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Centre for Nanotechnology & Regenerative Medicine,

UCL Division of Surgery & Interventional Science,

London,

UK

5.7.2016

Dear Editor,

RE: **The biomechanical characterisation of hard and soft tissues for the optimisation of generating tissue engineered organ replacements**

Thank you for reviewing our paper. We have now formatted the paper according the reviewer comments as below. We have shown our edits in red in the manuscript.

We look forward to hearing from you,

Best wishes

**Miss Michelle Griffin**

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**Editorial comments:**

.  1**. Please be consistent with the usage of indentor vs. indenter.**

Indenter has been used throughout.

**2. Formatting: -Please include commas between Last Name, First Name on the title page. -**

Commas have now been inserted.

**Sections 3 & 4 have the same heading. Please either combine the sections or use different headings. -**

Section 3 has been edited to ‘preparation of cartilage’.

**4.5 – Description of the indentor should appear much earlier in the protocol**,

The indentor description has been moved to 3.2.

**4.1. -All figure legends should contain a title and a brief description.**

The figures legends have been edited.

**3. Grammar: -Please copyedit the manuscript for typographical errors.**

The paper has now been checked for grammar throughout.

 -**Line 200 – Should be “Note:”**

This has now been corrected.

**-Line 283 – “Figures 4 and 5 provides” -**

This has now been corrected.

**Line 293 – “The absolute final level of relaxation is the final point in stress of MPa for example in this data set the level would be 0.03 MPa (Figure 4D).” – Please correct the run on sentence – it should be split into two. -**

This has now been corrected.

**Please use correct punctuation in the figure legends.**

This has now been corrected.

**-Line 389 – “still attached the underlying bone”**

This has now been corrected to ‘still attached to the underlying bone’.

**4. Additional detail is required: -From where are specimens obtained? This can be included as a note(s). -2.5,**

This has now been added under section 1.1.

**4.3 – How is the sample loaded? Is this manual or controlled by software? -3.3 – How was mechanical testing performed?**

Explanation that the process is governed by software is provided.

**5. Branding (Mach 1) should be removed from the Figure 2 legend.**

This has now been removed.

**6. Results: Figure 1 legend – Please highlight the panel labels in the figure legend identically. Panel A is missing.**

Panel A has now been added.

**Reviewer #1:**

1. **The abstract and introduction devote too much attention to motivation in the context of tissue engineering, including issues (e.g., stem cell differentiation on substrates of varied stiffness) with no connection to the tissue testing protocol described here. On the other hand, almost entirely missing is a discussion of relevant aspects of tissue mechanical behaviors, which aspects of these behaviors the proposed protocols can and cannot provide insights into, and the relationship between the proposed protocols and common, well established testing methods for tissues of interest. The paper would be stronger if it deleted the extraneous motivation and addressed these issues in detail.**

Thank you for your comment we have taken out non-relevant elements if the introduction and re-written the introduction to include more aspects of mechanical properties of tissue and the nature of different testing protocols.

**2. Line 70: any protocol that involves dissection of tissue samples cannot be described as "minimally invasive," which implies in vivo testing with minimal damage. Non- or minimally-destructive may be appropriate.**

It has been changed to minimally destructive.

**3. Lines 110-112: It is difficult to see how the proposed protocols are relevant to the "clinical setting," as they do not represent physiologic loading. For example, what is the physiological situation simulated by a 1.5 hour stress relaxation test? This does not mean that they are invalid as mechanical test procedures, but the proposed protocols seem to fall under this umbrella characterization.**

We have removed this sentence on line 110-112 as we have re-written the introduction. The decision to use 1.5 hour stress relaxation test was due to the time required to show minimal relaxation changes in the skin tissue as indicated later in the manuscript. This terminology and method has been previously peer reviewed and published by the senior author (Wood et al). As our research looks at the long-term subcutaneous implantation of implants long-term stress relaxation of 1.5 hours could be indicative of this. Furthermore, there are many occasions where there is prolonged stress relaxation including standing.

**4. Lines 118-122: These descriptions are not completely accurate (the descriptions summarized in the figure captions are better). In confined compression, the walls are rigid and impermeable but either the base or the platen must be porous and present minimal resistance to fluid flow; many authors have described systems with permeable bases and rigid, impermeable plungers. Unconfined compression is not "identical," as both the top and bottom platens are usually impermeable, forcing flow to be predominantly radial. It isn't clear what "axial deformation is not limited" is intended to convey, but it is not an accurate description of either confined or unconfined compression.**

Thank you for our comments, the reviewer is right. We have now re-written the definitions of the three test regimes and adjusted the figures.

5. **Lines 129-30: Three references are provided to support optimization of these protocols; one (#17) appears to be unrelated, as it uses a different protocol for a different tissue/organ. The others are single references (one for tension, one for compression), and do not convincingly establish that these protocols are in any way optimized, superior to the many alternative testing protocols that have been used for similar/identical tissues, or should be viewed as a gold standard (which, while not explicitly stated, appears to be the motivation for this methods manuscript). Again, this does not mean that the proposed methods are not reasonable, but promoting them as a standard procedure requires a higher level of justification and validation.**

We have removed the Boughton et al paper from our group. This was primarily included because it does use the same testing regime and principles of using a stress defined test to obtain a Young’s Elastic Modulus. However, we have now removed this to avoid confusion. We have rephrased the introduction and discussion to convey that we do not feel these should be the gold standard, just that these protocols are another technique by which to evaluate soft tissue mechanics that can be easily transferred to tissue-engineered constructs. We have also added another published paper to support our technique, which evaluates the mechanics properties of auricular cartilage.

 6. **The inclusion of two very different testing modes for tissues typically viewed as governed by different physical phenomena (elongation of fibrillar networks and intrinsic viscoelasticity for skin in tension; osmotic interactions and flow-dependent dissipation in cartilage) makes this protocol paper somewhat disjointed.**

Cartilage and skin are both soft tissues, so they can be included within the same paper. We have now indicated that the analysis is the same for both the tension and compression testing, to further support the inclusion of both cartilage and skin in the same paper.

**Also, the manuscript implies that these procedures can be directly applied to other tissues; this is not the case (as evidenced by the authors' own use of different protocols for other tissues).**

The same principles of testing were used in Boughton et al and thus demonstrating the testing could be applied to other tissues. However, we have removed this paper to avoid confusion. We have made it clear that these protocols could be used for characterising human soft tissues throughout the manuscript.

**Protocols:  7. 1.2: "use any size of skin": A sample that is too large will exhibit substantial inhomogeneity, and there are likely to be size effects. Some caveats, and discussions of relevant limitations, are called for.**

Thank you for the comment. ‘Use of any size of skin’ has been removed and discussion of relevant limitations has now been inserted.

**8. 1.2 and 2.3: Some guidance is required for the method of clamping and the clamping force, as it is possible to damage the sample and alter the observed behaviors. 2.3: It is quite easy to introduce a non-uniform gripping that results in non-uniform loading, with one side of the sample being stretched more than the other. Specific directions for properly mounting the samples are required.**

We have provided a note that a commercial jig was used to avoid damaging the sample and unequal gripping in section 2.3 ‘Note: A commercial jig was utlised to avoid non-uniform gripping and damaging the sample before testing’.

**10. 2.5: Here and later, the "load" should be given in units of force (N), not mass (g).**

We have changed the load to N throughout the manuscript.

**11. 2.5: It is not common to prescribe a load limit to a strain-controlled test. If comparing samples of different material properties, this will result in different peak strains, which may affect both the apparent elastic and viscoelastic behaviors. In this manner, the proposed protocols differ substantially from the vast majority of the tissue testing literature. Other than arguments of convenience (which is essentially the case made later in the manuscript), how is this justifiable?**

Yes the reviewer is right, it is not common to load limit and this justifies writing of our protocol. However we have demonstrated that we can use this technique to evaluate the compression and tension of soft tissues with three publications. We have highlighted that the usefulness of our protocol in the introduction on line 129-131 and in the first paragraph in the discussion. Our protocol still provides the researcher with a Youngs elastic modulus as shown in previous publications and in Figure 4 and 5. We have provided data to support that tissue engineered constructs can be tested in the same way in the provided figures. Our protocol is advantageous in that it provides ‘direct, non-destructive comparison of human soft tissues and tissue-engineered constructs’ and captures both the viscoelastic and relaxation properties of the tissue within the same test.

**12. 2.5: Also, it is most common to prescribe a defined tare load and measure deformation/strain relative to that point—the current protocol appears to take the unloaded grip-grip distance as the reference point, which is not as reliable or consistent (due in part to gripping issues noted above).**

The reviewer is correct that information will be collected while the indenter is moving onto the object. However, the analysis only includes data when the indenter has made contact with the sample. By applying small strains it is clear when contact has been made with the sample and thus no tare load is required. This information has been added as a note on line 208. The difference in the loaded specimen size is used for analysis, according to standard literature and we have made this clear on line 165 and line 222 and already in the analysis section.

**Finally, many authors employ preconditioning. All of these differences between this protocol (described in a single publication) and standard practices in the literature need to be examined and justified.**

Although some mechanical testing may advocate preconditioning, understanding the optimal cycles is still under debate. We have included this as a limitation of this protocol and suggested the researcher should consider including it depending on the reasons for conducting the test on each occasion on line 400-406 with a relevant reference.

**13. 2.6: While 1.5 hours is a substantial relaxation period, the results presented clearly indicate that the tissue is still relaxing and thus not at equilibrium..**

If the reviewer looks at the scale it is a very minimal amount of relaxation. Tissue will always continue to relax. We have rephrased the sentence to ‘a time-point at which there is minimal change in relaxation behaviour’ to make this clearer.

**Also, the manuscript should indicate that the displacement is being held constant, not the load**

Thank you for you comment. We have indicated that the displacement is being held constant, not the load as a note on line 201.

 14. 3.2: **Here and in some other places, it appears that the protocol describes a specific test that was previously performed, not instructions for future tests. Avoid past tense. 15.**

Past tense has been removed from the protocol section.

3.2: **What is the indenter size and geometry? This should be specified here, along with guidelines in case this size is not available**

The information on the indenter has been moved to section 3.2.

**16. 3.2 and later: In addition to being influenced by a cut edge if the sample is not large enough, the indentation stiffness can be affected by the boundary conditions at the base (attached to bone or free) and the sample thickness, as the penetration depth of the deformation field is related to the indenter diameter—if the tissue is not thick enough, tissue thickness will influence the measured response.**

Yes the reviewer is right. We have included that the thickness needs to be 8 times greater that the indentor on line 217.

 17**. Why do sections 3 and 4 have the same title?**

Section 3 and 4 now have different titles.

**18. 4.1: Cartilage samples should be equilibrated in PBS before testing, not just during testing—PBS does not exactly match the physiological bath, and there will be a transient adaptation that may affect the measured response.**

The reviewer is right, it was supposed to read before and during testing the sample is kept in PBS. We have added a note why PBS is justified as PBS allows the comparison of both materials and tissues to be tested.

**19. 4.3: Same comment about load limitation on a displacement-controlled test**.

This has been changed to N.

**20. 4.4 Again, the data indicate that 15 minutes is not adequate to reach equilibrium—change is slow, but ongoing, as could easily be seen by plotting stress vs. log time.**

Again if the reviewer looks at the scale it is very minimal amount of relaxation. Tissue will always continue to relax making the time limitations of testing regimes difficult to decide upon. However, we have rephrased ‘a time-point at which there is minimal change in relaxation behaviour’ to make this clearer.’

**21. 5.1 (lines 226-229): First, this statement does not make sense: a load cell is completely insensitive to strain because it does not measure strain, it measures load.**

This sentence has now been deleted due to making other corrections.

**Second, it appears that consistency of the strain history across samples is less valued than avoiding potential damage to an excised sample (which can easily be avoided by setting an appropriate strain limit).**

As discussed early we are applying a load limit to be able to perform a biomechanical test, which can be relevant to testing tissue engineered constructs and is the reason for writing this article.

**22. 5.2 Clearly indicate how the linear region is defined. Also, there appears to be an error here, as the determination of tensile and compressive properties seems to be mixed and this refers to a section that has not yet been presented (calculation of Young's modulus). In fact, the entire data analysis section seems to address the compressive tests of cartilage.**

The data analysis section has now been revised so it is clearer that the Youngs elastic modulus analysis is for indentation compression and tensile testing.

**23. Line 247: "Data" is a plural noun ("data are," not "data is"). It is not clear what this sentence means—what happens if all data are included and the r^2 value is less than 0.98? (this also applies to section 7.3)**

We have rephrased to include ‘all data points are’. Furthermore, we have stated what would happen if the r value is not less than >0.98. ‘If the R value is not >0.98 then the assumption of characterising linear viscoelastic behavior is invalid’.

24. **Lines 256-264: much of this is unnecessary.**

Most of this note has now been removed.

25. **The approach to determining viscoelastic properties is non-standard, and does not reflect common practices in the literature. Of what physical significance is the rate of relaxation after 1.5 hours of constant tensile strain, and how is this phenomenological measurement related to the viscoelastic material properties (which depend on the selection of a particular material model)**

Yes the reviewer is right, our method is not common and thus the reasons for writing this protocol as explained earlier. The 1.5 hours was chosen as dictated earlier to find a point at which there is a minimal change in relaxation, as tissues will always continue to relax. This time period has also been accepted in a peer reviewed biomechanics journal.

**26. 7.3: The stress after 15 minutes of compression is not the equilibrium stress, it is merely the final stress of the test**.

As highlighted earlier in the rebuttal letter ‘Again if the reviewer looks at the scale it is very minimal amount of relaxation. Tissue will always continue to relax making the time limitations of testing regimes difficult to determine. However, we have rephrased ‘a time-point at which there is minimal change in relaxation behaviour’ to make this clearer.’

**27. 7.4: Outside of the context of a specific experimental design, specification of a particular statistical model is not appropriate.**

Thank you for our comment. Section 7.4 has now been removed.

28. **Line 293: the rate should be MPa/s, not MPa.**

Thank you for our comment. This has now been corrected.

**29. Figure 2 caption (lines 320-324): This protocol should not be specific to any particular testing system, and there is no need to present/promote the use of the Mach-1 system for materials not even covered directly in this testing protocol.**

The references to the Mach-1 system have been removed.

 30. **Lines 355-356: what is the relevance of the comment on testing in multiple directions? How is that physiologic, and how would it be implemented In a minimally destructive test?**

It is well recognized that cartilage is anisotropic. Cartilage contains Hultkrantz lines, which are equivalent to Langer Lines. Thus it is important that researchers either have enough samples to account for this (as the reviewer is correct you cannot test the small sample in different directions) or allow for this limitation. We have made this clear on lines 388-397.

**31. Lines 380-384: Two points. First, as noted above, the comment about transducers being more sensitive to load than displacement is puzzling. The following statement seems to indicate that the protocols described in this manuscript are designed to work around limitations of the test system—if that is indeed the case, then this is a poor foundation for publishing a protocol (which should be based on best practices, not best ways to work around limitations of a specific system).**

As highlighted earlier, having a load limit is not a limitation of the mechanical system, it can be applied to other mechanical material testing machines. We have just described a protocol to obtain the Young’s modulus using stress that can be used to test the mechanical properties of tissues and synthetic materials. We have deleted this sentence as we agree with the reviewer that it may confuse the reader.

 32. **Little of the discussion addresses tensile testing.**

The discussion includes more about tensile testing on line 408-429.

**33. Figure 1: Should indicate whether parts are permeable or not.**

Thank you the figure 1 has now been edited to include which is permeable or not.

 34. **Figure 3: As noted above, g/kg are not units of force; in most cases, load cells are calibrated to provide output in N. The second line lacks units.**

Load has been changed to N. Strain units have been added to %. Force is now in units.

35. **Figure 3: For a hemispherical indenter, dividing the force by the cross-sectional area gives the nominal (average) stress, but not the peak stress. This should be noted.**

Thank you for this. This has been added under 6.2.

36. **Figures 4-5: Some of the subfigures are very similar to those in prior publications; if they are identical, then they should be regenerated for new data to avoid copyright issues**.

New samples were provided for this manuscript. They may be similar to previously published work as they are similar tissues with the same testing procedures so they would act similar demonstrating the accuracy of the protocol.

**Reviewer #2:**

***Major Concerns:* The manuscript is extremely simplistic and reads more like a standard operating procedure or a user guide for employees rather than a scientific manuscript.**

This is a methods paper and is not supposed to be set out as a scientific manuscript, we feel the reviewer may have not realized it is a method paper. We have made it read like a SOP for all users to be able to understand and repeat easily.

**Reviewer #3:**

**Title: a. The title is not appropriate for this manuscript. I suggest a title with a higher focus on the proposed protocol.**

The title has now been revised.

**2. Abstract: a. The short abstract is appropriate. However, the long abstract borrows a few sentences from the introduction, which shift the focus of the manuscript. I suggest editing the long abstract to focus more on the protocol and less on the potential application.**

The long abstract has now been revised to concentrate on the testing protocols.

**Introduction: a. The first paragraph is awkwardly phrased.**

The first paragraph has now been revised.

**b. Citation is needed to support claim in lines 86 and 87 c.**

Citations for this have now been inserted.

**In line 87, it is unclear the presence of "scaffold". A non-expert will be confused and miss the point.**

The introduction has now been revised.

**The sentence in 89-92 is confusing, and does not really explain the mechanical properties of auricular cartilage.**

This sentence has been rephrased ‘For example a material replacing auricular cartilage should have the appropriate mechanical properties to prevent being compressed by the overlying skin.’

**Citation needed in line 94 f.**

This has now been edited.

**Awkward phrasing in line 97 g.**

The introduction has now been revised.

**Substitute "tissues" with "cells" in line 98, as differentiated MSCs are still cells. h**.

This has now been deleted as the introduction has been re-written.

**The transition between tissue engineering, scaffolds, materials, and the need of appropriate testing protocols is not clear. I suggest to reframe the introduction to focus on the need of better testing setups, and less so about the importance of tissue engineering. 4.**

This has now been deleted as the introduction has been re-written due to comments from other reviewers.

**Methods: a. Please clarify what is meant with "appropriate testing machine" (Line 151).**

This has now been rephrased on line 152.

**Maybe providing an example would help in this case. b. Please suggest examples of rates that do not cause failure to skin (Line 167).**

The speed has been provided that does not cause failure of the skin on line 194.

**c. In the context of researchers that purchases a new testing machine, it would also be appropriate to suggest methods for benchmarking the equipment to guarantee that any variances in testing are not due to the equipment.**

We have provided a note to account for this in 3.1 ‘Note: All materials testing machine should be calibrated according to the manufacture guidelines prior to testing. ‘

1. **The word "suggested" is repeated twice (Line 361)**

This has now been edited.

. b. **Awkward phrasing in line 379**

This has now been rephrased.

**Reviewer #4:**

*Major Concerns:* **There are a large number of prior studies that examine the failure properties and viscoelastic properties of these two tissue types. In addition, there are a number of commercially available, non-invasive mechanical testers for examining skin biomechanics in vivo. It was not clear how the discussed equipment/protocol is an improvement over available technology.**

Yes there are many in vivo skin techniques to analyse skin mechanics. However, the major addition of our techniques is using minimally destructive protocols using small stresses and strains that be correlated to tissue engineered constructs and to gain information on the viscoelasticity and relaxation properties in a single test. This has now been highlighted in the introduction (119-142) and discussion (line 366-375).

*Minor Concerns*

**For example a material replacing auricular cartilage should have adequate mechanical properties to present the deformation of the skin but not be too stiff to cause extrusion through the skin due to the failure to interact with the other connective tissues, causing failure of the implant." Suggest rewording. How it is written is confusing and makes it sound as if the main function of the cartilage biomechanics is to deform but not penetrate the overlying skin.**

This sentence has been revised.

**"Several cell types have shown to respond to the scaffold's mechanical properties including fibroblasts, muscle cells and neurons by displaying different morphological and adhesion characteristics." Should read "have been shown" "**

This sentence has been deleted due to modifying the introduction.

**The size of the indentor should be at least 8 times the sample size to ensure the cartilage to react as if it were part of an indefinite sample." Is this supposed to read 1/8th?**

This is correct, however the sentence has been checked for grammar.

**Reviewer #5:**

***Major Concerns:* The major concern on this manuscript is that, to this reviewer's knowledge and experience, the range of strain that should be used for calculation of elasticity is the first portion of the stress-strain curve, corresponding to low strain regions where the material exhibits an elastic response. High strains, on the other side, correspond to the viscous properties of the material. The method for analyzing data in this manuscript indicates, however, that the Young's Modulus should be obtained from the data corresponding to higher strain data points. Please correct or explain why high strains are been used to calculate elasticity.**

The reviewer is right you should use the first portion of the stress-stain curve to calculate a Young’s Elastic Modulus. As the novelty of the manuscript is to calculate apply small stresses and strains we are calculating the Youngs Elastic modulus a low stain regions as normal literature dictates.

***Minor Concerns:* - The title feels too broad and does not indicate clearly what is described in the manuscript.**

The title has been changed to be more specific.

**- Please include units for strain (% or mm/mm)? -**

Thank you % has been added to the figures and throughout the text to strain.

**Please indicate which equations of stress and strain should be used for each testing method.**

The Data analysis section has now been revised to make it clear that the analysis is for tension and compression.

**Reviewer #6:** .

**Pages 4-5. A minimum number of specimens to be tested for each tissue and each testing method should be provided.**

This is highly dependent on what the researchers is trying to show and examine and thus cannot be given in a protocol. The researchers should be following general guidelines on performing scientific experiments and performing power calculations.

**Page 4, Point 3. Should it be Indentation Testing? The indentation speed should also be provided**.

It has been changed to compressive indentation testing. The speed has already been provided on line 239.

**Page 6. Line 283. "provides" should be "provide".**

This has now been changed.

**Page 7. Lines 346-347**. **Try to revise this sentence as the way to calculating the elastic moduli of synthetic viscoelastic materials is different from the method presented in this paper. It is typically calculated using a different equation from the unloading curve.**

This is not true these protocols could be used for synthetic materials, which is the essence of the paper and demonstrated in the analysis section. However this sentence has been revised to ‘Both protocols are easy and simple to implement and could be considered for the characterization of human soft tissues and tissue engineered constructs.’