-to-human heart xenotransplantation, refining the scope of the review to implantable cardiac grafts instead. We appreciate the reviewer bringing this to our attention and have removed the entire section. We hope this section now reads clearly.