nature portfolio

Peer Review File

Dental pulp stem cells-derived cannabidiol-treated organoid-like microspheroids show robust osteogenic potential via upregulation of WNT6

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Review of X et al., 2023
Communications Biology

In this paper the authors investigated the effect of cannabidiol (CBD) on the dental pulp stem cells (DPSC) derived micro-spheroids. They described that CBD pre-treated micro-spheroids have higher bone-regenerative capacity via upregulation of WNT6. CBD has already been shown to promote the osteogenic differentiation of DPSC in vitro. They took advantage of self-assembled DPSC micro-spheroids of 70um size using the microwell culture technique to investigate whether CBD has unaltered effect on osteogenic potential in micro-spheroids versus 2D culture.
They compared between DPSC and micro-spheroids treated with CBD or control and they assess the expression of different osteogenic markers showing higher osteogenic potential of micro-spheroids compared to 2D DPSC, and CBD treatment increases this potential further.
They showed that CBD-treated micro-spheroids induce abundant mineralized bone tissue formation and promote DPSC-based bone defect healing. After transcriptomic analysis on 2D DPSC and micro-spheroids with or without CBD treatment they concluded that CBD upregulates Wnt6 in micro-spheroids which promotes osteogenic potential via Wnt/ βcatenin signaling. Overall, the authors addressed the question effectively, and we believe that this work could be suitable for publication in Communications Biology with appropriate revisions.

Major revisions:
1- The bioinformatic analysis needs to be reconsidered. The Venn diagram shown in Fig 7A does not answer the question that has been asked about what makes the micro-spheroid pretreated with CBD better than the 2D DPSC cultures (in either treatment condition) in increasing the osteogenic potential. The differentially expressed genes that have been used for KEGG pathways analysis are the common DEGs between the 4 groups the authors should look specifically to the DEGs in the micro-spheroids treated with CBD.
2- The mechanism described needs to be completed by showing the whole set of osteogenic genes and not only Runx2 expression both on mRNA level and protein level.

Minor revisions
1- Fig 1D and 1F: could the authors show different magnifications of the staining to show the shape of the cells.
2- Fig 1G: representative image of crystal violet staining is not clear.
3- Fig 3B: mold has been misspelled.
4- “OM” abbreviation is used several times in the figures and was not explained.
5- Fig 3E: could the author point with an arrow to the red dead cells in the image?
6- Fig 4A: the statistical results are very complicated. Could the authors find a better way to separate the different comparisons they are talking about? Maybe a color code instead of different shapes.
7- Fig 4E: in addition to OCN immunostaining, could you show the protein expression of the

different osteogenic markers you describe in order to confirm that the effect is on both protein and mRNA levels.
8- Fig 6D is not mentioned in the text.
9- Fig S1 could the authors show the PCA with all the samples of the 4 different groups and how they segregate on the same plot?
Spheroids has been misspelled several times in many figures.

Reviewer #2 (Remarks to the Author):

The manuscript evaluates the osteogenic capacities of cannabidiol-pretreated DPSCs-micro-spheroids in vitro and in vivo. Although the study is novel, more solid and scientific results are recommended.

1. abstract, the description of the abstract is too brief, especially on lines 14~17, and lacks a description of in vivo results.
2. introduction, the fabrication or development of MSCs-3D-aggregates/spheroid has been discussed for several years. However, in the literature review, the introduction for the 3D-spheroids is too weak. More recent articles related to 3D-spheroids are recommended on lines 42~51.
3. On page 7, the proliferative effect of cannabidiol on DPSCs was evaluated using 0, 0.1, 0.5, 2.5, and 12.5 μM. Any preliminary result supported these concentrations, especially different for different cells. The concentrations (5, 10) may be more ideal for the study.
4. On page 10, line 189, why use the serum-free osteogenic medium in the 3D cell culture system rather than the 10% FBS? Please explain the differences.
5. In figure 3E, for CBD+OM at 14 days, more dead cells were observed in the image when compared to other groups. Please confirm the data and explain the differences or possible reasons. Moreover, the osteogenic differentiation of 3D-spheroids with and without cannabidiol treatment (different concentrations) is recommended.
6. In figure 4, the protein expressions of ALP, BMP2, OPN, and OCN are needed.
7. In figure 5, how to select the 14 days for the induction period?
8. Please extend the description and discussion regarding the GelMa materials.
9. In figure 6, please add the micro-ct parameters including trabecular number and separation.
10. How about the IF staining? For example, the proliferating cell nuclear antigen (PCNA) and osteogenic protein expression.
11. For the molecular transduction pathways, how about the GSK-3β and CK1 alpha?
12. The inhibition of WNT pathway should be considered.

Reviewer #3 (Remarks to the Author):

It is an honor to be invited to review this manuscript. The study presents self-assembled and injectable DPSC-derived micro-spheroids that promote bone regeneration both in vitro and in vivo. Additionally, the authors discovered that CBD-pretreated micro-spheroids exhibit a higher bone-regenerative capacity through the WNT6 signal pathway. Overall, the experimental design is scientifically sound. The use of CBD preprocessing in cranial bone regeneration is innovative and offers a new application for cannabis extract. Based on the scope and requirements of Communications Biology, I think there are several points that need to be revised.

1. In line 7, the authors state that " However, difficulties to control the cell number, size, and shape of DPSC-aggregates/spheroids cause cell necrosis and difficulties in homogenous seeding of aggregates/spheroids in the 3D-printed microporous bone grafts." I suggest that in the discussion section, authors should explain why the previous/3D-printing method failed to achieve homogeneous seeding and how authors' spheroid preparation method overcomes this challenge. Authors should also provide more data or references to support the claim made in line 7. Additionally, the innovative aspects of the authors' spheroid preparation method should be clearly elucidated.
2. The authors used a serum-free chemically defined medium to grow DPSC into spheroids in the preparation process and in Figure 3A-3C. However, most of the in vitro experiments in this manuscript were conducted using fetal bovine serum and adherent growth method. It would be useful for the authors to explain whether the osteogenic capacity and differentiation direction of DPSC will be changed when treated with serum-free chemically defined medium in spheroid formation process.
3. The manuscript needs to provide more specific details about the preparation of GelMA hydrogels. For example, did the authors use osteogenic medium or serum-free chemically defined medium as the solvent of GelMA hydrogels?
4. The labels and legend of Figure 7A could be misleading. It is not clear which two groups are being compared in the Venn diagram. Therefore, the authors should revise the figure legend and clarify which groups are being compared.
5. Based on Figure 7C-7E and the P values, it is unclear why the micro-spheroid-only group appears to be more effective in inducing osteogenesis than the CBD-only group. Therefore, the authors should provide additional explanations or hypotheses to account for this observation.
6. There are some grammatical errors and incoherent sentences. For example, the phrase "developed using" in line 13 makes the sentence difficult to comprehend. Similarly, "the direct in vivo drug application-related adverse effects" in line 21 is not well-constructed. Please carefully check the grammar and incoherence.

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Manuscript Title “Dental pulp stem cells-derived cannabidiol-pretreated micro-spheroids showed robust osteogenic potential via upregulation of WNT6” (Manuscript No. COMMSBIO-23-0509)

Dear reviewers,

On behalf of all the contributing authors, I would like to express our sincere appreciations of your letter and reviewers' constructive comments concerning our article entitled "Dental pulp stem cells-derived cannabidiol-pretreated micro-spheroids showed robust osteogenic potential via upregulation of WNT6" (Manuscript No: COMMSBIO-23-0509).

These comments are all valuable and helpful for improving our article. According to the associate editor and reviewers' comments, we have made extensive modifications to our manuscript and supplemented extra data to make our results convincing. The reviewer comments are laid out below and specific concerns have been numbered. Our response is given and changes/additions to the manuscript are given in the red text. Point-by-point responses to three nice reviewers are listed below this letter.

Respond to the Reviewer#1' comments:
Major revisions:
Comment 1. The bioinformatic analysis needs to be reconsidered. The Venn diagram shown in Fig 7A does not answer the question that has been asked about what makes the micro-spheroid pretreated with CBD better than the 2D DPSC cultures (in either treatment condition) in increasing the osteogenic potential. The differentially expressed genes that have been used for KEGG pathways analysis are the common DEGs between the 4 groups the authors should look specifically to the DEGs in the micro-spheroids treated with CBD.
Response: We re-analyzed the Venn diagram according to the editor's suggestions in Fig. 7A. The Venn diagram shows the number of common DEGs among the four groups. In addition to the common 1754 DEGs, we also performed KEGG enrichment analysis on the subset of 158 DEGs in the micro-spheroids treated with CBD following the reviewer's suggestion in Figure 7B.

Comment 2. The mechanism described needs to be completed by showing the whole set of osteogenic genes and not only Runx2 expression both on mRNA level and protein level.
Response: As suggested by the reviewer, we added late osteogenic osteocalcin (OCN) protein expression (Fig. 7H). We also analyzed WNT6 mRNA expression (Fig. 7E). Since it was the rescue experiment of mechanism confirmation, we chose key osteogenic markers instead of the whole set of osteogenic genes.

Minor revisions
Comment 1. Fig 1D and 1F: could the authors show different magnifications of the staining to show the shape of the cells.
Response： We have added magnified images in order to show the shape of cells in Figure 1D and Figure 1F.

Comment 2. Fig 1G: representative image of crystal violet staining is not clear.
Response: We have updated the images and added a magnified cell image in Figure 1G to clearly observe the cell aggregation.

Comment 3. Fig 3B: mold has been misspelled.
Response: We are sorry for our carelessness. In our resubmitted manuscript, the type is revised.

Comment 4. "OM" abbreviation is used several times in the figures and was not explained.
Response: We have checked this part carefully and added "osteogenic medium (OM)'' in INTRODUCTION part and figure legend.

Comment 5. Fig 3E: could the author point with an arrow to the red dead cells in the image?
Response: We have added red arrow to point to the red dead cells in Figure 3E and explained in revised manuscript and figure legend.

Comment 6. Fig 4A: the statistical results are very complicated. Could the authors find a better way to separate the different comparisons they are talking about? Maybe a color code instead of different shapes.
Response: We are sorry for the unclear expression. We attempted to use different color code and explained in figure legend as follows: "Significant difference compared different groups, *P<0.05, **P<0.01, and ***p<0.001; compared between DPSC and CBD+DPSC groups in blue; compared between DPSC and spheroids groups in red; compared between spheroids and CBD+spheroids groups in green; compared between CBD+DPSC and CBD+spheroids groups in purple."

Comment 7. Fig 4E: in addition to OCN immunostaining, could you show the protein expression of the different osteogenic markers you describe in order to confirm that the effect is on both protein and mRNA levels.
Response: We have detected the gene expression of the different osteogenic markers in Figure 4A and 4B and the protein expression of the different osteogenic markers in Figure 4C and 4D.

Comment 8. Fig 6D is not mentioned in the text.
Response: We are sorry for our carelessness and it is certificated at revised manuscript.

Comment 9. Fig S1 could the authors show the PCA with all the samples of the 4 different groups and how they segregate on the same plot?
Response: Based on your advice, we have replaced the Figure to show the PCA with all the samples in Figure S3A. We found that the groups treated with CBD has a higher level of similarity, while the 2D group and the 3D group have a larger dispersion, indicating better discriminability.

Comment 10. Spheroids has been misspelled several times in many figures.
Response: We are sorry for our carelessness. Based on your comments, we have made the corrections to make the word harmonized within the whole figures.

Respond to the Reviewer#2' comments:

Comment 1. abstract, the description of the abstract is too brief, especially on lines 14~17, and lacks a description of in vivo results.

Response: As suggested by the reviewer, we completely rewrote the abstract.

Comment 2. introduction, the fabrication or development of MSCs-3D-aggregates/spheroid has been discussed for several years. However, in the literature review, the introduction for the 3D-spheroids is too weak. More recent articles related to 3D-spheroids are recommended on lines 42~51.

Response: We sincerely appreciate the valuable comments We have checked the literature carefully and added more references for 3D-spheroids into INTRODUCTION part in the revised manuscript.

Comment 3. On page 7, the proliferative effect of cannabidiol on DPSCs was evaluated using 0, 0.1, 0.5, 2.5, and 12.5 μM. Any preliminary result supported these concentrations, especially different for different cells. The concentrations (5, 10) may be more ideal for the study.

Response: According to other literature, low concentrations of CBD do not show cytotoxicity. However high concentrations of CBD have been found to inhibit cell proliferation, with the inhibitory concentration for oral cells possibly exceeding 10 μmol/L (Pagano S et al. Biomedicine & pharmacotherapy. 2020). We choose concentrations 0.1, 05, 2.5, and 12.5 μmol/L with 5-fold increments in each group covering all the concentrations reported in the previous literature.

Comment 4. On page 10, line 189, why use the serum-free osteogenic medium in the 3D cell culture system rather than the 10% FBS? Please explain the differences.

Response: The use of 10% FBS hindered microspheroid formation in our developed agarose-gel microwell culture system. Cells were adhered on an agarose gel surface rather than aggregating. Therefore, we used a serum-free stem cell culture medium that contains certain growth factors fulfilling the function of FBS.

Comment 5. In figure 3E, for CBD+OM at 14 days, more dead cells were observed in the image when compared to other groups. Please confirm the data and explain the differences or possible reasons. Moreover, the osteogenic differentiation of 3D-spheroids with and without cannabidiol treatment (different concentrations) is recommended.

Response: We repeated the experiment and retook the images, we found there was no difference in the number of death cells between the groups. We have added the new figure 3E accordingly. As suggested by the reviewer, we added Western blot analysis of ALP and BMP2 (Fig. 4C-4D).

Comment 6. In figure 4, the protein expressions of ALP, BMP2, OPN, and OCN are needed.

Response: As suggested by the reviewer, we added Western blot analysis of ALP and BMP2 (Fig. 4C-4D).

Comment 7. In figure 5, how to select the 14 days for the induction period?
Response: Our in vitro studies indicated that 14 days of in vitro induction had the best osteogenic differentiation potential. Moreover, a previous study by (Miller, Henry et al. Cell biochemistry and function vol. 2021) cultured microspheroids using a similar technique found that 14 days of in vitro induction is the best for osteogenic application microspheroids based on in vitro and in vivo studies. According to our own in vitro results and report from the literature, we choose a 14-day in vitro induction period.

Comment 8. Please extend the description and discussion regarding the GelMa materials.
Response: We added the description of GelMa materials in the Materials and Methods section (page 12-13, line 246-261) as follows: "Preparation of GelMA hydrogels constructs: GelMA (EFL-GM-60) was purchased from Engineering For Life, Hangzhou, China. Following the manufacturer's instructions, a 0.05 g initiator was mixed with 20 mL PBS and dissolved in water at 45℃ to prepare a 0.25% (w/v) initiator standard solution. GelMA (2 g) was added to the initiator standard solution at 65℃ and mixed for 20-30 min to get 10% (w/v) GelMA hydrogel. Finally, the GelMA hydrogel was sterilized by filtering through a 0.22 μm syringe filter. Before implantation, both DPSC and microspheroids were cultured in an osteogenic medium with or without CBD for 2 weeks. Then, 2D-expanded DPSC were digested with 0.25% trypsin-EDTA. Microspheroids or 2D-expanded DPSC have resuspended in 10% (w/v) GelMA hydrogels. Microspheroids or 2D-expanded DPSC-loaded GelMA constructs (≈7.5×105 DPSC/3×103 microspheroids per construct, n=8) with a diameter of 3 mm and a depth of 1 mm were exposed to LAP 405 nm blue light (Engineering For Life, China) for 5 s as shown in Fig. 5A. DPSC and microspheroids constructs were cultured at 37°C incubator with 5% CO2 before implantation. To assess cell viability, the Live/Dead staining assay was performed on the GelMA hydrogel constructs cultured in vitro for 1, 3, and 7 days."

Comment 9. In figure 6, please add the micro-ct parameters including trabecular number and separation.
Response: We added the suggested parameters accordingly in Figure 6B.

Comment 10. How about the IF staining? For example, the proliferating cell nuclear antigen (PCNA) and osteogenic protein expression.
Response: Although we did not design the PCNA immunofluorescence staining for proliferation during this study, we have performed OCN immunofluorescence staining in Figure 4E-4F.

Comment 11. For the molecular transduction pathways, how about the GSK-3β and CK1 alpha?

Response: GSK-3β and CK1 are well-known key regulators of Wnt/β-catenin signaling. CBD has been reported to downregulate phosphorylation of GSK-3β in neuronal cells (Vallée, Alexandre et al. Acta biochimica et biophysica Sinica vol. 2017). We will analyze the in-depth mechanism of CBD-induced Wnt signaling in microspheroids in our ongoing study.

Comment 12. The inhibition of WNT pathway should be considered.
Response: In this study, the use of DDK1 was mainly aimed at Wnt inhibition. Wnt signaling inhibition by using DKK1 has been reported in the previous literature (Srikanth, M.P et al. Biomolecules 2020). Based on these facts, we used DKK1 to inhibit WNT signaling (Fig. 7H-I).

Respond to the Reviewer#3' comments:

Comment 1. In line 7, the authors state that " However, difficulties to control the cell number, size, and shape of DPSC-aggregates/spheroids cause cell necrosis and difficulties in homogenous seeding of aggregates/spheroids in the 3D-printed microporous bone grafts." I suggest that in the discussion section, authors should explain why the previous/3D-printing method failed to achieve homogeneous seeding and how authors' spheroid preparation method overcomes this challenge. Authors should also provide more data or references to support the claim made in line 7. Additionally, the innovative aspects of the authors' spheroid preparation method should be clearly elucidated.

Response: As suggested by the reviewer, we completely rewrote the DISCUSSION part in the revised manuscript.

Comment 2. The authors used a serum-free chemically defined medium to grow DPSC into spheroids in the preparation process and in Figure 3A-3C. However, most of the in vitro experiments in this manuscript were conducted using fetal bovine serum and adherent growth method. It would be useful for the authors to explain whether the osteogenic capacity and differentiation direction of DPSC will be changed when treated with serum-free chemically defined medium in spheroid formation process.

Response: According to the manufacturer's description, there is no difference in osteogenic differentiation of various mesenchymal stem cells using their serum-free medium and 10% FBS. As suggested by the editor, we also conducted an experiment and found similar expression patterns of osteogenic differentiation markers in 10% FBS and serum free-medium-containing osteogenic medium cultured DPSC (Fig. S1).

Comment 3. The manuscript needs to provide more specific details about the preparation of GelMA hydrogels. For example, did the authors use osteogenic medium or serum-free chemically defined medium as the solvent of GelMA hydrogels?

Response: We added the description of Gelma hydrogel preparation in the Materials and Methods section (page 12-13, line 246-261) as follows: "Preparation of GelMA hydrogels constructs: GelMA (EFL-GM-60) was purchased from Engineering For Life, Hangzhou, China. Following the manufacturer's instructions, a 0.05 g initiator was mixed with 20 mL PBS and dissolved in water at 45℃ to prepare a 0.25% (w/v) initiator standard solution. GelMA (2 g) was added to the initiator standard solution at 65℃ and mixed for 20-30 min to get 10% (w/v) GelMA hydrogel. Finally, the GelMA hydrogel was sterilized by filtering through a 0.22 μm syringe filter. Before implantation, both DPSC and microspheroids were cultured in an osteogenic medium with or without CBD for 2 weeks. Then, 2D-expanded DPSC were digested with 0.25% trypsin-EDTA. Microspheroids or 2D-expanded DPSC have resuspended in 10% (w/v) GelMA hydrogels. Microspheroids or 2D-expanded DPSC-loaded GelMA constructs (≈7.5×105 DPSC/3×103 microspheroids per construct, n=8) with a diameter of 3 mm and a depth of 1 mm were exposed to LAP 405 nm blue light (Engineering For Life, China) for 5 s as shown in Fig. 5A. DPSC and microspheroids constructs were cultured

at 37°C incubator with 5% CO2 before implantation. To assess cell viability, the Live/Dead staining assay was performed on the GelMA hydrogel constructs cultured in vitro for 1, 3, and 7 days."

Comment 4. The labels and legend of Figure 7A could be misleading. It is not clear which two groups are being compared in the Venn diagram. Therefore, the authors should revise the figure legend and clarify which groups are being compared.
Response: We have re-analyzed and changed the Venn diagram accordingly.

Comment 5. Based on Figure 7C-7E and the P values, it is unclear why the micro-spheroid-only group appears to be more effective in inducing osteogenesis than the CBD-only group. Therefore, the authors should provide additional explanations or hypotheses to account for this observation.
Response: The main aim of this study was to develop DPSC-based organoid-like osteogenic microspheroids and analyze their potential to translate in vitro stimulation during in vivo grafting. CBD was only used as a drug model for in vitro stimulation. WNT6 and osteogenic differentiation were prominently upregulated in microspheres itself, CBD only added extra effect on WNT6 stimulation over it. For better clarification, we added the following text in the discussion section (page 25-26, line 502-534).

Comment 6. There are some grammatical errors and incoherent sentences. For example, the phrase "developed using" in line 13 makes the sentence difficult to comprehend. Similarly, "the direct in vivo drug application-related adverse effects" in line 21 is not well-constructed. Please carefully check the grammar and incoherence.
Response: We are sorry for our incorrect grammar and incoherence and it is rectified in abstract.

Thank you very much for your attention and time. We appreciate for Editors and reviewers' warm work and hope the correction will meet with approval.

Yours sincerely

Jiang Li
Corresponding author

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

In this paper, the authors investigated the effect of cannabidiol (CBD) on the dental pulp stem cells (DPSC) derived micro-spheroids.
In this revised manuscript, the authors addressed most of the comments raised during the first round of revision.
They reanalyzed the Venn diagram which helped answering their question better, and they also performed KEGG enrichment and added the PCA with all samples and this showed a better discriminability. Concerning the mechanism they added the mRNA expression of OCN and WNT6. They switched many unclear images with new ones in better quality and found a new way to show the statistical results on their graphs. Finally, the authors revised the grammar and the spelling of the text.
Overall, the authors addressed the question effectively, and we believe that this work is now suitable for publication in Communications Biology.

Reviewer #2 (Remarks to the Author):

I accept the revised manuscript.