Journal of Clinical and Translational Research
Review process file 03.201702.001

Acellular porcine heart matrices: whole organ decellularization with 3D-bioscaffold & vascular preservation

Alice S. Ferng, Alana M. Connell, Katherine M. Marsh, Ning Qu, Annalisa O. Medina, Naing Bajaj, Daniel Palomares, Jessika Iwanski, Phat L. Tran, Kapil Lotun, Kitsie Johnson, Zain Khalpey

Corresponding author: Zain Khalpey, 1656 E. Mabel Street Medical Research Building Khalpey Lab, Room 126 Tucson, AZ 85724, United States

Handling editor:
Bote G. Bruinsma
Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, United States
Department of Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Review timeline:

Received: 4 October, 2016
Editorial decision: 27 November, 2016
Revision received: 27 February, 2017
Editorial decision: 10 March, 2017
Published online ahead of print: 15 March, 2017

1st Editorial Decision
Date: 27 Nov, 2016

Ref.: Ms. No. JCTRes-D-16-00033
Acellular Porcine Heart Matrices: Whole Organ Decellularization with 3D-Bioscaffold & Vascular Preservation
Journal of Clinical and Translational Research

Dear Dr. Khalpey,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. A major concern raised with your submission is the qualitative nature of your analysis and it's insufficiency in standardizing the decellularization process. Other concerns raised pertain to the interpretation and presentation of your data. I share these concerns and would suggest you address them in full should you decide to resubmit.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Journal of Clinical and Translational Research
Review process file 03.201702.001

Your revision is due by February 27th, 2017. Should you need more time to complete a revision, please let us know.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Dr. Bote G. Bruinsma
Editorial Board Member
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1:

This is a manuscript on investigating different decellularizing agents and perfusion pressures for porcine heart decellularization. The results were compared by analysis of physical appearance, hydroxyproline content, and elastic modulus. The best condition was further analyzed for DNA content, histological stains (H&E and Masson's trichrome), ultrastructure (SEM, TEM), and vascular architecture. While interesting, the claims are overly qualitative, the results are not organized well and the novelty of the work is minimal because decellularization of large scale hearts with SDS has been shown before.

The authors claim that their aim is to standardize the decellularization protocol for porcine hearts, by comparing the perfusion pressures and the type of detergents and find an optimal combination of both. Standardization requires quantification of the end results which they fail to do. The optimal pressure is determined qualitatively by describing the physical appearance of the scaffolds at the end of the protocol (Table 1). The optimal detergent type and concentration was determined through visual inspection of the color of resulting scaffolds (Figure 1, table 1). While these observations are ok, they need to be confirmed by quantitative data (like DNA content, or ECM components). Their only quantitative data comparing different detergents are given in Table 2 in terms of elastic modulus which suggest that the hearts that are not decellularized completely have the least elasticity. In my opinion, this is an unexpected result and the authors do not explain or discuss these findings.

It would be interesting to see how different decellularization techniques affect the recellularization.

Finally, the figures do not have captions. In section 2.2, the authors refer to Figure 1 as showing the decellularization apparatus, but the figure only shows the native and decellularized organs.

Reviewer #2:

Journal of Clinical and Translational Research
Review process file 03.201702.001

In the manuscript entitled, "Acellular Porcine Heart Matrices: Whole Organ Decellularization with 3D-Bioscaffold & Vascular Preservation", the authors systematically compare different decellularization methods of porcine hearts with respect to detergent type and perfusion pressure. In comparing detergents, the authors tested different concentrations of SDS (3, 5, 10%), as well as CHAPS, 1% OGP, and 3% Triton X-100. In testing perfusion pressures, the authors applied retrograde aortic perfusion ranging from 70-140 mmHg. The general outcomes from this systematic comparison are summarized in Table 1, with closer evaluation of definitive decellularization metrics in subsequent experiments.
The premise of the work is highly appreciated. While other groups have systematically evaluated detergents and perfusion pressures in decellularization of other organs (lungs, kidneys, livers), this type of comparison is rather limited in whole-heart decellularization, especially in large hearts. In addition, evaluating the mechanical properties of the acellular cardiac scaffolds resulting from different decellularization methods will have a profound impact with regards to material integrity, cell attachment/integration, cell maturation/function, and using the matrix in cardiac tissue engineering applications.
With this being said, the manuscript is in need of major revision before I can recommend publication in the Journal of Clinical and Translational Research. There are a number of major concerns ranging from missing or limited citations, to methodologies, to representation of results, to interpretation. The manuscript has merit and promise, but these areas of concern must be addressed to satisfy the rigor and scrutiny of the current state of the field in organ decellularization.

Major concerns:

1. There appears to be a limited number of citations with respect to how large the field of whole-organ decellularization has grown over the last 5+ years. The manuscript should include references on other groups that have done systematic work to evaluate decellularization methods, such as:
a. Sullivan, D.C. et al, “Decellularization methods of porcine kidneys for whole organ engineering using a high-throughput system. Biomaterials 33, 7756–7764 (2012).
b. Uygun, B.E. et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat. Med. 16, 814–820 (2010).
c. Gilpin, S. E. et al. Perfusion decellularization of human and porcine lungs: bringing the matrix to clinical scale. The Journal of heart and lung transplantation 33, 298-308 (2014).
d. Jensen, T. et al. A rapid lung de-cellularization protocol supports embryonic stem cell differentiation in vitro and following implantation. Tissue Eng. Part C Methods 18, 632–646 (2012).
e. He, M. & Callanan, A. Comparison of methods for whole-organ decellularization in tissue engineering of bioartificial organs. Tissue Eng. Part B Rev. 19, 194–208 (2013).

The manuscript should also include some of the newest relevant whole-heart decellularization papers that have helped to shape the field, such as:

f. Guyette, J. P. et al. Bioengineering Human Myocardium on Native Extracellular Matrix. Circ Res 118, 56-72 (2016).
g. Sanchez, P. L. et al. Acellular human heart matrix: A critical step toward whole heart grafts. Biomaterials 61, 279-289(2015).
h. Oberwallner, B. et al. Preparation of cardiac extracellular matrix scaffolds by decellularization of human myocardium. J Biomed Mater Res A 102, 3263-3272 (2014).
i. Lu, T. Y. et al. Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. Nature communications 4, (2013).

The authors should also consider referencing some of the broader statements in the text, which are significant contributions to the field and deserve recognition. Some examples include:

j. Page 4, second paragraph of the Introduction Section, line 55: “Far from an inert scaffold, the role of ECM has been increasingly recognized in cell signaling, differentiation and tissue homeostasis.”
k. Page 7, Introduction Section, line 14: “In particular, CHAPS retained ECM and mechanical elasticity following lung decellularization fairly robustly…” – What does this mean? And who showed it?
l. Page 15, Discussion Section, line 29: “Though decellularization techniques have thus far been unable to remove 100% of cell material in larger animal model and human hearts…” – Add appropriate human heart references.

2. In Section 2.1 of the Materials and Methods, the values for whole-animal and procured porcine heart weights appear to be too imprecise or generic. If possible, the values should be represented as mean +/- standard deviation, with significant digits. Presumably the data exists for these values. If full data sets are not available, perhaps a subset would suffice as a representation of for both animal and heart weights.
3. In Section 2.2 of the Materials and Methods, the first sentence suggests that there is an image of the custom-built apparatus used for decellularization in Figure 1. Figure 1 does not contain an image of the apparatus.
4. In the histological assessment, Figure 2D does not represent a decellularized sample as the red staining depicts a large number of cardiomyocytes or muscle fibers still embedded in the matrix. Please choose a better representative image more indicative of a heart decellularized using SDS (like the heart scaffold depicted in Figure 1B).
5. The histological assessment would be more convincing of your premise if you included more comparative samples from different decellularization protocols. Could you generate a composite of histology similar to the composite of Figure 1?
6. In the SEMs, it is unclear which features in Figure 3A (native) are comparable to Figure 3B (decell). Please elaborate with respect to fiber structure, pore size, tertiary matrix structures. Perhaps a combination of magnifications will help to depict these features. Also, in the SEM methods, can you clarify which decell regimen did you used for the analysis?
7. DNA quantification data should be back-calculated and normalized to the wet weight of the

original tissue sample. DNA concentrations represented as ng/uL by read-out of the NanoDrop are not comparable head-to-head, and are not conventional compared to other works in the field (Crapo 2011, Wainwright 2009, Guyette 2016, etc.). Native and decell tissue samples of approximately the same size will have vastly different weights, as decellularized samples are now void of the "cellular weight". Normalization to wet tissue weight is necessary – it will allow you to compare samples head-to-head, and also allow you to compare values to other studies in the field. The use of the NanoDrop for this study is somewhat unconventional. While the NanoDrop is widely used in molecular biology for dsDNA, ssDNA, and RNA - I am unsure of how to back-calculate and normalize to the wet tissue weight. If this becomes challenging, I suggest using the Quant-iT PicoGreen dsDNA assay kit.
8. In Figure 4 of the DNA analysis, what is "Percent Recovery" in the chart below the graph? dsDNA was lost, not recovered, right?
9. The protocol using SDS and 120 mmHg was used to show removal of DNA, but it is then later concluded that 90 mmHg is sufficient/complete decellularization. How do you determine this? And can you provide both DNA and histological data for the SDS/90 mmHg protocol?
10. For collagen content, please state in the methods and in the results that the hydroxyproline assay was used to determine "insoluble collagen". To get a full representation of collagen content, you would need to quantify the "soluble collagen" content by Sircol assay (Biocolor, UK).
11. Similar to the DNA analysis, collagen content data needs to be back-calculated and normalized to the wet weight of the original tissue sample. It is difficult to draw conclusions from Figure 5 because we do not know if the variation is due to tissue/sample weight. This normalization step is necessary, and is the convention in the field.
12. For the angiograms, which decell method was used to render the tested scaffolds? And what was the flow rate used for injection? It would be helpful to add these details to the Materials and Methods section.
13. There appears to be more evidence or data that compares the different detergents. Can you highlight in one of your figures how different perfusion pressures contributed to the decellularization process? Probably best shown visually by histology, but you could also show comparisons of DNA or collagen content. Gross anatomy may also suffice, but it may not be as clear. This is a highlight of the manuscript, and it would be further supported by data that could accompany the summarized results in Table 1.
14. Please add N#'s to Table 1
15. The mechanical testing is a great addition. Please indicate the N#'s for each group, and please add standard deviations to Table 2.
16. Please add Figure captions – they appear to be missing from the version I received.
17. Some references appear to be incomplete. Citations for both Momtahan (2015) and Orlando (2011) should include the list of coauthors.

******Authors'rebuttal******

Journal of Clinical and Translational Research
Review process file 03.201702.001

Zain Khalpey
1501 N. Campbell Avenue
P.O. Box 245071
Tucson, AZ 85724
520.626.7806
zkhalpey@surgery.arizona.edu

Arizona, USA, 26 February 2017

Re: revision JCTRes-D-16-00033

Dear Dr. Bote G. Bruinsma,

Thank you for giving us the opportunity to resubmit a revised version of our manuscript entitled "Acellular Porcine Heart Matrices: Whole Organ Decellularization with 3D-Bioscaffold & Vascular Preservation."

We have majorly revised our manuscript from the last submission to include one additional figure depicting a schematic of our experimental setup, and another figure with histological data of the experiments performed using the detergents OGP and CHAPS. Furthermore, we included histology of heart tissue decellularized with the preferred detergent (3% SDS) at 90, 120, and 140 mmHg. We feel that this additional histologic data using both the H&E and Masson's trichrome stains clearly demonstrates both the nuclear/cellular content remaining in cardiac tissue and the collagen matrix structure for each experimental condition following decellularization.

We have addressed all of the comments of the reviewers to the best of our ability, and tracked these changes in a Word document (attached as supplementary material not for publication). Each point of revision or rebuttal for each reviewer comment has been detailed per comment as bullet points written in italicized red font.

We are grateful for the very detailed review of our previous manuscript and feel confident that addressing the reviewer's comments has considerably improved the quality of this manuscript. On behalf of all the authors, we would like to thank the reviewers and editors for their tremendous efforts in offering suggestions and thoughtful responses.

Best regards,

Dr. Alice Ferng
Division of Cardiothoracic Surgery

Journal of Clinical and Translational Research
Review process file 03.201702.001

Department of Surgery
University of Arizona College of Medicine
Tucson, AZ, USA

Reviewers' comments:

Reviewer #1:

This is a manuscript on investigating different decellularizing agents and perfusion pressures for porcine heart decellularization. The results were compared by analysis of physical appearance, hydroxyproline content, and elastic modulus. The best condition was further analyzed for DNA content, histological stains (H&E and Masson's trichrome), ultrastructure (SEM, TEM), and vascular architecture. While interesting, the claims are overly qualitative, the results are not organized well and the novelty of the work is minimal because decellularization of large scale hearts with SDS has been shown before.

- *As Reviewer #2 writes: "While other groups have systematically evaluated detergents and perfusion pressures in decellularization of other organs (lungs, kidneys, livers), this type of comparison is rather limited in whole-heart decellularization, especially in large hearts. In addition, evaluating the mechanical properties of the acellular cardiac scaffolds resulting from different decellularization methods will have a profound impact with regards to material integrity, cell attachment/integration, cell maturation/function, and using the matrix in cardiac tissue engineering applications." We agree with this assessment and believe it speaks to the novelty of the findings in this manuscript.*

The authors claim that their aim is to standardize the decellularization protocol for porcine hearts, by comparing the perfusion pressures and the type of detergents and find an optimal combination of both. Standardization requires quantification of the end results which they fail to do.

- *In this experiment, the concept of "standardization" is modeled off of our experimental observations, our laboratory's previous work, and the published results of numerous other groups on both the micro- and macro-scale. From this foundation of research, it was apparent that on the macro-level, decellularization was considered unsuccessful if the organs did not lose their coloration grossly and become more white/opaque. We initially did process a few of these grossly subpar organs to confirm, and subsequently deduced that it was both unreasonable and wasteful to do this for many dozens of organs if we could categorize organs as being incompletely decellularized early on in the project flow. However on a micro-scale, as Reviewer #1 noted, DNA content, histological stains, ultrastructure and vascular architecture were analyzed for the organs that did appear grossly decellularized. In this sense, end results were quantified. Both the micro- and macro-scale findings are how we based the definition of a successful decellularization. Of course, arguably this definition will vary depending on the utilization of the tissue and application (e.g. recellularization or reparative patches).*

The optimal pressure is determined qualitatively by describing the physical appearance of the scaffolds at the end of the protocol (Table 1).

- *This is correct. We first describe the physical appearance of the scaffolds qualitatively, prior to committing to a battery of further quantitative and qualitative measurements and analyses to examine the degree of decellularization as described above.*

The optimal detergent type and concentration was determined through visual inspection of the color of resulting scaffolds (Figure 1, table 1). While these observations are ok, they need to be confirmed by quantitative data (like DNA content, or ECM components). Their only quantitative data comparing different detergents are given in Table 2 in terms of elastic modulus which suggest that the hearts that are not decellularized completely have the least elasticity. In my opinion, this is an unexpected result and the authors do not explain or discuss these findings.

- *We consider the process of optimization and standardization as requiring both a qualitative and quantitative approach. Qualitatively, it is necessary to first determine if the final results are likely to be successful, and if further investigation is needed. If we could clearly identify undecellularized tissue, then we did not see the necessity to further confirm the same results quantitatively. However, for the samples that required quantitative processing, we provided multiple quantitative measurements including DNA content and collagen content. Additionally, histological stains (H&E and Masson's trichrome), ultrastructure (SEM, TEM), and vascular architecture were obtained for further results. The elastic modulus was performed to assess functionality, with higher values corresponding to higher resistance. Since more resistance (i.e. less elasticity) is seen in native hearts, the results seen in the study were the expected results. Clarification was added to a sentence in section 3.6 Cardiac Tissue Mechanics: "The native heart demonstrated the largest modulus of elasticity when compared with the decellularized heart, suggesting the native hearts possessed the most resistance and least elasticity."*

It would be interesting to see how different decellularization techniques affect the recellularization.

- *Yes, we agree that this would be very interesting and valuable to see. This is object of future research.*

Finally, the figures do not have captions. In section 2.2, the authors refer to Figure 1 as showing the decellularization apparatus, but the figure only shows the native and decellularized organs.

- *We had attached the Figure and Table legends in a separate file for the original submission, but have now included the legends at the end of this document so that they will not be left out for your further review.*
- *Thank you for your attention to detail, especially regarding the figure numbers and descriptions. Figure 1 has been correctly attached, and all of the labels updated throughout.*

Journal of Clinical and Translational Research
Review process file 03.201702.001

Reviewer #2:

In the manuscript entitled, "Acellular Porcine Heart Matrices: Whole Organ Decellularization with 3D-Bioscaffold & Vascular Preservation", the authors systematically compare different decellularization methods of porcine hearts with respect to detergent type and perfusion pressure. In comparing detergents, the authors tested different concentrations of SDS (3, 5, 10%), as well as CHAPS, 1% OGP, and 3% Triton X-100. In testing perfusion pressures, the authors applied retrograde aortic perfusion ranging from 70-140 mmHg. The general outcomes from this systematic comparison are summarized in Table 1, with closer evaluation of definitive decellularization metrics in subsequent experiments.

The premise of the work is highly appreciated. While other groups have systematically evaluated detergents and perfusion pressures in decellularization of other organs (lungs, kidneys, livers), this type of comparison is rather limited in whole-heart decellularization, especially in large hearts. In addition, evaluating the mechanical properties of the acellular cardiac scaffolds resulting from different decellularization methods will have a profound impact with regards to material integrity, cell attachment/integration, cell maturation/function, and using the matrix in cardiac tissue engineering applications.

With this being said, the manuscript is in need of major revision before I can recommend publication in the Journal of Clinical and Translational Research. There are a number of major concerns ranging from missing or limited citations, to methodologies, to representation of results, to interpretation. The manuscript has merit and promise, but these areas of concern must be addressed to satisfy the rigor and scrutiny of the current state of the field in organ decellularization.

Major concerns:

1. There appears to be a limited number of citations with respect to how large the field of whole-organ decellularization has grown over the last 5+ years. The manuscript should include references on other groups that have done systematic work to evaluate decellularization methods, such as:
a. Sullivan, D.C. et al, “Decellularization methods of porcine kidneys for whole organ engineering using a high-throughput system. Biomaterials 33, 7756–7764 (2012).
b. Uygun, B.E. et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat. Med. 16, 814–820 (2010).
c. Gilpin, S. E. et al. Perfusion decellularization of human and porcine lungs: bringing the matrix to clinical scale. The Journal of heart and lung transplantation 33, 298-308 (2014).
d. Jensen, T. et al. A rapid lung de-cellularization protocol supports embryonic stem cell differentiation in vitro and following implantation. Tissue Eng. Part C Methods 18, 632–646 (2012).

e. He, M. & Callanan, A. Comparison of methods for whole-organ decellularization in tissue engineering of bioartificial organs. Tissue Eng. Part B Rev. 19, 194–208 (2013).

The manuscript should also include some of the newest relevant whole-heart decellularization papers that have helped to shape the field, such as:

f. Guyette, J. P. et al. Bioengineering Human Myocardium on Native Extracellular Matrix. Circ Res 118, 56-72 (2016).
g. Sanchez, P. L. et al. Acellular human heart matrix: A critical step toward whole heart grafts. Biomaterials 61, 279-289(2015).
h. Oberwallner, B. et al. Preparation of cardiac extracellular matrix scaffolds by decellularization of human myocardium. J Biomed Mater Res A 102, 3263-3272 (2014).
i. Lu, T. Y. et al. Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. Nature communications 4, (2013).

The authors should also consider referencing some of the broader statements in the text, which are significant contributions to the field and deserve recognition. Some examples include:

j. Page 4, second paragraph of the Introduction Section, line 55: “Far from an inert scaffold, the role of ECM has been increasingly recognized in cell signaling, differentiation and tissue homeostasis.”
k. Page 7, Introduction Section, line 14: “In particular, CHAPS retained ECM and mechanical elasticity following lung decellularization fairly robustly…” – What does this mean? And who showed it?
l. Page 15, Discussion Section, line 29: “Though decellularization techniques have thus far been unable to remove 100% of cell material in larger animal model and human hearts…” – Add appropriate human heart references.

- *Thank you very much for your helpful suggestions regarding citations. Each of these citations has been included in the appropriate locations as carefully noted by the reviewer.*

2. In Section 2.1 of the Materials and Methods, the values for whole-animal and procured porcine heart weights appear to be too imprecise or generic. If possible, the values should be represented as mean +/- standard deviation, with significant digits. Presumably the data exists for these values. If full data sets are not available, perhaps a subset would suffice as a representation of for both animal and heart weights.

- *All porcine hearts were obtained from the University of Arizona Food Products and Safety Laboratory. Given that we could not determine the size of hearts prior to harvesting, we could only control for similar weights of the animal prior to organ harvesting. As reported, animals were gated within the weight bracket of 130 +/- 10 kg, and due to naturally occurring physiological differences, the procured hearts had a large range of sizes and weights.*

3. In Section 2.2 of the Materials and Methods, the first sentence suggests that there is an image of the custom-built apparatus used for decellularization in Figure 1. Figure 1 does not contain an image of the apparatus.

- *Thank you for noticing this. We have now added Figure 1 and made sure that figure legends are included as well at the end of the Word document.*

4. In the histological assessment, Figure 2D does not represent a decellularized sample as the red staining depicts a large number of cardiomyocytes or muscle fibers still embedded in the matrix. Please choose a better representative image more indicative of a heart decellularized using SDS (like the heart scaffold depicted in Figure 1B).

- *We consulted with our pathology department and had their help in analyzing and taking additional histology images for this paper.*
- *Additional images were provided for multiple decellularization experiments that used 3% SDS at 3 different perfusion pressures (new Figure 3). As we make the argument for in the paper – the ideal decellularization pressure will be between 90 to 120 mmHg. While there are some nuclei present after decellularization with 90 mmHg, we have confirmed with the pathologists that the cells are dead and preserved in a collagen meshwork, as demonstrated by the trichrome stain. Whereas, at 120 mmHg, the nuclei of cells is more fully removed, and the collagen meshwork is less dense. Arguably, it is not yet known whether dense or very loose collagen meshwork is more ideal for recellularization. It may be that a more dense matrix with a more preserved meshwork will be ideal, since cells involved in recellularization (stem cells, fibroblasts, etc.) will be unable to lay down extracellular matrix in a way that tightens up the collagen meshwork if it has been too far stretched apart, such as under 140 mmHg of perfusion pressure (Figure 3G and 3H).*

5. The histological assessment would be more convincing of your premise if you included more comparative samples from different decellularization protocols. Could you generate a composite of histology similar to the composite of Figure 1?

- *We agree with this, and have added additional histology to the paper. There is a new figure 3 and figure 4 that shows comparative samples from multiple decellularization experiments.*

6. In the SEMs, it is unclear which features in Figure 3A (native) are comparable to Figure 3B (decell). Please elaborate with respect to fiber structure, pore size, tertiary matrix structures. Perhaps a combination of magnifications will help to depict these features. Also, in the SEM methods, can you clarify which decell regimen did you used for the analysis?

- *We have added more detailed descriptions in the figure legend for the SEM samples (Figure 4). Notably, we mentioned that the fibrillar and junctional meshwork was intact between panels A and B, and that the cells seen on the native tissue surface (C) were removed in the decellularized heart (D). Your suggested wording was a helpful addition, and was also included.*
- *The decell regimen used (3% SDS) was added to the SEM methods.*

7. DNA quantification data should be back-calculated and normalized to the wet weight of the original tissue sample. DNA concentrations represented as ng/uL by read-out of the NanoDrop are not comparable head-to-head, and are not conventional compared to other works in the field (Crapo 2011, Wainwright 2009, Guyette 2016, etc.). Native and decell tissue samples of approximately the same size will have vastly different weights, as decellularized samples are now void of the "cellular weight". Normalization to wet tissue weight is necessary – it will allow you to compare samples head-to-head, and also allow you to compare values to other studies in the field. The use of the NanoDrop for this study is somewhat unconventional. While the NanoDrop is widely used in molecular biology for dsDNA, ssDNA, and RNA - I am unsure of how to back-calculate and normalize to the wet tissue weight. If this becomes challenging, I suggest using the Quant-iT PicoGreen dsDNA assay kit.

- *Thank you for pointing this out. We did in fact use the wet tissue weight when we processed these samples, thus back-calculation is not required. We however failed to detail this in our methods section previously and have now added this detail to the methods section under DNA quantification.*

8. In Figure 4 of the DNA analysis, what is "Percent Recovery" in the chart below the graph? dsDNA was lost, not recovered, right?

- *Correct - this mistake has been changed in the figure from "Percent Recovery" to "Percent Removal".*

9. The protocol using SDS and 120 mmHg was used to show removal of DNA, but it is then later concluded that 90 mmHg is sufficient/complete decellularization. How do you determine this? And can you provide both DNA and histological data for the SDS/90 mmHg protocol?

- *The statement that 90 mmHg is sufficient for decellularization was based on histological analysis showing that the nuclei were no longer viable, while the collagen meshwork was well-preserved. We have concluded instead that a perfusion pressure between 90 to 120mmHg is likely the ideal range. Through some preliminary additional work we have started, we have been able to recellularize some decellularized tissue at 90 mmHg using 3% SDS. However, these are only preliminary, and part of future experiments, so they are not included in the immediate scope of this paper, which was to compare different perfusion pressures and detergents. Figure 3 now includes additional histology for both 90 and 120mmHg experiments.*

10. For collagen content, please state in the methods and in the results that the hydroxyproline assay was used to determine "insoluble collagen". To get a full representation of collagen content, you would need to quantify the "soluble collagen" content by Sircol assay (Biocolor, UK).

- *Thank you for pointing this out. This detail was added in the section.*

11. Similar to the DNA analysis, collagen content data needs to be back-calculated and normalized to the wet weight of the original tissue sample. It is difficult to draw conclusions

from Figure 5 because we do not know if the variation is due to tissue/sample weight. This normalization step is necessary, and is the convention in the field.

- *Similar to DNA analysis, we neglected to mention in the text that the wet weight was used and have now added this to the manuscript. Additionally, the wet weight was used for the materials analysis, and both size and weight was used to normalize samples prior to elasticity measurements.*

12. For the angiograms, which decell method was used to render the tested scaffolds? And what was the flow rate used for injection? It would be helpful to add these details to the Materials and Methods section.

- *The 3% SDS method at a 120 mmHg flow rate was used. This detail has been added to the appropriate section.*

13. There appears to be more evidence or data that compares the different detergents. Can you highlight in one of your figures how different perfusion pressures contributed to the decellularization process? Probably best shown visually by histology, but you could also show comparisons of DNA or collagen content. Gross anatomy may also suffice, but it may not be as clear. This is a highlight of the manuscript, and it would be further supported by data that could accompany the summarized results in Table 1.

- *As stated in earlier points (4, 5, and 9), additional histology has been provided as requested. Figure 3 and 4 now compare histology between the different perfusion pressures and detergents.*

14. Please add N#'s to Table 1

- *We expressed that each experiment had an n=3 in the table legends, but it seems that all figure and table legends written had not been included in the document that was reviewed. They are found after the References section in this document.*

15. The mechanical testing is a great addition. Please indicate the N#'s for each group, and please add standard deviations to Table 2.

- *The N=3 for each group and the standard error mean was added and indicated in Table 2 and its associated legend.*

16. Please add Figure captions – they appear to be missing from the version I received.

- *Yes, thank you. They have been added after the References section so that they will less likely be separated from the main document.*

17. Some references appear to be incomplete. Citations for both Momtahan (2015) and Orlando (2011) should include the list of coauthors.

- *This has been corrected in the References section.*

Journal of Clinical and Translational Research
Review process file 03.201702.001

2nd Editorial decision
Date: 10 Mar, 2017

Ref.: Ms. No. JCTRes-D-16-00033R1
Acellular Porcine Heart Matrices: Whole Organ Decellularization with 3D-Bioscaffold & Vascular Preservation
Journal of Clinical and Translational Research

Dear Dr Khalpey,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

B. G. Bruinsma
Editorial Board Member
Journal of Clinical and Translational Research

****