eric priyo prasetyo <eric-p-p@fkg.unair.ac.id>

Pesquisa Brasileira em Odontopediatria e Clínica Integrada - Manuscript ID PBOCI-2020-0044

1 message

Pesquisa Brasileira em Odontopediatria e Clínica Integrada <onbehalfof@manuscriptcentral.com>
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Tue, Mar 10, 2020 at 12:13 AM

09-Mar-2020

Dear Dr. Prasetyo:

Your manuscript entitled "Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells" has been successfully submitted online and is presently being given full consideration for publication in the Pesquisa Brasileira em Odontopediatria e Clínica Integrada.

Your manuscript ID is PBOCI-2020-0044.

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Thank you for submitting your manuscript to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada.

Sincerely,
Pesquisa Brasileira em Odontopediatria e Clínica Integrada Editorial Office

eric priyo prasetyo <eric-p-p@fkg.unair.ac.id>

Pesquisa Brasileira em Odontopediatria e Clínica Integrada - Decision on Manuscript ID PBOCI-2020-0044

1 message

Alessandro Cavalcanti <onbehalfof@manuscriptcentral.com> Thu, Apr 23, 2020 at 8:05 PM
Reply-To: alessandrouepb@gmail.com
To: eric-p-p@fkg.unair.ac.id

23-Apr-2020

Dear Dr. Prasetyo:

Manuscript ID PBOCI-2020-0044 entitled "Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells" which you submitted to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended consideration for publication, but also suggested some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript, in order for it to be able to be considered for acceptance.

To revise your manuscript, log into https://mc04.manuscriptcentral.com/pboci-scielo and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

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IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada, your revised manuscript should be submitted by 23-Jun-2020. If it is not possible for you to submit your revision by this date, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada and I look forward to receiving your revision.

Sincerely,
Prof. Alessandro Cavalcanti
Editor-in-Chief, Pesquisa Brasileira em Odontopediatria e Clínica Integrada
alessandrouepb@gmail.com

Associate Editor
Comments to the Author:
(There are no comments.)

Entire Scoresheet:
Reviewer: 1

Recommendation: Major Revision

Comments:
Thank you for submitting the above manuscript to Brazilian Research in Pediatric Dentistry and Integrated Clinic. This article is interesting; however, some points still remain to be solved. The suggestions and questions are posted attach.
All my best.

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: Yes

Does the Abstract (Summary) clearly and accurately describe the content of the article?: Yes

Is the problem significant and concisely stated?: No

Are the methods described comprehensively?: Yes

Are the interpretations and conclusions justified by the results?: Yes

Is adequate reference made to other work in the field?: Yes

Length of article is: Adequate

Number of tables is: Too many

Number of figures is: Adequate

Please state any conflict(s) of interest that you have in relation to the review of this paper (state “none” if this is not applicable).: None

Rating:

Interest: 3. Average

Quality: 4. Below Average

Originality: 2. Good

Overall: 3. Average

Reviewer: 2

Recommendation: Major Revision

Comments:
(There are no comments.)

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: Not applicable

Does the Abstract (Summary) clearly and accurately describe the content of the article?: No

Is the problem significant and concisely stated?: Yes

Are the methods described comprehensively?: No

Are the interpretations and conclusions justified by the results?: No

Is adequate reference made to other work in the field?: Yes

Length of article is: Too short

Number of tables is: Adequate

Number of figures is: Too few

Please state any conflict(s) of interest that you have in relation to the review of this paper (state “none” if this is not applicable).: none

Rating:

Interest: 2. Good

Quality: 5. Poor

Originality: 2. Good

Overall: 5. Poor

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3 attachments

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37K

Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord.pdf
109K

PBOCI-2020-0044-1.pdf
269K

Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells

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Abstract

Objective: The purpose of this study was to investigate the cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells (HUCMSC) in order to understand the characteristics for use in regenerative procedures. **Methods:** HUCMSC was isolated, cultured, and confirmed by flow cytometry. The biological characteristics such as cell morphology, proliferation, and protein expression were screened. To check the cytotoxicity, HUCMSC was cultured and divided into two groups, the control group and calcium hydroxide group. Methyl-thiazole-tetrazolium (MTT) assay was done on different concentrations of calcium hydroxide (0.39 to 25 µg/mL) and the cells were observed and counted. Obtained data were analyzed statistically by ANOVA with $p< 0.05$ was considered statistically significant. **Results:** The results show that significant difference was found between concentration of 6.25 and 3.125 µg/mL ($p=0.004$; $p<0.05$). There was no significant difference between 6.25, 12.5 and 25 µg/mL concentrations. There was no significant difference between 0.39, 0.78, 1.56, and 3.125 µg/mL concentrations. **Conclusion:** calcium hydroxide decreases the viability of HUCMSC. The lower the concentration of calcium hydroxide, the higher the viability of HUCMSC.

Keywords: Calcium hydroxide, Cytotoxicity, umbilical cord, Mesenchymal stem cells, Cell survival

Introduction

There are several mesenchymal stem cells (MSC) sources for clinical applications. Human umbilical cord mesenchymal stem cells (HUCMSCs) are MSC found in the umbilical cords and may be an attractive candidate for use in tissue regeneration [1]. Compared to other sources of stem cells from adult tissues, HUCMSCs are more primitive, non-invasive procedure of collection, provide high proliferation potential, high differentiation potential, immune privileged, immunosuppressive, rich in stemness [2].

Calcium hydroxide (CH), an odorless white powder, is classified as a strong alkali or base [3]. It has long been used in various treatment modalities in conservative dentistry, including as intracanal dressings, root canal sealers and pulp-capping material [4]. Calcium hydroxide is an essential alkaline material used in protocols of a successful regenerative endodontic treatment regimen [5] and the material of choice of all pulp therapy [4]. The mineralizing action of calcium hydroxide is determined by its high pH level, and the hydroxyl group is reckoned as the most important part as it gives an alkaline milieu, which promotes healing and mineralization [3].

Many researches have examined the cytotoxicity of calcium hydroxide, but few studies have used HUCMSC. Cell cultures are generally done to evaluate the biocompatibility of many materials [6,7]. In vitro experiments of cell cultures are usually performed to explore mechanism and biological responses in certain conditions. Even though results from in vitro experiments cannot immediately be deduced to clinical human conditions, they are clinically pertinent as they regard a model for screening of various material properties and risks [8].

Stem cells viability and response may differ depending on the time variation of contact from available calcium hydroxide. Therefore, the objective of this study was to investigate the cytotoxicity of calcium hydroxide on the viability HUCMSC, through MTT assay.

Materials and Methods

Preparation of HUCMSC

This study was approved by the Health Research Ethical Clearance Commission, Universitas Airlangga Faculty of Dental Medicine, Surabaya, Indonesia (Clearance number 059/HRECC.FODM/II/2020), and written informed consent was obtained before specimen collection. Umbilical cord tissue from donor was cleaned and washed from blood or debris with sterile Dulbecco's Phosphate Buffered Saline (Gibco, Paisley, UK), and then finely cut. Collagenase Type 4 (Worthington Biochemical Corporation, New Jersey, USA) was added to collect stem cells from the umbilical tissue.

Incubation was done for 45 minutes at 37°C. The cells and medium were centrifuged to form a pellet of cells. Minimum essential medium (MEM) alpha was added to the pellet and planted in 100 mm tissue culture plate (Iwaki, Asahi, Japan) and then incubated at 37°C. The plate was checked on a daily basis until it form 80-90% monolayer, the cells were split to form the next passage. Cells from the fourth passage was characterized at Stem Cell Research and Development Center Universitas Airlangga (Surabaya, Jawa Timur, Indonesia).

A total of 2 x 10^6 HUCMSC were taken for flow cytometric analysis. Flow cytometric analysis confirmed positive of CD73, CD90, CD105, negative of CD45 and CD34. All antibodies were purchased from BD Biosciences. HUCMSC were analyzed using a FACS Calibur Becton-Dickinson (BD Biosciences, USA), and the data were analyzed using CellQuest software (BD Biosciences, USA).

Calcium hydroxide preparation for HUCMSC

Calcium hydroxide medium was prepared by mixing the calcium hydroxide powder (EMSURE®Merck, Darmstadt, Germany) with minimal essential medium (MEM) alpha

(Gibco, Paisley, UK). We use calcium hydroxide concentration of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 μg/mL to be compared in this experiment.

MTT assay of calcium hydroxide on HUCMSC

The viability of HUCMSCs on Calcium hydroxide was determined by methyl-thiazol-tetrazolium (MTT) assay. The fifth passage HUCMSCs were seeded at a density of 5,000 cells per well in a 96-well microtiter plate (Iwaki, Asahi, Japan) and treated with calcium hydroxide at concentrations ranging from 0.39-25 μg/mL for 24 hours.

After incubation, MTT was added to each well and incubated for 3 hours, the process was stopped by the addition of dimethyl sulfoxide. Cell viability was analyzed by measuring the optical density (OD) detected by an automatic microplate reader (GloMax®Explorer, Wisconsin, USA) at a wavelength of 595 nm. The assessment was done in triplicates of n=3.

Statistical analysis

Data were provided as mean ± standard deviation. Statistical analysis were performed using SPSS20.0 package (SPSS Inc., Chicago, Illinois, USA). All data were tested for normality. One-way ANOVA test was used for comparisons of 3 or more groups (among control and different concentrations of calcium hydroxide groups).

Results

Isolation and identification of HUCMSC

The presence of mesenchymal stem cells taken from human umbilical cord was established and identified by means of antibodies for CD105, CD90, CD73, CD45, and CD34. The flow cytometry recorded more than 95% positivity of CD105, CD90, CD73, negativity of CD45 and CD34. This result confirmed the characterized cells are HUCMSC (Fig.1).

Cytotoxicity assessment of calcium hydroxide on HUCMSC

Viable cells seen under inverted microscope are available on figure 1. The mean and standard deviation (SD) of control and calcium hydroxide groups are shown on table 1. Data on the result was distributed normally (P>0.05), and homogeneity test yield a homogeny data (P>0.05). The significance is shown on table 2.

MTT assay result of different calcium hydroxide (CH) concentrations showed no significant difference among 6.25, 12.5 and 25 μg/mL. There were also no significant difference among 0.39, 0.78, 1.56, and 3.125 μg/mL. The significant difference was found between 3.125 and 6.25 μg/mL (P= 0.004).

Table 1. Mean and Standard Deviation (SD) of control and CH groups.

Group	Percentage of cell viability mean ± SD	n
Control	100 ± 0.00	3
CH 25	56.1013 ± 3.22481	3
CH 12.5	58.0903 ± 6.76619	3
CH 6.25	58.4880 ± 3.06637	3
CH 3.125	69.0950 ± 2.50740	3
CH 1.56	69.8520 ± 2.27282	3
CH 0.78	70.0930 ± 3.26168	3
CH 0.39	70.2317 ± 3.04301	3

Table 2. Significance (*P* value) among groups of different CH concentrations.

CH Concentrations	25	12.5	6.25	3.125	1.56	0.78	0.39
25	-	0.523	0.445	0.001	0.000	0.000	0.000
12.5	0.523	-	0.898	0.003	0.002	0.001	0.001
6.25	0.445	0.898	-	0.004	0.002	0.002	0.002
3.125	0.001	0.003	0.004	-	0.807	0.747	0.714
1.56	0.000	0.002	0.002	0.807	-	0.938	0.902
0.78	0.000	0.001	0.002	0.747	0.938	-	0.964
0.39	0.000	0.001	0.002	0.714	0.902	0.964	-

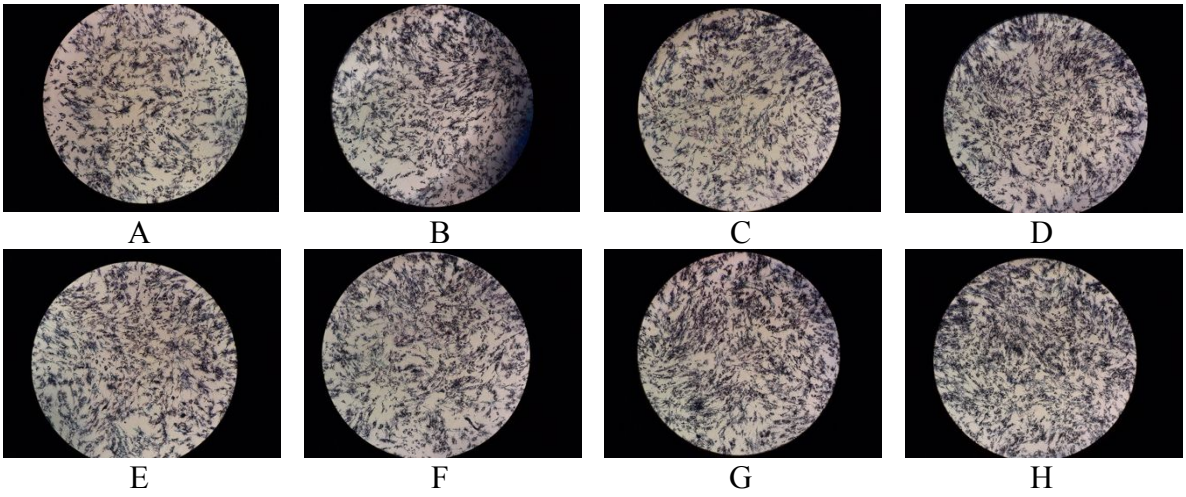

Figure 1. The viable cells seen with inverted microscope: control (A), CH concentration of 25 μg/mL (B), 12.5 μg/mL (C), 6.25 μg/mL (D), 3.125 μg/mL (E), 1.56 μg/mL (F), 0.78 μg/mL (G), and 0.39 μg/mL (H).

Discussion

The interest in finding alternative sources of mesenchymal stem cells (MSC) and exploring its potential in dentistry is increasing. There are studies about other stem cell origins, such as human bone marrow mesenchymal stem cells, stem cells of the apical papillae, dental pulp stem cells, periodontal ligament stem cells, etc., but little information was available regarding HUCMSC and its potential use in regeneration.

Although human bone marrow mesenchymal stem cells are more popular, there are disadvantages regarding the invasive procedure involved [9]. Human umbilical cord mesenchymal stem cells (HUCMSC) are used in this study because they can be isolated and expanded easily in large quantities in vitro [2].

Biocompatibility is an essential factor that must be contemplated when choosing a material for therapeutic purposes. In this study, HUCMSC were cultured in Minimum Essential Medium Alpha (MEM-A) added calcium hydroxide in normal culture condition, incubated at

37°C and 5% CO_2. We used MTT assay to determine cell viability and cytotoxicity under calcium hydroxide influence.

MTT assay is the most common method to determine cytotoxicity and proven to be more accurate and time saving. The principle of MTT assay is to break tetrazolium ring (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide) to produce insoluble blue-purple formazan product. The formazan production can be measured by optical density spectrophotometry. The lower the optical density means the lower living cells to metabolize the MTT.

Calcium hydroxide is chemically categorized as a strong alkali with a high pH (about 12.5 – 12.8) and its core effects originated from ionic separation of Ca^{++} and OH^- ions [3]. Calcium ion plays an important function in the intrinsic pathway of apoptosis. The upregulation of calcium into mitochondrion results in the release of cytochrome c. In cytoplasm, the cytochrome c binds to apoptotic protease-activating factor-1 (APAF-1), a cytoplasmic protein, and forms the apoptosome which turn and activates procaspase-9 into caspase-9 and subsequently activates other caspases to carry out cell death [10]. Calcium hydroxide may possibly upregulate the expression of caspase-9, but further research is important to determine the effect of time and duration of calcium hydroxide application.

In this study, the significant decrease in cell viability might be caused by apoptosis. Apoptosis is a programmed cell death mechanism that engage in the homeostatic management of cell population, with no inflammatory reaction occurring [7]. Apoptosis is initiated by either an intrinsic or an extrinsic pathway.

Calcium hydroxide is well tolerated by bone and pulpal tissue. However, it will create a zone of necrosis if in direct contact with tissue, altering the physicochemical form of cell to cell material which through the break of glycoproteins, causing protein alteration [3]. This is in parallel with the result of this study which found calcium hydroxide effects the viability of HUCMSC.

Conclusion

These results show CH effects the viability of HUCMSC depending on the concentration used. In conclusion, this study provide evidence that CH can decrease the viability of HUCMSC on longer period. The lower the concentration of Calcium hydroxide, the higher the viability of HUCMSC. Further studies will be required to reveal more novel mechanisms of HUCMSC for regenerative endodontic purposes.

Author's Contributions

EPP	0000-0001-9962-235X	Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project administration, Resources, Writing-original draft preparation, Writing-review and editing
IW	0000-0002-5928-7005	Funding Acquisition, Investigation, Resources, Supervision
FC	0000-0001-6363-8093	Investigation, Methodology, Project administration, Resources
MK	0000-0002-3869-754X	Investigation, Methodology, Project administration, Resources, Writing-original draft
NH	0000-0002-9261-3626	Investigation, Methodology, Project administration, Resources, Supervision

NH	0000-0003-0807-0081	Investigation, Methodology, Project administration, Resources, Supervision
ERW	0000-0002-9554-6051	Investigation, Methodology, Project Administration, Writing-original draft, Writing-review & editing
APN	0000-0001-7427-7561	Investigation, Methodology, Project Administration, Writing-original draft, Writing-review & editing
SG	0000-0003-2398-0016	Project Administration, Writing-original draft, Writing-review & editing
HS	0000-0002-3358-5231	Project Administration, Writing-original draft, Writing-review & editing
EH	0000-0001-9723-8098	Data Curation, Formal Analysis, Investigation, Methodology, Software, Validation
FAR	0000-0001-8182-1465	Investigation, Methodology, Project administration, Resources, Supervision

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest
The authors declare no conflicts of interest.

Acknowledgement
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References

1. Meguid EA, Ke Y, Ji J, El-Hashash AHK. Stem cells applications in bone and tooth repair and regeneration: New insights, tools, and hopes. J Cell Physiol. 2017; 9999:1-11. https://doi.org/10.1002/jcp.25940.
2. Li T, Xia M, Gao Y, Chen Y, Xu Y. Human umbilical cord mesenchymal stem cells: an overview of their potential in cell-based therapy. Expert Opin Biol Ther. 2015; 15:1293-1306. https://doi.org/10.1517/14712598.2015.1051528.
3. Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. International Endodontic Journal. 2011; 44: 697-730. https://doi.org/10.1111/j.1365-2591.2011.01886x.
4. Ba-Hattab R, Al-Jamie M, Aldreib H, Alessa L, Alonazi M. Calcium hydroxide in endodontics: an overview. Open journal of stomatology. 2016; 6: 274-289. https://doi.org/10.4236/ojst.2016.612033.
5. Kahler B, Chugal N, Lin LM. Alkaline materials and regenerative endodontics: a review. Materials. 2017; 10: 1389. https://doi.org/10.3390/ma10121389.
6. Peters OA. Research that matters-biocompatibility and cytotoxicity screening. International Endodontic Journal 2013: 46; 195-7. https://doi.org/10.1111/iej.12047.
7. Collado-Gonzales M, Garcia-Bernal D, Onate-Sanchez RE, Ortolani-Seltenerich PS, Alvarez-Muro T, Lozano A, Forner L, Llena C, Moraleda JM, Rodriguez-Lozano FJ. Cytotoxicity and bioactivity of various pulpotomy materials on stem cells from human

exfoliated primary teeth. International Endodontic Journal 2017: 50; e19-e30. https://doi.org/10.1111/iej.12751.
8. Pires CW, Botton G, Cadona FC, Machado AK, Azzolin VF, da cruz IBM, et al. Induction of cytotoxicity, oxidative stress and genotoxicity by root filling pastes used in primary teeth. International Endodontic Journal 2016: 49; 737-45. https://doi.org/10.1111/iej.12502.
9. Hendrijantini N. comparative in vitro study of the cytotoxicity of gelatin and alginate to human umbilical cord mesenchymal stem cells. Dental Journal (Majalah Kedokteran Gigi) 2019; 52(1): 36-40. https://doi.org/10.20473/j/djmkg.v52.i1.
10. Kontogiannis TG, Tosios KI, Kerezoudis NP. Effect of calcium hydroxide as intracanal medicament on the expression of caspase-9 located within the radicular cyst epithelium. Australian Endodontic Journal. 2019; 1-5. https://doi.org/10.1111/aej.12325.

Stem cells can be derived from various parts of the umbilical cord; including Wharton's jelly, cord lining, and the perivascular region.it is not clear , from which part are they isolated?

abstract :

You said that" The protein expression of stem cells were screened", but in result section This has not been done.

Introduction

No previous related studies have been reported.

Methods

What is the volume of added Ca(OH)2?

fourth passage or fifth passage? In the article, Both are mentioned.

Have authors seen Viable cells under inverted microscope? It is not correct.

Result

the fig 1 did not show characterization of HUCMSC, but the authors have mentioned it.

discussion

Discussion is too weak. Most sentences do not have a reference. In addition, the results need to be further discussed .

Thank you for submitting the above manuscript to Brazilian Research in Pediatric Dentistry and Integrated Clinic. This article is interesting; however, some points still remain to be solved. The suggestions and questions are posted bellow:

ABSTRACT:

- The authors should emphasize the dentistry application in the objective, e.g. “use in regenerative procedures in dentistry”.

- In the sentence “To check the cytotoxicity, HUCMSC was cultured and divided into two groups, the control group and calcium hydroxide group”, please, include what were used as control.

- The authors should include in the ‘Results’ section data about morphology, proliferation and protein expression of cells.

- Conclusions: I suggest include briefly some clinical relevance.

- Keywords: ‘Cytotocity’ and ‘Human umbilical cord’ are not registered at Medical Subject Headings of the U.S. National Library of Medicine (https://meshb.nlm.nih.gov).

INTRODUCTION:

- The authors should explore some points to justify the research, as follow: What is the innovation of the study? Why it is important explore the use of HUCMSC in dentistry? Why does this need to be tested?

- In addition, I suggest include some information about the HUCMSC have been used in others regenerative procedures or investigations, as Alzheimer’s disease, rheumatoid arthritis or multiple sclerosis.

MATERIAL AND METHODS:

- I would like to suggest change the topic ‘Preparation of HUCMSC’ to ‘Isolation, expansion and cultivation of HUC-MSCs’.

- Please include some references about the isolation and cultivation of cells.

- What parameters are chosen for calcium hydroxide concentration of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 μg/mL ?

- The authors should in this section include some information about the methods used to visualize the cells.

- I also would like to suggest change the topic ‘MTT assay of calcium hydroxide on HUCMSC’ to ‘Viability of HUCMS on calcium hydroxide’.

- Please include some reference about the MTT assay.

RESULTS

- In the sentence ‘This result confirmed the characterized cells are HUCMSC (Fig.1)’. Although the Figure 1 shows the morphology of the cells, it does not correspond to their characterization. Figure 1 represents the architecture/morphology of viable cells according to viability.

- The authors should consider improve this section with details about characterization of cells in the Figure 1.

- I suggest clustering the information from Tables 1 and 2 in one table. Moreover, highlight or use with special characters for the $p < 0.05$ value.

DISCUSSION:

- In the sentence 'Although human bone marrow mesenchymal stem cells are more popular, there are disadvantages regarding the invasive procedure involved [9]': please include what are these disadvantages.

- Is there any difference between performing the cytotoxicity test with HUCMSC or other cells? This need to be clear in discussion section.

- I suggest to the authors include some explanations about the findings (morphology, proliferation, protein expression and cytotoxicity), besides that new directions/possibilities regarding the use of HUCMSC in dentistry.

- I also suggest include the limitations of the findings and future directions.

Response to Reviewer 1 taken from the attachment:
Revisions are provided in yellow highlights.

Thank you for the review and valuable suggestions provided to us for the improvement of our manuscript.

ABSTRACT:
-Emphasis has been added, for regenerative dentistry (endodontics).
-The control used has been included, which is MEM alpha only, without the addition of Calcium hydroxide.
-Results has been revised. Morphology and expression has been included (positive and negative CD).
-Keywords has been revised according to Medical Subject Headings of the US National Library of Medicine, Human umbilical cord changed to Umbilical cord, and cytotoxicity changed to Cell viability.

INTRODUCTION:
-This need to be explored because study of HUCMSC in dentistry is still limited.
-Information is included about the HUCMSC have been used in other regenerative therapy, e.g. diabetes and its complications.

MATERIALS AND METHODS:
-The topic has been changed as suggested.
-Isolation and other methods is according to the standard of protocol used by Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia.
-The method to visualize the cell is through viewing from inverted microscope, according to the manufacturer's direction.
-The topic has been changed as suggested.

RESULTS:
-We apologize that there is a typographical error on the sentence. "(Fig.1)" was not supposed to be typed.
-We thank you for the suggestion. For the $p<0.05$ value, we have added asterisks (*) at the back of each significant result.

DISCUSSION:
-We have included some disadvantages, such as the need of general anesthesia, invasive procedure and multiple procedures needed to take the bone marrow stem cells.
-We have included the specificity of using HUCMSC in this study, instead of other cells.
-Limitations of the study has been added in conclusion.

Response to Reviewer 2 taken from the attachment:
Revisions are provided in yellow highlights.

Thank you for the review and valuable suggestions provided to us for the improvement of our manuscript.

-We used HUCMSC derived from Wharton's Jelly, and we have added in the manuscript.

ABSTRACT:
-We have added the protein expression of positive and negative CD in the abstract.

INTRODUCTION:
-Unfortunately no previous studies have been reported, and this study was meant to be the first stage of exploration.

MATERIALS AND METHODS:
-As suggested, we have included the volume, which was 200 microliter. We use the same volume of medium for both control and treatment groups.
-The fourth passage cells were used for screening of CDs, and while we waited for the result, the cells were grown and expanded to become the fifth passage, and we used this fifth passage HUCMSC for analysis.
-Cells morphology and distribution were observed through inverted microscope, while viable cells were determined by optical density (OD) from automated microplate reader.

RESULT:
-We apologize that there is a typographical error on the sentence. "(Fig.1)" was not supposed to be typed.

DISCUSSION:
-We have revised and added the discussion session.

Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells

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Abstract

Objective: The purpose of this study was to examine the cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells (HUCMSC) in order to understand the characteristics for use in procedures of regenerative dentistry, especially regenerative endodontics. **Methods:** HUCMSC was isolated, cultured, and confirmed by flow cytometry. The biological characteristics such as cell morphology, proliferation, and protein expression were screened. To check the cytotoxicity, HUCMSC was cultured and divided into two groups, the control group (cultured in minimum essential medium (MEM) alpha) and calcium hydroxide group (cultured in MEM alpha and calcium hydroxide). Methyl-thiazole-tetrazolium (MTT) assay was done on different concentrations of calcium hydroxide (0.39 to 25 μg/mL) and the cells were observed and counted. Obtained data were analyzed statistically, with *P* value < 0.05 was considered statistically significant. **Results:** Flow cytometric analysis confirmed positive of CD73, CD90, CD105, negative of CD45 and CD34.The results show that significant difference was found between concentration of 6.25 and 3.125 μg/mL (*P*=0.004). There were no significant difference among 6.25, 12.5 and 25 μg/mL concentrations. There were also no significant difference among 0.39, 0.78, 1.56, and 3.125 μg/mL concentrations. **Conclusion:** Even though calcium hydroxide is a medicament of choice in clinical endodontics, it decreases the viability of HUCMSC. The lower the concentration of calcium hydroxide, the higher the viability of HUCMSC.

Keywords: Calcium hydroxide, Umbilical cord, Mesenchymal stem cells, Cell viability

Introduction

There are several mesenchymal stem cells (MSC) sources for clinical applications. Human umbilical cord mesenchymal stem cells (HUCMSCs) are MSC found in the umbilical cords and may be an attractive candidate for use in tissue regeneration [1]. Compared to other sources of stem cells from adult tissues, HUCMSCs are more primitive, non-invasive procedure of collection, provide high proliferation potential, high differentiation potential, immune privileged, immunosuppressive, rich in stemness [2]. Currently, HUCMSC is vastly studied to be used for degenerative therapy, e.g. diabetes and its complications.

Calcium hydroxide (CH), an odorless white powder, is classified as a strong alkali or base [3]. It has long been used in various treatment modalities in conservative dentistry, including as intracanal dressings, root canal sealers and pulp-capping material [4]. Calcium hydroxide is an essential alkaline material used in protocols of a successful regenerative endodontic treatment regimen [5] and the material of choice of all pulp therapy [4]. The mineralizing action of calcium hydroxide is determined by its high pH level, and the hydroxyl group is reckoned as the most important part as it gives an alkaline milieu, which promotes healing and mineralization [3].

The use of HUCMSC in dentistry is still limited and therefore need to be explored. Many researches have examined the cytotoxicity of calcium hydroxide, but few studies have used HUCMSC. Cell cultures are generally done to evaluate the biocompatibility of many materials [6,7]. In vitro experiments of cell cultures are usually performed to explore mechanism and biological responses in certain conditions. Even though results from in vitro experiments cannot immediately be deduced to clinical human conditions, they are clinically pertinent as they regard a model for screening of various material properties and risks [8].

Stem cells viability and response may differ depending on the time variation of contact from available calcium hydroxide. To further explore the potency of calcium hydroxide on HUCMSC, viability testing is important to be done. Therefore the objective of this study was to investigate the cytotoxicity of calcium hydroxide on the viability HUCMSC, through MTT assay.

Materials and Methods

Isolation, expansion and cultivation of HUCMSC

This study was approved by the Health Research Ethical Clearance Commission, Universitas Airlangga Faculty of Dental Medicine, Surabaya, Indonesia (Clearance number 059/HRECC.FODM/II/2020), and written informed consent was obtained before specimen collection. The isolation, expansion and cultivation of HUCMSC is according to the protocol of Stem Cell Research and Development Center Universitas Airlangga (Surabaya, Jawa Timur, Indonesia). Umbilical cord tissue from donor was cleaned and washed from blood or debris with sterile Dulbecco's Phosphate Buffered Saline (Gibco, Paisley, UK), the Wharton's Jelly was taken and then finely cut. Collagenase Type 4 (Worthington Biochemical Corporation, New Jersey, USA) was added to collect stem cells from the umbilical tissue.

Incubation was done for 45 minutes at 37°C. The cells and medium were centrifuged to form a pellet of cells. Minimum essential medium (MEM) alpha was added to the pellet and planted in 100 mm tissue culture plate (Iwaki, Asahi, Japan) and then incubated at 37°C. The plate was checked on a daily basis until it form 80-90% monolayer, the cells were split to form the next passage. Cells from the fourth passage was characterized at Stem Cell Research and Development Center Universitas Airlangga (Surabaya, Jawa Timur, Indonesia).

A total of 2 x 10^6 HUCMSC were taken for flow cytometric analysis. Flow cytometric analysis confirmed positive of CD73, CD90, CD105, negative of CD45 and CD34. All antibodies were purchased from BD Biosciences. HUCMSC were analyzed using a FACS Calibur Becton-Dickinson (BD Biosciences, USA), and the data were analyzed using CellQuest software (BD Biosciences, USA).

Calcium hydroxide preparation for HUCMSC

Calcium hydroxide medium was prepared by mixing the calcium hydroxide powder (EMSURE®Merck, Darmstadt, Germany) with minimal essential medium (MEM) alpha (Gibco, Paisley, UK). We use calcium hydroxide concentration of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 μg/mL to be compared in this experiment.

Viability of HUCMSC on calcium hydroxide

The viability of HUCMSCs on Calcium hydroxide was determined by methyl-thiazol-tetrazolium (MTT) assay. The fifth passage HUCMSCs were seeded at a density of 5,000 cells per well in a 96-well microtiter plate (Iwaki, Asahi, Japan) and treated with calcium hydroxide at concentrations ranging from 0.39-25 μg/mL for 24 hours. Each well was given 200 microliter medium containing calcium hydroxide according to the explored concentrations.

After incubation, MTT was added to each well and incubated for 3 hours, the process was stopped by the addition of dimethyl sulfoxide. Cell viability was analyzed by measuring the optical density (OD) detected by an automatic microplate reader (GloMax®Explorer, Wisconsin, USA) at a wavelength of 595 nm. The assessment was done in triplicates of n=3.

Statistical analysis

Data were provided as mean ± standard deviation. Statistical analysis were performed using SPSS20.0 package (SPSS Inc., Chicago, Illinois, USA). All data were tested for normality. One-way ANOVA test was used for comparisons of 3 or more groups (among control and different concentrations of calcium hydroxide groups).

Results

Isolation and identification of HUCMSC

The presence of mesenchymal stem cells taken from human umbilical cord was established and identified by means of antibodies for CD105, CD90, CD73, CD45, and CD34. The flow cytometry recorded more than 95% positivity of CD105, CD90, CD73, negativity of CD45 and CD34. This result confirmed the characterized cells are HUCMSC.

Cytotoxicity assessment of calcium hydroxide on HUCMSC

Viable cells seen under inverted microscope are available in figure 1. The mean and standard deviation (SD) of control and calcium hydroxide groups are shown in table 1. Data in the result was distributed normally (P>0.05), and homogeneity test yield a homogeny data (P>0.05). The significance is shown in table 2.

MTT assay result of different calcium hydroxide (CH) concentrations showed no significant difference among 6.25, 12.5 and 25 μg/mL. There were also no significant

difference among 0.39, 0.78, 1.56, and 3.125 μg/mL. The significant difference was found between 3.125 and 6.25 μg/mL (*P*= 0.004).

Table 1. Mean and Standard Deviation (SD) of control and CH groups.

Group	Percentage of cell viability mean ± SD	n
Control	100 ± 0.00	3
CH 25	56.1013 ± 3.22481	3
CH 12.5	58.0903 ± 6.76619	3
CH 6.25	58.4880 ± 3.06637	3
CH 3.125	69.0950 ± 2.50740	3
CH 1.56	69.8520 ± 2.27282	3
CH 0.78	70.0930 ± 3.26168	3
CH 0.39	70.2317 ± 3.04301	3

Table 2. Significance (*P* value) among groups of different CH concentrations.

CH Concentration (μg/mL)	25	12.5	6.25	3.125	1.56	0.78	0.39
25	-	0.523	0.445	0.001*	0.000*	0.000*	0.000*
12.5	0.523	-	0.898	0.003*	0.002*	0.001*	0.001*
6.25	0.445	0.898	-	0.004*	0.002*	0.002*	0.002*
3.125	0.001*	0.003*	0.004*	-	0.807	0.747	0.714
1.56	0.000*	0.002*	0.002*	0.807	-	0.938	0.902
0.78	0.000*	0.001*	0.002*	0.747	0.938	-	0.964
0.39	0.000*	0.001*	0.002*	0.714	0.902	0.964	-

*Significant (*P*<0.05)

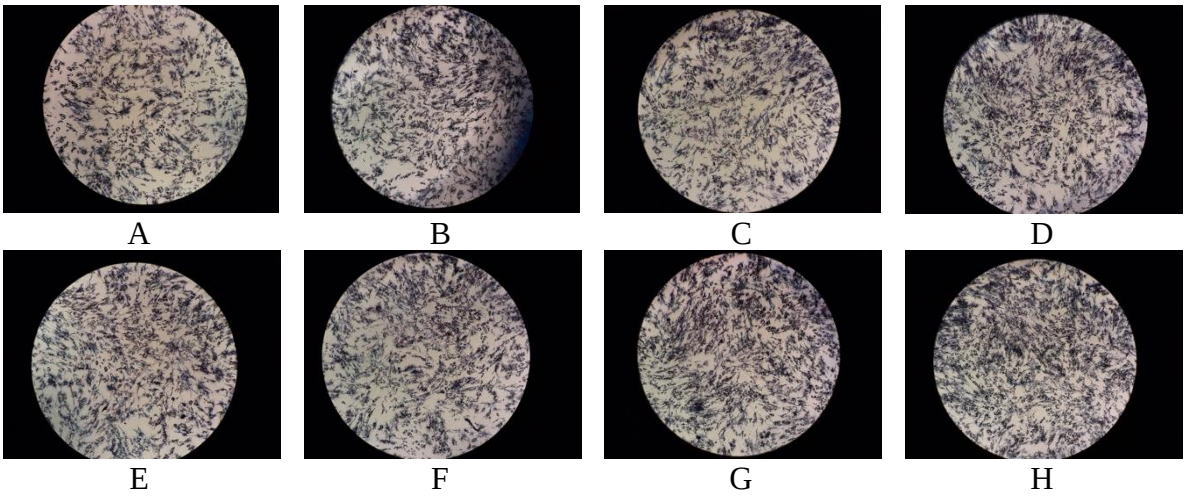

Figure 1. The viable cells seen with inverted microscope: control (A), CH concentration of 25 μg/mL (B), 12.5 μg/mL (C), 6.25 μg/mL (D), 3.125 μg/mL (E), 1.56 μg/mL (F), 0.78 μg/mL (G), and 0.39 μg/mL (H).

Discussion

The interest in finding alternative sources of mesenchymal stem cells (MSC) and exploring its potential in dentistry is increasing. There are studies about other stem cell origins, such as human bone marrow mesenchymal stem cells, stem cells of the apical papillae, dental pulp stem cells, periodontal ligament stem cells, etc., but little information was available regarding HUCMSC and its potential use in regeneration.

Although human bone marrow mesenchymal stem cells are more popular, there are disadvantages, such as the requirement of general anesthesia regarding the invasive surgical procedure involved, and multiple procedures may be needed to acquire sufficient amount of cells [9]. Human umbilical cord mesenchymal stem cells (HUCMSC) are used in this study because they can be isolated and expanded easily in large quantities in vitro [2].

Biocompatibility is an essential factor that must be contemplated when choosing a material for therapeutic purposes. In this study, HUCMSC were cultured in Minimum Essential Medium Alpha (MEM-A) added calcium hydroxide in normal culture condition, incubated at 37°C and 5% CO_2. We used MTT assay to determine cell viability and cytotoxicity under calcium hydroxide influence. HUCMSC is used instead of other cells, because stem cells are different in its nature and character compared to other matured cells. Although in morphology HUCMSC is similar to fibroblast, HUCMSC has specific protein expressions that fibroblast don't have, such as the positivity of CD105, CD90, CD73, negativity of CD45 and CD34.

MTT assay is the most common method to determine cytotoxicity and proven to be more accurate and time saving. The principle of MTT assay is to break tetrazolium ring (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide) to produce insoluble blue-purple formazan product. The formazan production can be measured by optical density spectrophotometry. The lower the optical density means the lower living cells to metabolize the MTT.

Calcium hydroxide is chemically categorized as a strong alkali with a high pH (about 12.5 – 12.8) and its core effects originated from ionic separation of Ca^{++} and OH^{-} ions [3]. Calcium ion plays an important function in the intrinsic pathway of apoptosis. The upregulation of calcium into mitochondrion results in the release of cytochrome c. In cytoplasm, the cytochrome c binds to apoptotic protease-activating factor-1 (APAF-1), a cytoplasmic protein, and forms the apoptosome which turn and activates procaspase-9 into caspase-9 and subsequently activates other caspases to carry out cell death [10]. Calcium hydroxide may possibly upregulate the expression of caspase-9, but further research is important to determine the effect of time and duration of calcium hydroxide application.

In this study, the significant decrease in cell viability might be caused by apoptosis. Apoptosis is a programmed cell death mechanism that engage in the homeostatic management of cell population, with no inflammatory reaction occurring [7]. Apoptosis is initiated by either an intrinsic or an extrinsic pathway.

Calcium hydroxide is well tolerated by bone and pulpal tissue. However, it will create a zone of necrosis if in direct contact with tissue, altering the physicochemical form of cell to cell material which through the break of glycoproteins, causing protein alteration [3]. This is in parallel with the result of this study which found calcium hydroxide effects the viability of HUCMSC.

Conclusion

These results show CH effects the viability of HUCMSC depending on the concentration used. In conclusion, this study provide evidence that CH can decrease the viability of HUCMSC on longer period. The lower the concentration of Calcium hydroxide, the higher the viability of HUCMSC. Since this study is the first step of HUCMSC exploration on CH, further studies will be required to reveal more novel mechanisms of HUCMSC for regenerative endodontic purposes.

Author's Contributions

EPP	0000-0001-9962-235X	Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project administration, Resources, Writing-original draft preparation, Writing-review and editing
IW	0000-0002-5928-7005	Funding Acquisition, Investigation, Resources, Supervision
FC	0000-0001-6363-8093	Investigation, Methodology, Project administration, Resources
MK	0000-0002-3869-754X	Investigation, Methodology, Project administration, Resources, Writing-original draft
NH	0000-0002-9261-3626	Investigation, Methodology, Project administration, Resources, Supervision
NH	0000-0003-0807-0081	Investigation, Methodology, Project administration, Resources, Supervision
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APN	0000-0001-7427-7561	Investigation, Methodology, Project Administration, Writing-original draft, Writing-review & editing
SG	0000-0003-2398-0016	Project Administration, Writing-original draft, Writing-review & editing
HS	0000-0002-3358-5231	Project Administration, Writing-original draft, Writing-review & editing
EH	0000-0001-9723-8098	Data Curation, Formal Analysis, Investigation, Methodology, Software, Validation
FAR	0000-0001-8182-1465	Investigation, Methodology, Project administration, Resources, Supervision

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

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The authors thank the Publication Center, Faculty of Dental Medicine for technical supports.

References

1. Meguid EA, Ke Y, Ji J, El-Hashash AHK. Stem cells applications in bone and tooth repair and regeneration: New insights, tools, and hopes. J Cell Physiol. 2017; 9999:1-11. https://doi.org/10.1002/jcp.25940.
2. Li T, Xia M, Gao Y, Chen Y, Xu Y. Human umbilical cord mesenchymal stem cells: an overview of their potential in cell-based therapy. Expert Opin Biol Ther. 2015; 15:1293-1306. https://doi.org/10.1517/14712598.2015.1051528.
3. Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. International Endodontic Journal. 2011; 44: 697-730. https://doi.org/10.1111/j.1365-2591.2011.01886x.
4. Ba-Hattab R, Al-Jamie M, Aldreib H, Alessa L, Alonazi M. Calcium hydroxide in endodontics: an overview. Open journal of stomatology. 2016; 6: 274-289. https://doi.org/10.4236/ojst.2016.612033.
5. Kahler B, Chugal N, Lin LM. Alkaline materials and regenerative endodontics: a review. Materials. 2017; 10: 1389. https://doi.org/10.3390/ma10121389.
6. Peters OA. Research that matters-biocompatibility and cytotoxicity screening. International Endodontic Journal 2013: 46; 195-7. https://doi.org/10.1111/iej.12047.
7. Collado-Gonzales M, Garcia-Bernal D, Onate-Sanchez RE, Ortolani-Seltenerich PS, Alvarez-Muro T, Lozano A, Forner L, Llena C, Moraleda JM, Rodriguez-Lozano FJ. Cytotoxicity and bioactivity of various pulpotomy materials on stem cells from human exfoliated primary teeth. International Endodontic Journal 2017: 50; e19-e30. https://doi.org/10.1111/iej.12751.
8. Pires CW, Botton G, Cadona FC, Machado AK, Azzolin VF, da cruz IBM, et al. Induction of cytotoxicity, oxidative stress and genotoxicity by root filling pastes used in primary teeth. International Endodontic Journal 2016: 49; 737-45. https://doi.org/10.1111/iej.12502.
9. Hendrijantini N. comparative in vitro study of the cytotoxicity of gelatin and alginate to human umbilical cord mesenchymal stem cells. Dental Journal (Majalah Kedokteran Gigi) 2019; 52(1): 36-40. https://doi.org/10.20473/j/djmkg.v52.i1.
10. Kontogiannis TG, Tosios KI, Kerezoudis NP. Effect of calcium hydroxide as intracanal medicament on the expression of caspase-9 located within the radicular cyst epithelium. Australian Endodontic Journal. 2019; 1-5. https://doi.org/10.1111/aej.12325.

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1 message

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Tue, Apr 28, 2020 at 2:16 AM

27-Apr-2020

Dear Dr. Prasetyo:

Your manuscript entitled "Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells" has been successfully submitted online and is presently being given full consideration for publication in the Pesquisa Brasileira em Odontopediatria e Clínica Integrada.

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Thank you for submitting your manuscript to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada.

Sincerely,
Pesquisa Brasileira em Odontopediatria e Clínica Integrada Editorial Office

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1 message

Alessandro Cavalcanti <onbehalfof@manuscriptcentral.com> Thu, May 7, 2020 at 2:27 AM
Reply-To: alessandrouepb@gmail.com
To: eric-p-p@fkg.unair.ac.id

06-May-2020

Dear Dr. Prasetyo:

Manuscript ID PBOCI-2020-0044.R1 entitled "Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells" which you submitted to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended consideration for publication, but also suggested some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript, in order for it to be able to be considered for acceptance.

To revise your manuscript, log into https://mc04.manuscriptcentral.com/pboci-scielo and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

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Because we are trying to facilitate timely publication of manuscripts submitted to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada, your revised manuscript should be submitted by 06-Jun-2020. If it is not possible for you to submit your revision by this date, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Pesquisa Brasileira em Odontopediatria e Clínica Integrada and I look forward to receiving your revision.

Sincerely,
Prof. Alessandro Cavalcanti
Editor-in-Chief, Pesquisa Brasileira em Odontopediatria e Clínica Integrada
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Associate Editor
Comments to the Author:
There are still few comments from the reviewers. Please consider them in a revised version of the manuscript.

Entire Scoresheet:
Reviewer: 1

Recommendation: Major Revision

Comments:
Dear author
Thank you for submitting your manuscript entitled "Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells" to Pesquisa Brasileira em Odontopediatria e Clínica Integrada. Some topics were not well elucidated, as follows:

Please refer any previous studies that evaluated cytotoxicity by means of HUCMSC in the filed of Dentistry, and how this would correlate with dental pulp stem cells.

The authors state that few studies have used HUCMSC to evaluate the cytotoxicity of calcium hydroxide but does not provide any reference to ensure that.

Please discuss the results of this study with previous work that have used a well-established cell line to assess the cytotoxicity of materials in the field of regenerative dentistry

Give a reasonable explanation why the authors chose 24 hours of exposition to calcium hydroxide. If the results were evaluated at lower exposure times, does the authors expected to have the same results?

The results and discussion showed which groups differed from each other, but it does not justify which value can be considered relevant. How was cytotoxicity estimated? The IC50 was calculated?

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: No

Does the Abstract (Summary) clearly and accurately describe the content of the article?: Yes

Is the problem significant and concisely stated?: No

Are the methods described comprehensively?: Yes

Are the interpretations and conclusions justified by the results?: No

Is adequate reference made to other work in the field?: Yes

Length of article is: Adequate

Number of tables is: Adequate

Number of figures is: Adequate

Please state any conflict(s) of interest that you have in relation to the review of this paper (state “none” if this is not applicable).: None.

Rating:

Interest: 3. Average

Quality: 3. Average

Originality: 5. Poor

Overall: 3. Average

Reviewer: 2

Recommendation: Accept

Comments:
Thank you for correct appropriately the manuscript. However, is still important to insert some references about the methods used (e.g. cell culture and the MTT assay). Please include in the ‘methods’ section.

Additional Questions:
Does the manuscript contain new and significant information to justify publication?: Yes

Does the Abstract (Summary) clearly and accurately describe the content of the article?: Yes

Is the problem significant and concisely stated?: Yes

Are the methods described comprehensively?: Yes

Are the interpretations and conclusions justified by the results?: Yes

Is adequate reference made to other work in the field?: Yes

Length of article is: Adequate

Number of tables is: Adequate

Number of figures is: Adequate

Please state any conflict(s) of interest that you have in relation to the review of this paper (state “none” if this is not applicable).: None

Rating:

Interest: 2. Good

Quality: 3. Average

Originality: 2. Good

Overall: 3. Average

Response for Reviewer 1:

Revisions are provided in yellow highlights.

Thank you for the review and valuable suggestions provided to us for our manuscript.

- Previous study was included and referred in reference no 9. To properly correlate HUCMSC and DPSC, further research needs to be done to objectively compare them.

- Few studies use HUCMSC to evaluate CH cytotoxicity. About this matter, we were having difficulty in searching by the keywords of HUCMSC, Calcium hydroxide, viability and cytotoxicity altogether from Google Scholar for scholarly articles. It would be very kind and we would certainly appreciate if you could guide us and give some examples in finding such references.

- We have added discussion about the result of this study and previous work with a well-established cell line and referred in reference no 12.

- We chose 24 hours of CH exposure to extrapolate the minimum feasible clinical application. CH was meant for long term exposure. Duration of less than 24 hours would be irrelevant with its use in clinical practice. It's hard to say, but lower exposure time would probably make CH less toxic or perhaps non-toxic, but to confirm this, another research needs to be done.

- In our study, cytotoxicity was measured with MTT assay. IC50 was neither conducted nor calculated.

Response for Reviewer 2:

Revisions are provided in yellow highlights.

Thank you for your review and sincere recommendation of accepting our manuscript.

- Reference about the methods used (cell culture and MTT assay) was added, and referred in reference no 9.

Cytotoxicity of Calcium Hydroxide on Human Umbilical Cord Mesenchymal Stem Cells

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Abstract

Objective: The purpose of this study was to examine the cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells (HUCMSC) in order to understand the characteristics for use in procedures of regenerative dentistry, especially regenerative endodontics. **Methods:** HUCMSC was isolated, cultured, and confirmed by flow cytometry. The biological characteristics such as cell morphology, proliferation, and protein expression were screened. To check the cytotoxicity, HUCMSC was cultured and divided into two groups, the control group (cultured in minimum essential medium (MEM) alpha) and calcium hydroxide group (cultured in MEM alpha and calcium hydroxide). Methyl-thiazole-tetrazolium (MTT) assay was done on different concentrations of calcium hydroxide (0.39 to 25 μg/mL) and the cells were observed and counted. Obtained data were analyzed statistically, with P value < 0.05 was considered statistically significant. **Results:** Flow cytometric analysis confirmed positive of CD73, CD90, CD105, negative of CD45 and CD34.The results show that significant difference was found between concentration of 6.25 and 3.125 μg/mL (P=0.004). There was no significant difference among 6.25, 12.5 and 25 μg/mL concentrations. There was also no significant difference among 0.39, 0.78, 1.56, and 3.125 μg/mL concentrations. **Conclusion:** Even though calcium hydroxide is a medicament of choice in clinical endodontics, it decreases the viability of HUCMSC. The lower the concentration of calcium hydroxide, the higher the viability of HUCMSC.

Keywords: Calcium hydroxide, Umbilical cord, Mesenchymal stem cells, Cell viability

Introduction

There are several mesenchymal stem cells (MSC) sources for clinical applications. Human umbilical cord mesenchymal stem cells (HUCMSCs) are MSC found in the umbilical cords and may be an attractive candidate for use in tissue regeneration [1]. Compared to other sources of stem cells from adult tissues, HUCMSCs are more primitive, non-invasive procedure of collection, provide high proliferation potential, high differentiation potential, immune privileged, immunosuppressive, rich in stemness [2]. Currently, HUCMSC is vastly studied to be used for degenerative therapy, e.g. diabetes and its complications.

Calcium hydroxide (CH), an odorless white powder, is classified as a strong alkali or base [3]. It has long been used in various treatment modalities in conservative dentistry, including as intracanal dressings, root canal sealers and pulp-capping material [4]. Calcium hydroxide is an essential alkaline material used in protocols of a successful regenerative endodontic treatment regimen [5] and the material of choice of all pulp therapy [4]. The mineralizing action of calcium hydroxide is determined by its high pH level, and the hydroxyl group is reckoned as the most important part as it gives an alkaline milieu, which promotes healing and mineralization [3].

The use of HUCMSC in dentistry is still limited and therefore need to be explored. Many researches have examined the cytotoxicity of calcium hydroxide, but few studies have used HUCMSC. Cell cultures are generally done to evaluate the biocompatibility of many materials [6,7]. In vitro experiments of cell cultures are usually performed to explore mechanism and biological responses in certain conditions. Even though results from in vitro experiments cannot immediately be deduced to clinical human conditions, they are clinically pertinent as they regard a model for screening of various material properties and risks [8]. HUCMSC was studied to evaluate cytotoxicity of scaffold material for regenerative tissue engineering [9].

Stem cells viability and response may differ depending on the time variation of contact from available calcium hydroxide. To further explore the potency of calcium hydroxide on HUCMSC, viability testing is important to be done. Therefore, the objective of this study was to investigate the cytotoxicity of calcium hydroxide on the viability HUCMSC, through MTT assay.

Materials and Methods

Isolation, expansion and cultivation of HUCMSC

This study was approved by the Health Research Ethical Clearance Commission, Universitas Airlangga Faculty of Dental Medicine, Surabaya, Indonesia (Clearance number 059/HRECC.FODM/II/2020), and written informed consent was obtained before specimen collection. The isolation, expansion and cultivation of HUCMSC is according to the protocol of Stem Cell Research and Development Center Universitas Airlangga [9]. Umbilical cord tissue from donor was cleaned and washed from blood or debris with sterile Dulbecco's Phosphate Buffered Saline (Gibco, Paisley, UK), the Wharton's Jelly was taken and then finely cut. Collagenase Type 4 (Worthington Biochemical Corporation, New Jersey, USA) was added to collect stem cells from the umbilical tissue.

Incubation was done for 45 minutes at 37°C. The cells and medium were centrifuged to form a pellet of cells. Minimum essential medium (MEM) alpha was added to the pellet and planted in 100 mm tissue culture plate (Iwaki, Asahi, Japan) and then incubated at 37°C. The plate was checked on a daily basis until it forms 80-90% monolayer, the cells were split to form

the next passage. Cells from the fourth passage was characterized at Stem Cell Research and Development Center Universitas Airlangga (Surabaya, Jawa Timur, Indonesia).

A total of 2 x 10^6 HUCMSC were taken for flow cytometric analysis. Flow cytometric analysis confirmed positive of CD73, CD90, CD105, negative of CD45 and CD34. All antibodies were purchased from BD Biosciences. HUCMSC were analyzed using a FACS Calibur Becton-Dickinson (BD Biosciences, USA), and the data were analyzed using CellQuest software (BD Biosciences, USA).

Calcium hydroxide preparation for HUCMSC

Calcium hydroxide medium was prepared by mixing the calcium hydroxide powder (EMSURE®Merck, Darmstadt, Germany) with minimal essential medium (MEM) alpha (Gibco, Paisley, UK). We use calcium hydroxide concentration of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 μg/mL to be compared in this experiment.

Viability of HUCMSC on calcium hydroxide

The viability of HUCMSCs on Calcium hydroxide was determined by methyl-thiazol-tetrazolium (MTT) assay [9]. The fifth passage HUCMSCs were seeded at a density of 5,000 cells per well in a 96-well microtiter plate (Iwaki, Asahi, Japan) and treated with calcium hydroxide at concentrations ranging from 0.39-25 μg/mL for 24 hours. Each well was given 200 microliter medium containing calcium hydroxide according to the explored concentrations.

After incubation, MTT was added to each well and incubated for 3 hours, the process was stopped by the addition of dimethyl sulfoxide. Cell viability was analyzed by measuring the optical density (OD) detected by an automatic microplate reader (GloMax®Explorer, Wisconsin, USA) at a wavelength of 595 nm. The assessment was done in triplicates of n=3.

Statistical analysis

Data were provided as mean $\pm$ standard deviation. Statistical analysis were performed using SPSS20.0 package (SPSS Inc., Chicago, Illinois, USA). All data were tested for normality. One-way ANOVA test was used for comparisons of 3 or more groups (among control and different concentrations of calcium hydroxide groups).

Results

Isolation and identification of HUCMSC

The presence of mesenchymal stem cells taken from human umbilical cord was established and identified by means of antibodies for CD105, CD90, CD73, CD45, and CD34. The flow cytometry recorded more than 95% positivity of CD105, CD90, CD73, negativity of CD45 and CD34. This result confirmed the characterized cells are HUCMSC.

Cytotoxicity assessment of calcium hydroxide on HUCMSC

Viable cells seen under inverted microscope are available in figure 1. The mean and standard deviation (SD) of control and calcium hydroxide groups are shown in table 1. Data in the result was distributed normally (P>0.05), and homogeneity test yield a homogeny data (P>0.05). The significance is shown in table 2.

MTT assay result of different calcium hydroxide (CH) concentrations showed no significant difference among 6.25, 12.5 and 25 μg/mL. There was also no significant difference among 0.39, 0.78, 1.56, and 3.125 μg/mL. The significant difference was found between 3.125 and 6.25 μg/mL (*P*= 0.004).

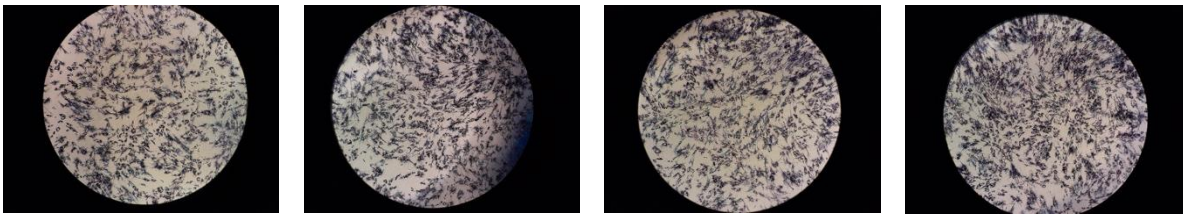
Table 1. Mean and Standard Deviation (SD) of control and CH groups.

Group	Percentage of cell viability mean ± SD	n
Control	100 ± 0.00	3
CH 25	56.1013 ± 3.22481	3
CH 12.5	58.0903 ± 6.76619	3
CH 6.25	58.4880 ± 3.06637	3
CH 3.125	69.0950 ± 2.50740	3
CH 1.56	69.8520 ± 2.27282	3
CH 0.78	70.0930 ± 3.26168	3
CH 0.39	70.2317 ± 3.04301	3

Table 2. Significance (*P* value) among groups of different CH concentrations.

CH Concentration (μg/mL)	25	12.5	6.25	3.125	1.56	0.78	0.39
25	-	0.523	0.445	0.001*	0.000*	0.000*	0.000*
12.5	0.523	-	0.898	0.003*	0.002*	0.001*	0.001*
6.25	0.445	0.898	-	0.004*	0.002*	0.002*	0.002*
3.125	0.001*	0.003*	0.004*	-	0.807	0.747	0.714
1.56	0.000*	0.002*	0.002*	0.807	-	0.938	0.902
0.78	0.000*	0.001*	0.002*	0.747	0.938	-	0.964
0.39	0.000*	0.001*	0.002*	0.714	0.902	0.964	-

*Significant (*P*<0.05)



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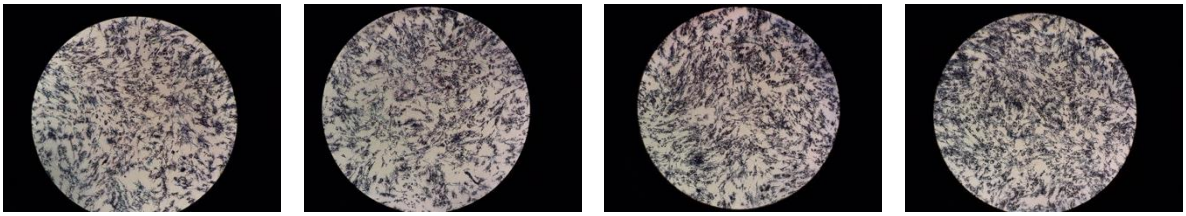

E F G H

Figure 1. The viable cells seen with inverted microscope: control (A), CH concentration of 25 μg/mL (B), 12.5 μg/mL (C), 6.25 μg/mL (D), 3.125 μg/mL (E), 1.56 μg/mL (F), 0.78 μg/mL (G), and 0.39 μg/mL (H).

Discussion

The interest in finding alternative sources of mesenchymal stem cells (MSC) and exploring its potential in dentistry is increasing. There are studies about other stem cell origins, such as human bone marrow mesenchymal stem cells, stem cells of the apical papillae, dental pulp stem cells, periodontal ligament stem cells, etc., but little information was available regarding HUCMSC and its potential use in regeneration.

Although human bone marrow mesenchymal stem cells are more popular, there are disadvantages, such as the requirement of general anesthesia regarding the invasive surgical procedure involved, and multiple procedures may be needed to acquire sufficient number of cells [10]. Human umbilical cord mesenchymal stem cells (HUCMSC) are used in this study because they can be isolated and expanded easily in large quantities in vitro [2].

Biocompatibility is an essential factor that must be contemplated when choosing a material for therapeutic purposes. In this study, HUCMSC were cultured in Minimum Essential Medium Alpha (MEM-A) added calcium hydroxide in normal culture condition, incubated at 37°C and 5% CO_2. We used MTT assay to determine cell viability and cytotoxicity under calcium hydroxide influence. HUCMSC is used instead of other cells, because stem cells are different in its nature and character compared to other matured cells. Although in morphology HUCMSC is similar to fibroblast, HUCMSC has specific protein expressions that fibroblast doesn't have, such as the positivity of CD105, CD90, CD73, negativity of CD45 and CD34.

MTT assay is the most common method to determine cytotoxicity and proven to be more accurate and time saving. The principle of MTT assay is to break tetrazolium ring (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide) to produce insoluble blue-purple formazan product. The formazan production can be measured by optical density spectrophotometry. The lower the optical density means the lower living cells to metabolize the MTT. This study used 24-hour exposure of CH, instead of lower exposure times, in order to extrapolate that clinically CH was applied for long term exposure.

Calcium hydroxide is chemically categorized as a strong alkali with a high pH (about 12.5 – 12.8) and its core effects originated from ionic separation of Ca^{++} and OH^{-} ions [3]. Calcium ion plays an important function in the intrinsic pathway of apoptosis. The upregulation of calcium into mitochondrion results in the release of cytochrome c. In cytoplasm, the cytochrome c binds to apoptotic protease-activating factor-1 (APAF-1), a cytoplasmic protein, and forms the apoptosome which turn and activates procaspase-9 into caspase-9 and subsequently activates other caspases to carry out cell death [11]. Calcium hydroxide may possibly upregulate the expression of caspase-9, but further research is important to determine the effect of time and duration of calcium hydroxide application.

In this study, the significant decrease in cell viability might be caused by apoptosis. Apoptosis is a programmed cell death mechanism that engage in the homeostatic management

of cell population, with no inflammatory reaction occurring [7]. Apoptosis is initiated by either an intrinsic or an extrinsic pathway.

Calcium hydroxide is well tolerated by bone and pulpal tissue. Some studies used a well-established cell line to assess the cytotoxicity of CH, such as fibroblasts from various origins and reported a good biocompatibility [12]. However, CH will create a zone of necrosis if in direct contact with tissue, altering the physicochemical form of cell to cell material which through the break of glycoproteins, causing protein alteration [3]. This is in parallel with the result of this study which found calcium hydroxide effects the viability of HUCMSC.

Conclusion

These results show CH effects the viability of HUCMSC depending on the concentration used. In conclusion, this study provide evidence that CH can decrease the viability of HUCMSC on longer period. The lower the concentration of Calcium hydroxide, the higher the viability of HUCMSC. Since this study is the first step of HUCMSC exploration on CH, further studies will be required to reveal more novel mechanisms of HUCMSC for regenerative endodontic purposes.

Author's Contributions

EPP	0000-0001-9962-235X	Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project administration, Resources, Writing-original draft preparation, Writing-review and editing
IW	0000-0002-5928-7005	Funding Acquisition, Investigation, Resources, Supervision
FC	0000-0001-6363-8093	Investigation, Methodology, Project administration, Resources
MK	0000-0002-3869-754X	Investigation, Methodology, Project administration, Resources, Writing-original draft
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EH	0000-0001-9723-8098	Data Curation, Formal Analysis, Investigation, Methodology, Software, Validation
FAR	0000-0001-8182-1465	Investigation, Methodology, Project administration, Resources, Supervision

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest
The authors declare no conflicts of interest.

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References

1. Meguid EA, Ke Y, Ji J, El-Hashash AHK. Stem cells applications in bone and tooth repair and regeneration: New insights, tools, and hopes. J Cell Physiol. 2017; 9999:1-11. https://doi.org/10.1002/jcp.25940.
2. Li T, Xia M, Gao Y, Chen Y, Xu Y. Human umbilical cord mesenchymal stem cells: an overview of their potential in cell-based therapy. Expert Opin Biol Ther. 2015; 15:1293-1306. https://doi.org/10.1517/14712598.2015.1051528.
3. Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. International Endodontic Journal. 2011; 44: 697-730. https://doi.org/10.1111/j.1365-2591.2011.01886x.
4. Ba-Hattab R, Al-Jamie M, Aldreib H, Alessa L, Alonazi M. Calcium hydroxide in endodontics: an overview. Open journal of stomatology. 2016; 6: 274-289. https://doi.org/10.4236/ojst.2016.612033.
5. Kahler B, Chugal N, Lin LM. Alkaline materials and regenerative endodontics: a review. Materials. 2017; 10: 1389. https://doi.org/10.3390/ma10121389.
6. Peters OA. Research that matters-biocompatibility and cytotoxicity screening. International Endodontic Journal 2013: 46; 195-7. https://doi.org/10.1111/iej.12047.
7. Collado-Gonzales M, Garcia-Bernal D, Onate-Sanchez RE, Ortolani-Seltenerich PS, Alvarez-Muro T, Lozano A, Forner L, Llena C, Moraleda JM, Rodriguez-Lozano FJ. Cytotoxicity and bioactivity of various pulpotomy materials on stem cells from human exfoliated primary teeth. International Endodontic Journal 2017: 50; e19-e30. https://doi.org/10.1111/iej.12751.
8. Pires CW, Botton G, Cadona FC, Machado AK, Azzolin VF, da cruz IBM, et al. Induction of cytotoxicity, oxidative stress and genotoxicity by root filling pastes used in primary teeth. International Endodontic Journal 2016: 49; 737-45. https://doi.org/10.1111/iej.12502.
9. Hendrijantini N, Kresnoadi U, Salim S, Agustono B, Retnowati E, Syahrial I, Mulawardhana P, Wardhana MP, Pramono C, Rantam FA. Study biocompatibility and osteogenic differentiation potential of human umbilical cord mesenchymal stem cells (hUCMSCs) with gelatin solvent. Journal of Biomedical Science and Engineering 2015: 8; 420-8. https://doi.org/10.4236/jbise.2015.87039.
10. Hendrijantini N. Comparative in vitro study of the cytotoxicity of gelatin and alginate to human umbilical cord mesenchymal stem cells. Dental Journal (Majalah Kedokteran Gigi) 2019; 52(1): 36-40. https://doi.org/10.20473/j/djmkg.v52.i1.

11. Kontogiannis TG, Tosios KI, Kerezoudis NP. Effect of calcium hydroxide as intracanal medicament on the expression of caspase-9 located within the radicular cyst epithelium. Australian Endodontic Journal. 2019; 1-5. https://doi.org/10.1111/aej.12325.
12. Modena KCS, Casas-Apayco LC, Atta MT, Costa CAS, Hebling J, Sipert CR, Navarro MFL, Santos CF. Cytotoxicity and biocompatibility of direct and indirect pulp capping materials. Journal of Applied Oral Science 2009; 17(6):544-54. https://doi.org/10.1590/S1678-77572009000600002.

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