Centre for Atherothrombosis and
Metabolic Disease

Hull York Medical School

Hull
University of Hull
Hull, HU6 7RX, UK

York
University of York
York, YO10 5DD, UK

www.hyms.ac.uk

4th December 2020

Dear Editor,

We are delighted that our enclosed manuscript '*Human Ex Vivo Wound Model and Whole-Mount Staining Approach to Accurately Evaluate Skin Repair*' has been favourably reviewed for publication in *The Journal of Visualized Experiments*. Please find below our rebuttal to the editorial and peer review comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Please ensure that citations follow an ascending chronology E.g. Line 534.

3. Line 103: Please specify the chemical(s) used.

4. Check lines 150-153. Are a total of 3 rinsing steps involved?

5. Line 186: Specify the humidity level.

6. Line 324: "Collect Z stacks.." instead of "Z stacks could be.."

7. Please include a single space between the quantity and its unit E.g. Line 377, 511: "3 mm" instead of "3mm".

8. Please do not use personal pronouns such as "we", "our" etc. in the protocol section.

9. Please include a single line spacing between the protocol steps. Furthermore, any text that cannot be written in the imperative tense may be added as a "Note." E.g.: Line 322-323

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. NovaRed, Alexa Fluor, Zeiss etc.

Response: We have now made the requested editorial changes to the manuscript. For point 4 we have also amended the text for clarification that there are 4 rinsing steps (2 x HBSS plus Abs, 1 x HBSS without antibiotics and 1 x DPBS).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The submitted manuscript by Wilkinson et al., describes the development of an ex vivo 3D human skin wound healing model and whole mount staining approach, for the quantification of wound healing responses in skin. The validation and potential usefulness of this model were confirmed by the impaired 'wound' re-epithelialization observed in skin derived from diabetic patients compared to normal skin, akin to non-healing apparent in chronic wounds such as diabetic foot ulcers, which are a major issue for healthcare providers worldwide. Consequently, by using human skin samples, this model not only supports the 3Rs remit in biomedical research, but could further be utilised in future for the routine screening of new therapeutic entities, in terms of identifying their respective wound healing efficacies in promoting diabetic skin wound re-epithelialisation and their mechanisms of action. As a considerable limitation to current chronic wound therapy development involves the inadequate nature of existing 3D organotypic and ex vivo models, the current model described here may aid the successful translation of novel therapeutics into approved clinical use in future. Overall, this is an interesting and well-performed study, which builds on previous ex vivo wound healing model work published by the Authors. Thus, the manuscript is concise and generally well-written, whilst providing sufficient and straightforward to follow details on the methods and techniques used, in order to allow this experimental model to be reproduced in other laboratories. Consequently, I only have relatively limited number of minor comments for the Authors to address:-

1. Protocol (page 4). It would be useful if further details were included on the patients from which the non-diabetic and diabetic skin biopsies were collected? This is particularly true for the diabetic skin and how these patients were selected?

Response: We thank the reviewer for raising this point. Non-diabetic skin was collected from patients undergoing routine surgery (mean age = 68). Diabetic skin was selected from donors who had established type II diabetes and a history of ulceration (mean age = 81). We have updated the text to include this information.

2. Related to this, it appears that the Human Skin Growth Media used was high glucose DMEM (presumably at 25 mM concentrations)? Could the Authors clarify whether both non-diabetic and diabetic ex vivo cultures were maintained in high glucose DMEM, as I would assume that non-diabetic cultures were maintained in more normal glucose concentrations (i.e. 5.5 mM glucose-containing DMEM)? If this is the case, details of the normal glucose medium needs to be included here. if not, could the Authors justify why both non-diabetic and diabetic ex vivo cultures were maintained in high glucose DMEM?

Response: All cultures were maintained in high glucose DMEM. The rationale for this is to allow evaluation of intrinsic differences in cellular behaviour rather than differences that may in fact be due to culturing each group in different glucose levels. Indeed, the fact that we see a difference between non-diabetic and diabetic skin when both are cultured in the same media conditions demonstrates that there are intrinsic cellular differences in the diabetic skin that contribute to delayed healing. It would be interesting to explore the role of these intrinsic versus extrinsic factors in skin culture in the future.

3. Protocol (page 6). Could the Authors please provide details on to what extent 'wound' formation by punch biopsy can vary between patient donors and skin sites used? i.e. Could this lead to potential problems with variability in wound healing responses within experimental groups and between different experimental groups?

Response: The reviewer raises an important point. In our extensive experience, we do observe donor differences at the point of wounding. For example, aged skin generally requires less force to generate wounds and skin with significant stretch marks can be

challenging to use. However, in general, most skin is suitable for ex vivo wounding. We have not directly compared the healing potential of ex vivo skin derived from different body sites, but we have successfully used skin from different body sites for ex vivo wound healing studies.

As with all human research, there is inherent variability between donors. There are also many external factors that can influence ex vivo healing, a significant one being the time from surgical tissue collection to ex vivo wound set up. For this very reason, we never split treatment groups across multiple donors/sites. We have updated the discussion text to include some of this information.

4. Results (page 11). The Authors very nicely show enhanced wound closure and re-epithelialization in normal skin 'wounds' over 7 days in culture. However, have the Authors extended these cultures beyond 7 days and if so, does the model show any evidence of keratinocyte differentiation/stratification within this air/medium interface model, once wound repair is complete?

Response: We have not extended ex vivo skin cultures beyond 7 days as we primarily use this model to evaluate wound re-epithelialisation. We agree that it would be interesting in the future to explore keratinisation/stratification of the re-epithelialised tissue.

5. Blood vessel assessment (page 13 and Figure 3). Could the Authors please explain the choice of a-SMA as a blood vessel marker, over more established angiogenic markers, e.g. CD31?

Response: We have optimised the α-SMA monoclonal from Abcam for both histological and whole-mount wound samples. We thank the reviewer for the suggestion of exploring CD31 and will do so in the future.

6. Figures 3 and 4 (pages 13 and 14). Could the Authors please clarify which ex vivo skin samples were used to generate the data presented in Figures 3 and 4? I presume that these are derived from normal skin ex vivo cultures, but need to be confirmed within the text and the Figure legends. Related to this, could the Authors explain why data for both normal and diabetic skin were not included here, as characterisation of these additional parameters would provide added strength to the ex vivo model, in terms of the additional differences between normal and diabetic skin wound healing observed.

Response: The data presented in Figure 3 and 4 pertains to normal (non-diabetic) skin. We have updated both text and Figure legends to clarify this. We agree that detailed characterisation of the ex vivo diabetic healing model is important, but we feel this is beyond the scope of this current methods paper. Our approach was to include diabetic skin data in Figure 2 to demonstrate the utility of the ex vivo model to show differences in overall wound closure. By contrast, the focus of Figure 3 was to illustrate the diversity of markers that can be evaluated. Nevertheless, we thank the reviewer for this suggestion and we plan to address it in the future.

Reviewer #2:

Manuscript Summary:

This study describes a human ex vivo wound model and whole mount staining for the performance of high throughput re-epithelialization measurements. The manuscript is well written and touches on the main strength and weaknesses of the model.

Minor Concerns:

The authors did not elaborate on the possibilities to use these samples for traditional histological analyses that could complement their whole mount evaluation. Can this be done? If so, it may be interesting to indicate how. It would have been interesting to know if findings on the whole mount correlates with findings on sections.

Response: It is absolutely possible to process (wax or OCT) and section whole-mount samples for histology. This allows direct comparison of whole-mount measured versus histological measured healing. We have not attempted to perform immunohistochemistry on samples that have been whole-mount stained, but in theory this should be possible. We have updated the manuscript discussion to emphasise the possibility of combining whole-mount staining with subsequent traditional histological analysis.

What is the minimum size of human tissue required for these studies? Understanding that it will warry from one experimental design to another, it may be useful for the audience to have an appreciation of how much tissue is needed, considering that human samples may not be trivial to acquire.

Response: We agree with the point the reviewer makes that access to human skin is not trivial. For a time course experiment as shown (using n=6 per group), a 5x5 cm piece of skin should suffice when using a biopsy diameter of 6mm.

Figure 2, panel D, last panel on the right. It is indicated: Loss of K14. This is somewhat inaccurate, as K14 is not loss, but the visualization of K14 is impaired because of lack of penetration of the antibody. At a minimum, it should say Loss of K14 staining. To this point, careful wording is warranted when discussing this issue in the manuscript, to make sure that it is the loss of the staining, and not the loss of the protein that is occurring with healing.

Response: We thank the reviewer for making this point. We have now carefully amended the figure and text for clarification.

Figure 3, panels B and C are hard to interpret. What is the orientation? What are we looking at?

Response: Both panels B and C are ex vivo wounds imaged in the same orientation as A (i.e. wounded epidermis side up). Panel B illustrates keratin localisation in migrating epidermis at the edge of the wound at high power. Panel C shows Langerhans cell staining (CD1a) in the epidermis of a fully re-epithelialised wound.

Figure 4, panel A, extracellular panel: I would recommend using COL I and Fn .

Response: We have amended the text as suggested.

Figure 4, panel B. By itself, this panel is hard to understand. One suggestion would be to not separate the two parts of the panel, but rather having one common title (whole-mount staining versus histological staining), with two downward arrows to their respective staining. The "measures entire wound area" and "measures specific…" should be placed at the same level to highlight the contrast. "Measures specific wound region orientation and sectioning variability" is confusing, may be could say "Measures Specific Wound Region Highly Variable" .

Response. We thank the reviewer for their insight and we have amended the panel as suggested.

Minor details:

2mm and 6mm punches: throughout the manuscript, there is no space between the number and the unit. Typically, the scientific language would require a space. Is it common rule to omit the space in this case?

P4, line 95, the use of "theatre" is awkward

P5, line 134, igG should be capitalized

P10, line 333, "petri" should be capitalize

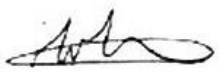
P12, line 408, "wound K14 levels" is awkward

P15, line 518, should say "reduced"

Response: We thank the reviewer for highlighting these points and we have made amendments as suggested (red text where appropriate).

Thank you again for considering our work.

Yours Sincerely,



Dr Holly N Wilkinson
Lecturer in Wound Healing
Hull York Medical School
T: +44 (0)1482 461879
E: h.n.wilkinson@hull.ac.uk