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Assessment of angiogenic potential of mesenchymal stem cells derived conditioned media from various oral sources

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Handling editor:
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Ref.: Ms. No. JCTRes-D-22-00025
Assessment of angiogenic potential of mesenchymal stem cells derived conditioned media from various oral sources.
Journal of Clinical and Translational Research

Dear Dr. Kheur,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. Particularly the comments of reviewer 1 are valid and important and will require you to perform additional analyses. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below and attached to this decision letter.

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 submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by May 20, 2022.

To submit a revision, go to https://www.editorialmanager.com/jctres/ 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

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The aim of this study was to compare the levels of angiogenic growth factors in conditioned medium obtained from various oral tissue-derived mesenchymal stem cell (MSC) cultures. The authors found that conditioned medium (CM) from dental pulp-derived MSC (DP-MSC) contained larger amount of hepatocyte growth factor (HGF) than these from other MSCs. Additionally, CM from buccal fat-derived MSC (BFMSC) demonstrated higher levels of HGF, platelet derived growth factor (PDGF)-AA, and vascular endothelial cell growth factor (VEGF) than other MSC-derived CMs. It is interesting that angiogenic growth factor production was different among oral tissue MSCs. However, this study needs further explanation and reconsideration as follows.

General comment
Angiogenesis is one of the important steps in wound healing, and angiogenic factors produced from MSC may be important when considering regenerative medicine and other therapeutic use of MSCs. However, angiogenesis is a highly complex biological process involving a wide variety of growth factors and cells such as vascular endothelial cells, pericytes, vascular smooth muscle, etc. Therefore, it is difficult to determine the outcome of angiogenesis based solely on the amount of angiogenic factor in the culture supernatant of cultured cells. In this paper, MSCs derived from four oral tissues were cultured, the culture supernatants were collected, and the amounts of eight growth factors or cytokines associated with angiogenesis were measured and compared. It is interesting to note the difference in measured quantities of some angiogenic factors among cells. However, given that angiogenesis is a complex process involving various molecules and cells, as described above, it is essential to study angiogenesis using functional assays. The current format of the paper is simply a quantitative comparison of angiogenic factors, and the impact of the data obtained is small. A functional study of angiogenesis is highly recommended.

Specific point
Introduction
1. Cluster differentiation (CD) should be "cluster of differentiation". Please check throughout the manuscript.
2. When abbreviations are used, the name and abbreviation should be specified when they first appear. Need to check on the entire paper.

Materials and Methods
1. Insufficient information on tissue donors. How many samples were used? Age and sex of

donors.
2. Information was lacking regarding how each tissue was collected for MSC culture. The authors should describe the details of the procedure. Nothing is written about SHED. Additional information needs to be added and described.
3．Isotype control is not used in FACS analysis? It should be described.
4．Insufficient description of staining methods for Alizarin red, Oil red O, and Alucian blue staining. Staining methods should be described.
5．In the growth factor analysis section, material suppliers are not described. Information should be added.

Results
For Figures 7 and 8, the asterisks indicate significant differences, but where are these significant differences detected? It is necessary to indicate clearly between which groups are significantly different.

Reviewer #2: Overall, the team has done a good job in describing the various expressions of angiogenic proteins. The study is indeed useful considering the cell-free approach we are currently looking in the field of regenerative medicine.
However, there are a couple of concerns which require some attentions, as listed below:

1. The explant culture methodology seems unclear. How were the tissues reduced to small sections measuring 1-2mm? What was the media per T25 flask?
2. How many patients were the samples derived from? Were there deviations among the donors?
3. The source of alpha MEM, FBS, trypsin, Alcian Blue, Oil Red O and anti-anti is missing. Kindly provide to ensure the study is replicable by researchers elsewhere.
4. What was the seeding density of cells prior to CM collection?
5. What was the control used to derive the protein content from LEGENDplex Multianalyte flow assay kit? Was there a standard curve? How many replicates per sample source were used? What was the model of flow cytometry used to capture these expressions?
6. The images of Figure 1 are lacking scale bar. Without it, assessing the morphology appears tricky.
7. For Figure 2-5, there are similarities in the FACS plot across a few dental sources. For instance, the CD45 plot for BFMSC appears to be the same as the CD45 plot for SHED. The same pattern is also seen in all surface markers except for CD34. Ideally, each source should have separate plots to describe the surface marker expression. Perhaps attaching the raw data as supporting documents would increase the credibility of the data.
8. The images for Figure 6 are lacking scale bar for proper comparison.
9. Since trilineage differentiation is rather a semi-qualitative assay, perhaps the surface area of stained area could be calculated and presented in bar graphs for better comparison.
10. The adipogenic stain for BFMSC seems rather low despite being adipose origin. Could the team explain why?
11. The p-value presented here showed significance. But it is unsure if the significance is against the culture media or that of other dental sources. Kindly clarify.
12. The study showed that DPSC-CM shows the maximum presence of the critical factors for angiogenesis. It would be convincing if functional assays such as tube formation or using commercial kits could be added.

13. In the discussion, adding a few examples that utilize conditioned media in clinical trials would be appreciated. Based on these, future strategies can be derived for further improvement.

Reviewer #3: The text in the Abstract / Graphical Abstract Results section need rewording to make it clearer

There are some missing spacings throughout the manuscript

In section 2.5 it would be worth including how long it take for a T25 to become 80% confluent with cells

In section 2.9 some more detail on the method of flow cytometry would be warranted

Section 3.4 needs to be written in a more coherent way to better represent what is being shown sequentially in Figures 7 and 8. Also a mention of concentration ranges would be useful for comparison to other literature.

In the Discussion, referencing authors in text need to be corected by removing first names.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

Authors' response

Ref.: Ms. No. JCTRes-D-22-00025

Assessment of angiogenic potential of mesenchymal stem cells derived conditioned media from various oral sources.

Journal of Clinical and Translational Research
Reviewers' comments:

Reviewer #1
This study aimed to compare the levels of angiogenic growth factors in a conditioned medium obtained from various oral tissue-derived mesenchymal stem cell (MSC) cultures. The authors found that conditioned medium (CM) from dental pulp-derived MSC (DP-MSC) contained a larger amount of hepatocyte growth factor (HGF) than those from other MSCs. Additionally, CM from buccal fat-derived MSC (BFMSC) demonstrated higher levels of HGF, platelet-derived growth factor (PDGF)-AA, and vascular endothelial cell growth factor (VEGF) than other MSC-derived CMs. Interestingly, angiogenic growth factor production was different among oral tissue MSCs. However, this study needs further explanation and reconsideration as follows.

General comment

Angiogenesis is one of the critical steps in wound healing, and angiogenic factors produced from MSC may be necessary when considering regenerative medicine and other therapeutic use of MSCs. However, angiogenesis is a highly complex biological process involving many growth factors and cells such as vascular endothelial cells, pericytes, vascular smooth muscle, etc. Therefore, it is difficult to determine the outcome of angiogenesis based solely on the amount of angiogenic factor in the culture supernatant of cultured cells. In this paper, MSCs derived from four oral tissues were cultured, the culture supernatants were collected, and the amounts of eight growth factors or cytokines associated with angiogenesis were measured and compared. Interestingly, the difference in measured quantities of some angiogenic factors among cells is intriguing. However, given that angiogenesis is a complex process involving various molecules and cells, as described above, it is essential to study angiogenesis using functional assays. The current format of the paper is simply a quantitative comparison of angiogenic factors, and the impact of the data obtained is small. A functional study of angiogenesis is highly recommended.
Response:
We appreciate the concerns raised by the reviewers. We agree that the mere presence of high levels of growth factors may not result in the increased potential of angiogenesis. Therefore, as per your suggestion, we assessed the angiogenesis in the Chick Yolk Sac Membrane (YSM) assay. The results are consistent with our previous conclusion that DPMSCs possess higher angiogenic potential than the other sources of MSCs. The results of the YSM assay have been included in the revised manuscript (Line no: 239-249, Figure No: 8).

Specific point

Introduction
1. Cluster differentiation (CD) should be a "cluster of differentiation." Please check throughout the manuscript.
Response:
Cluster differentiation (CD) is changed to Cluster of differentiation throughout the manuscript. (Changes are highlighted)
2. When abbreviations are used, the name and abbreviation should be specified when they first appear. You need to check on the entire paper.
Response:
Required corrections are made. Name and abbreviations are specified when they appeared first in the manuscript.

Materials and Methods

1. Insufficient information on tissue donors. How many samples were used? Age and sex of donors.
Response: Thank you for your valuable suggestion. The details of the donors have been included in the supplementary files.
2. Information was lacking regarding how each tissue was collected for MSC culture. The authors should describe the details of the procedure. Nothing is written about SHED. Additional information needs to be added and described.
Response: Thank you for the suggestions. The details regarding the same are now addressed in the revised manuscript (Section 2.4 and 2.5, Line No: 115 to 144).

3 . Isotype control not used in FACS analysis? It should be described.

Response: We routinely use the isotype control to characterize the MSCs from all the sources. Although due to space constraints, we had not included it in the prevision version. The details of the isotype control (mouse IgG-PE isotype control) are now included in the supplementary file.

4 . Insufficient description of staining methods for Alizarin red, Oil red O, and Alcian blue staining. Staining methods should be described.
Response: We have elaborated the description of various staining methods used during the differentiation of MSCs. The details have been highlighted (Section no: 2.9 Line No: 181 to 214

5 . In the growth factor analysis section, material suppliers are not described. Information should be added.
Response:
As per your suggestion, the make and catalog number of the materials used during the experiment have been added to the revised manuscript (Section 2.11, Line No: 225).

Results
The asterisks indicate significant differences for Figures 7 and 8, but where are these significant differences detected? It is necessary to indicate clearly which groups are significantly different.
Response:
Thank you for pointing out the mistake. We have done the necessary corrections in figure 7.

Reviewer #2:
Overall, the team has done a good job of describing the various expressions of angiogenic proteins. The study is indeed useful considering the cell-free approach we are currently looking in the field of regenerative medicine.
Response:
We thank you the reviewer for his/her encouraging comments.

However, there are a couple of concerns that require some attention, as listed below:

1. The explant culture methodology seems unclear. How were the tissues reduced to small sections measuring 1-2mm? What was the media per T25 flask?
Response:
The explant culture is a non-enzymatic primary cell isolation and culture method. In our laboratory, various types of MSCs are isolated and cultured by this method and the protocol for the same is well established and published in an international journal (Patil VR, Kharat AH, Kulkarni DG, Kheur SM, Bhonde RR. Long-term explant culture for harvesting a homogeneous population of human dental pulp stem cells. Cell Biol Int. 2018;42(12):1602-1610. doi:10.1002/cbin.11065). Once the tissue is disinfected, it is cut into small pieces, approximately 1 to 2 mm, using a sterile surgical blade. Furthermore, to collect the conditioned medium in the T25 flask, 4-5 ml of α-MEM along with 1% antibiotic-antimycotic solution was added.
2. How many patients were the samples derived from? Were there deviations among the donors?
Response: For each source of MSCs, six samples were collected from donors in the age range of 18 to 25 years to avoid the influence of age on the secretory potential of MSCs. We did not

find any significant deviations among the group in the secretory profile of the MSCs. The details of the donors of the tissue samples are listed in the supplementary files.

3. The source of alpha MEM, FBS, trypsin, Alcian Blue, Oil Red O and anti-anti is missing. Kindly provide to ensure the study is replicable by researchers elsewhere.
Response: Thank you for your suggestion. The source for each reagent, culture media, and supplement is added to the revised manuscript (Changes are highlighted).

4. What was the seeding density of cells prior to CM collection?
Response:
Cells in each source category were seeded at the density of 1 x 104 cells per T25 flask. The correction has been done in the revised manuscript (Section 2.10, Line No:217).

5. What was the control used to derive the protein content from the LEGENDplexTM Multianalyte flow assay kit? Was there a standard curve? How many replicates per sample source were used? What was the model of flow cytometry used to capture these expressions?
Response: Cell culture medium (MEM-α) without preconditioning with MSCs was used to control growth factors analysis. Standard curves for each growth factor were established, which are shown below. The analysis was performed in triplicates for each source. Attune NxT Flow Cytometer (Life Technologies) analyzed the growth factors.
6. The images in Figure 1 lack a scale bar. Without it, assessing the morphology appears tricky.
Response: Thank you for your suggestion. Scale bars are now added to the revised manuscript for convenience (Figure No:1)

7. For Figures 2-5, there are similarities in the FACS plot across a few dental sources. For instance, the CD45 plot for BFMSC appears to be the same as the CD45 plot for SHED. The same pattern is also seen in all surface markers except for CD34. Each source should have separate plots to describe the surface marker expression. Perhaps attaching the raw data as supporting documents would increase the credibility of the data.
Response: Thank you for pointing out this inadvertent error. We have rectified and revised the figure for the characterization of the MSCs (Figures 2,3,4 and 5)

8. The images for Figure 6 lack a scale bar for proper comparison.
Response: Thank you for your suggestion. A scale bar has been added to the revised manuscript.

9. Since trilineage differentiation is instead a semi-qualitative assay, perhaps the surface area of the stained area could be calculated and presented in bar graphs for better comparison.
Response: Thank you for your suggestion. We have now included a quantitative analysis of the stained area to assess the extent of tri-lineage differentiation by using Image J analysis. (Figure 6). Raw data of the analysis has been provided in the supplementary file.
10. The adipogenic stain for BFMSC seems relatively low despite being of adipose origin. Could the team explain why?
Response: Thank you for this thought-provoking question. The buccal fat pad is present in the submucosal area of the buccal cavity (Cheek). Physiologically, the fat content of this region remains unaffected (fat constant). Also, it does not increase or decrease in size in situ. Therefore, MSCs from tissue sources may be relatively resistant to adipogenic differentiation.

Hence, BFMSCs may have relatively more minor adipogenic potential. Although, it is an excellent research question and can be studied in detail in the future.

11. The p-value presented here showed significance. But it is unsure if the significance is against the cultural media or that of other dental sources. Kindly clarify.

Response:

The significance level is shown against control, and intra-group significance is also depicted. (Figure No:7)

12. The study showed that DPSC-CM shows the maximum presence of the critical factors for angiogenesis. It would be convincing if functional assays such as tube formation or commercial kits could be added.

Response: Thank you for your suggestion. The functionality of the conditioned media is tested in the Chick Yolk Sac Membrane assay. The results of the same have been added to the revised manuscript. (highlighted) (Line no: 239-249, Figure No: 8)

13. In the Discussion, adding a few examples that utilize conditioned media in clinical trials would be appreciated. Based on these, future strategies can be derived for further improvement.

Response: Thank you for your suggestion; as mentioned above, we have included a few clinical study examples in the discussion (highlighted) (Line no: 446-458)

Reviewer #3:

1. The text in the Abstract / Graphical Abstract Results section needs rewording to make it clearer.

Response: The abstract is reframed as per your suggestion. Now it looks appropriate.

2. There are some missing spacings throughout the manuscript.

Response: Thank you for your suggestion; the missing spaces have been corrected throughout the manuscript.

3. Section 2.5 would be worth including how long it takes for a T25 to become 80% confluent with cells.

Response: It takes around 48 hours for a T25 flask to become 80% confluent with cells.

4. In section 2.9, some more detail on the flow cytometry method would be warranted.

Response: Thank you for your suggestion. The revised manuscript has incorporated the detailed flow cytometry methodology (highlighted) (Line no: 225-238, Section 2.11).

5. Section 3.4 needs to be written more coherently to represent what is shown sequentially in Figures 7 and 8. Also, a mention of concentration ranges would be useful for comparison to other literature.

Response: Thank you for your suggestion. A detailed description of the growth factor analysis result is provided in section 3.4 in the revised manuscript. (highlighted) (Line no:313-326 Section No: 3.4)

6. In the discussion, referencing authors in the text need to be corrected by removing first names.

Response: Thank you for your keen observation; the required corrections are made in the revised manuscript and highlighted.

2nd Editorial decision
20-May-2022

Ref.: Ms. No. JCTRes-D-22-00025R1
Assessment of angiogenic potential of mesenchymal stem cells derived conditioned media from various oral sources.
Journal of Clinical and Translational Research

Dear authors,

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

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Please implement the following changes in the proofs:

- remove all trademark symbols (TM) behind brands and brand names
- insert a space between value and unit (e.g., 3 mL instead of 3mL)
- CO2 -> 2 should be in subscript
- correct grammar/spelling errors and inconsistencies; e.g., ...antimycotic solution(CELLcloneTM... -> space between solution and (, cluster of differentiation instead of Cluster of differentiation, etc.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: