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Research Article

Calcium Hydroxide Upregulates Interleukin-10 Expression in Time Dependent Exposure and Induces Osteogenic Differentiation of Human Umbilical Cord Mesenchymal Stem Cells

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ABSTRACT

Background: Human umbilical cord mesenchymal stem cells (HUCMSCs) as one of mesenchymal stem cells source, have a promising potential for regenerative dentistry. Calcium hydroxide, a widely administered medicament for endodontic therapy, is used for its numerous benefits. The presence of calcium hydroxide is expected to bring a positive outcome on HUCMSCs.

Purpose: This study aims to evaluate the anti-inflammatory effect through Interleukin-10 expression and osteogenic differentiation of calcium hydroxide on HUCMSCs.

Methods: This study is an experimental laboratory research with controlled group design. HUCMSCs were grown in Minimum Essential Medium (MEM) Alpha containing 0.1 µg/ml of calcium hydroxide. Observation of interleukin-10 expression were conducted in 24 hours, 72 hours, and 168 hours. Osteogenic differentiation was observed with Alizarin Red S staining. Obtained data were analyzed with analysis of variance (ANOVA). The level of significance was set at 0.05.

Results: Calcium hydroxide increases Interleukin-10 expression of HUCMSCs (P 0.000). Exposure time of 24 hours, 72 hours, and 168 hours exhibit increased expression. Exposure of 168 hours displayed the highest increase, followed by 72 hours and 24 hours as the lowest. Calcium hydroxide also induce osteogenic differentiation of HUCMSCs.

Conclusions:

Calcium hydroxide increases the expression of Interleukin-10 in time dependent exposure and induces osteogenic differentiation of HUCMSCs. The longer the exposure of calcium hydroxide to HUCMSCs, the higher the expression of interleukin-10. These findings support the use of calcium hydroxide on HUCMSCs for pulpal and periapical tissue regeneration.

Keywords: calcium hydroxide, differentiation, immunoregulation, mesenchymal stem cells, umbilical cord

INTRODUCTION

Calcium hydroxide, a white and odorless powder, is chemically classified as a strong base [1]. The mineralizing action of calcium hydroxide is influenced by its high pH, and the hydroxyl group is considered to be the most important component as it provides an alkaline environment which support repair and active calcification [2]. Calcium hydroxide

is the material of choice for all pulpal and periapical therapy [3].

Mesenchymal stem cells from umbilical cords might be an option for use in pulpal, periapical and bone tissue regeneration [4]. The use of human umbilical cord mesenchymal stem cells (HUCMSCs) in dentistry is still limited and not as popular as dental pulp stem cells, stem cells from the apical papilla, gingival stem cells, and stem cell from exfoliated deciduous teeth. Stem cells from external source are needed especially when the surrounding pulpal, periapical, and bone tissues failed to regenerate. Failure of regeneration is usually related to systemic degenerative conditions, such as diabetes and its complications [5,6].

Previous report on HUCMSCs showed a promising proliferation and differentiation potential, therefore they are appropriate for regenerative therapy. Stem cell proliferation and differentiation are crucial for the regenerative process. Immune modulatory is an important healing factor in regeneration. Interleukin-10 is a pleomorphic cytokine with various phenotypic effects, with core actions of potent anti-inflammatory, regulatory, and homeostasis through paracrine and autocrine mechanisms at both local and systemic level [7]. HUCMSCs reaction may differ with regard to time of contact from calcium hydroxide [5]. Osteogenic differentiation of HUCMSCs is an important point for application on hard tissues, such as alveolar bone, immature root formation and periapical tissues. Accordingly, the purpose of this study was to evaluate the effect of calcium hydroxide on immune modulation through interleukin-10 expression in timely dependent exposure of 24 hours, 72 hours, and 168 hours period and the osteogenic differentiation.

MATERIALS AND METHODS

HUCMSCs Culture and Calcium Hydroxide Preparation

This research was given ethical clearance by Health Research Ethical Clearance Commission, Universitas Airlangga Faculty of Dental Medicine, Surabaya, Indonesia (Approval document 059/HRECC.FODM/II/2020), and written informed consents was collected beforehand the procedure. This research was conducted in accordance with ethical standards of experiments.

Isolation and culture of HUCMSCs was done following procedures in previous study [5]. Wharton's jelly from the umbilical cord tissue from donor was collected from full-term births by cesarean section. The tissue was obtained and cut into small pieces, and given collagenase type 4 (Worthington Biochemical Corporation, New Jersey, USA) to obtain stem cells from the umbilical tissue. Cells were incubated for 45 minutes at 37ºC, and then centrifuged to form a cell pellet. Minimum essential medium (MEM) alpha was added and the pellet was plated on 100 mm culture plate (Iwaki, Japan) and incubated at 37ºC and checked until they form a monolayer to be split for the next passage.

Cells from the third passage was characterized at Stem Cell Research and Development Center Universitas Airlangga (Surabaya, Indonesia). The cells were taken for flow cytometric analysis. All antibodies were obtained from BD Biosciences, USA. The cells were confirmed as HUCMSCs. Results of the flow cytometry were positive for CD73, CD90, CD105, and negative for CD45 and CD34. HUCMSCs from the fourth passage was used in this experiment.

Calcium hydroxide was prepared by mixing the powder (EMSURE Merck, Germany) with MEM alpha medium (Gibco, UK). This study uses calcium hydroxide concentration of 0.1 μg/ml and HUCMSCs was divided into 3 groups, each group consists of

Assessment of HUCMSCs Immune Modulation

We used 24-well tissue dishes (Iwaki Asahi, Japan) and they were divided into six groups, consisted of three control groups and three calcium hydroxide groups based on different exposure time of 24, 72, and 168 hours. Every well was seeded with 250.000 HUCMSCs in 1 ml media. The control groups used only MEM alpha medium, while the treatment groups used MEM alpha medium containing 0.1 μg/ml calcium hydroxide. Both groups were incubated at 37ºC and 5% CO2 for 24, 72, and 168 hours.

Immune modulation capacity of HUCMSC was observed through interleukin-10 (Bioss Antibodies, USA) expression on 24, 72, and 168 hours for both control and treatment groups. Interleukin-10 expression assessment was done according to the manufacturer's protocol. The expressions were observed using polyclonal antibody with fluorescence isothiocyanate (FITC) label (Bioss Antibodies, USA). The results were observed using fluorescence microscope (Nikon, Japan) with imaging system at 100x magnification. The images were processed in ImageJ software for fluorescence quantification (National Institute of Health, USA) [8].

Assessment of HUCMSCs Osteogenic Differentiation

Osteogenic differentiation of HUCMSCs was observed on HUCMSCs cultures through mineralization formation on the cultures. This assessment was performed on control group without calcium hydroxide and treatment group with calcium hydroxide using 40 mmol/l Alizarin Red S (Sigma-Aldrich, USA) staining according to the manufacturer's protocol.

HUCMSCs were planted and incubated at room temperature in a 24-well tissue dishes for 21 days, with medium changed every 2 days. On day 21, the mineralization was checked for positivity or negativity. Results were taken on a light microscope (Nikon, Japan) with 100x magnification. The results were photographed and observed for any red calcifications.

Statistical Analysis

The assessment was conducted in triplicates. We presented the data as mean ± standard deviation. Statistical analysis was performed using SPSS 20 software package for Windows (SPSS Inc, USA). Level of significance was set at 0.05. Normal distribution of all data was checked with Kolmogorov-Smirnov test. Differences between two groups was analyzed with t-test. Comparisons of three groups among exposure times of 24, 72, and 168 hours were analyzed with one-way analysis of variance (ANOVA).

RESULTS

Calcium Hydroxide induce Interleukin-10 expression of HUCMSCs

Calcium hydroxide induced HUCMSCs' interleukin-10 expression aggressively. This result corresponds with the observed time of exposure (Figure 1). In the unstimulated cultures of control groups, the expression of interleukin-10 was low. At the beginning (24 hours), interleukin-10 expression was low on both control and calcium hydroxide groups. Over time, the control group showed a gradual increase of interleukin-10 expression, but not as high as the calcium hydroxide group. (P 0.000)

Significant increase (P 0.000) was found on 72 hours and 168 hours on calcium hydroxide groups. Interleukin-10 expression was the highest on 168 hours of calcium hydroxide exposure. These findings showed that calcium hydroxide increases anti-inflammatory potency of HUCMSCs.

Calcium Hydroxide induce Osteogenic Differentiation of HUCMSCs

Osteogenic differentiation ability of HUCMSCs was observed for the indicated time of 21 days with Alizarin Red S assessment. HUCMSCs did not show any mineralization in the control plate where only MEM alpha medium was available, without any presence of calcium hydroxide. The treatment group of calcium hydroxide showed increased HUCMSCs mineralization. Calcium hydroxide concentration of 0.1 μg/ml increased mineralization after 21 days of culture (Figure 2).

DISCUSSION

There is growing interest in finding alternative sources of mesenchymal stem cells and exploring its potential. As stem cells in human body is strictly limited, methods to increase their proliferations are needed to be developed [9]. HUCMSCs is interesting because they can be isolated and expanded in large quantities in vitro [10]. There are studies about other stem cell sources, such as stem cells of the apical papilla, dental pulp stem cells, periodontal ligament stem cells, but limited information is available regarding HUCMSCs and its potential use in pulpal and periapical regeneration.

In this study, HUCMSCs from Wharton's jelly were cultured under normal condition. In this study, HUCMSCs are in line with the standardization of MSCs morphology and cluster of differentiation surface markers [11]. We used calcium hydroxide concentration of 0.1 μg/ml based on MTT assay conducted previously on HUCMSCs which resulted above 60% cells viability. In this study, we found that 0.1 μg/ml promotes interleukin-10 expression of HUCMSCs. This increase can modulate the immune system, with direct action, as one of its clinical benefit. Therefore, calcium hydroxide can enhance HUCMSCs to act as direct regulator of the immune response.

Interleukin-10 is a representative of strong anti-inflammatory cytokine through immune regulation and the most prominent among anti-inflammatory cytokines derived from MSCs [12,13]. It is also the most important cytokine with anti-inflammatory properties besides TGF-beta and Interleukin-35 [14]. Interleukin-10 comprises of a noncovalent homodimer of polypeptide chains consists of six alpha-helix and connecting loops [15]. Interleukin-10 plays a significant role in lowering inflammatory response, and reduced the expression of pro-inflammatory cytokines [12].

Previous studies have stated that interleukin-10 have positive effects on inflammation and novel delivery

tools for interleukin-10, including cell-based therapy to directly or indirectly modulate interleukin-10 signaling has been developed, how it signals and interacts with effector cells [7]. Delivering interleukin-10, a strong immune modulation through cell therapy is promising because the cytokines have a short half-life for use in the clinics [16]. Recent study provide that interleukin-10 delivered by MSC have a neuroprotective effect in traumatic brain injury [17]. Interleukin-10 is also essential to promote osteoblast maturation and reduce bone loss in diabetic osteoporosis [18].

Calcium hydroxide has a special potential to stimulate tissue mineralization, but how the process is still not clear [19]. Alizarin Red S staining was used to assess the calcified nodule formation and the amount of calcium to detect HUCMSCs mineralization in vitro. In this study we found that 0.1 μg/ml calcium hydroxide can increase the formation of mineralization nodules in HUCMSCs. The number of calcified nodules and the amount of calcium were significantly higher and visible in medium containing calcium hydroxide.

Calcium hydroxide supplies calcium ions which are a potent regulator for cell functions and have an important role in mineralization by supporting fibronectin gene expression [20]. A pH of 8.6 to 10.3 is needed for biological action of calcium hydroxide to activate alkaline phosphatase enzyme which allows phosphate to react with calcium ions then forming calcium phosphate which is related to the mineralization process [21]. Mineralization is not only affected by the exogenous phosphates, but also cell density [22]. Higher HUCMSCs density would lead to more mineralization. This is in line with previous study that HUCMSCs from Wharton's jelly provides more osteogenic differentiation compared to other compartments of the umbilical cord [23].

These results showed HUCMSCs act as a good candidate for use in regenerative dental procedures. Interleukin-10 is among newly studied cytokines and few researches have been conducted in dentistry [24]. The development of HUCMSCs as cell-based or tissue engineering therapy that target interleukin-10 may have great implications for patients. However, a deeper insight of interlukin-10 signaling and interaction will be needed for further studies and it is a necessity to obtain more and farther results with regard to HUCMSCs and calcium hydroxide, for the purpose of regenerative dentistry.

CONCLUSION

In summary, this study provide evidence that calcium hydroxide can be used to enhance HUCMSCs to increase their anti-inflammatory and osteogenic differentiation capacity, as they could produce mineralized nodules. These findings can support HUCMSCs use for regenerative dentistry, but further studies will be required to reveal more novel mechanisms of HUCMSCs for regeneration.

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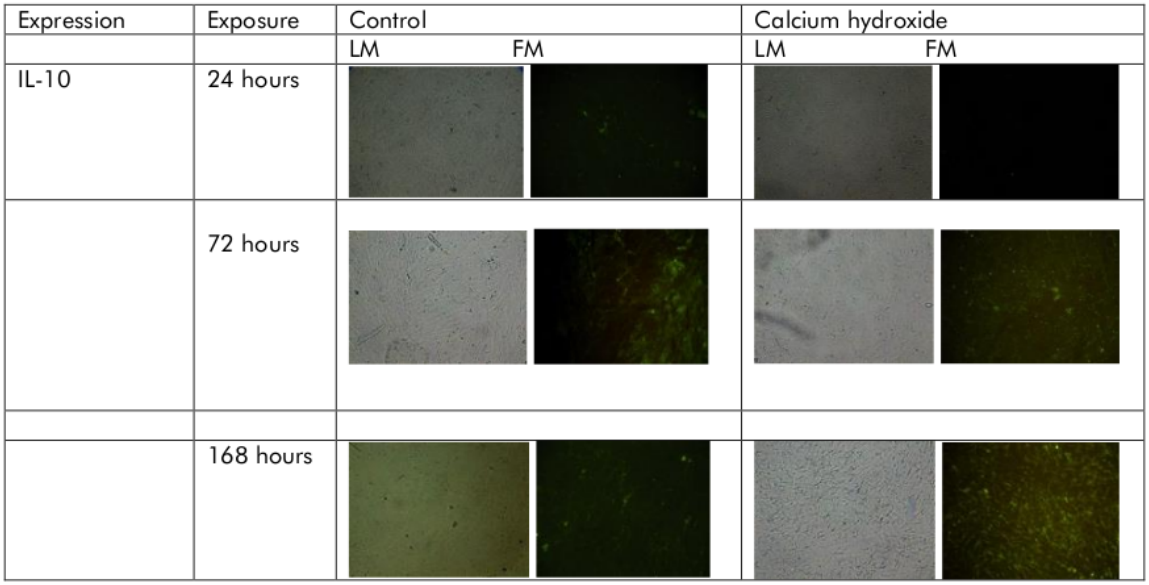
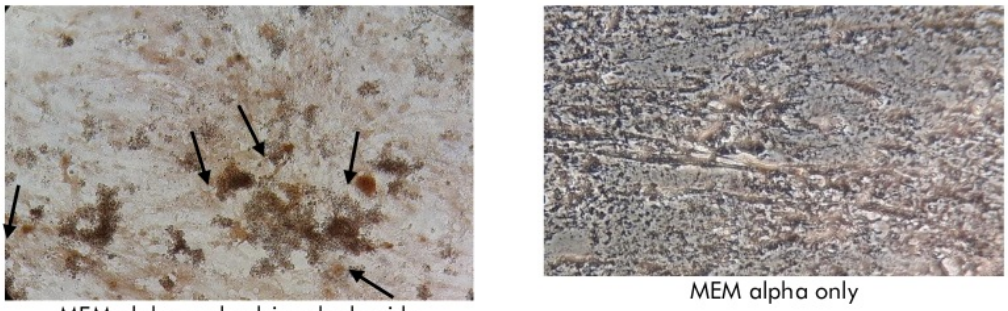


Fig.1: HUCMSCs culture and fluorescence expressions of interleukin-10 (IL-10) observed on 24 hours, 72 hours, and 168 hours between control groups and calcium hydroxide groups. The cultures were seen under light microscope (LM) and fluorescence microscope (FM).



MEM alpha and calcium hydroxide

MEM alpha only

Fig.2: Mineralization assessments on day 21. Mineralization is found on HUCMSCs cultured in calcium hydroxide added MEM alpha (treatment group). Calcification nodules are shown by the arrows. No mineralization is found on HUCMSCs cultured in MEM alpha only (control group).

Table 1: Mean and Standard Deviation (SD) of interleukin-10 expression from HUCMSCs in control groups and calcium hydroxide groups.

Groups & Time of Exposures	Interleukin-10 Expression Mean ± SD
Control 24 hours	3.5200 ± 0.4330
Control 72 hours	11.8600 ± 0.4563
Control 168 hours	13.9800 ± 0.3669
Calcium hydroxide 24 hours	1.5473 ± 0.3383
Calcium hydroxide 72 hours	22.2513 ± 2.6025
Calcium hydroxide 168 hours	27.5833 ± 3.4579

Table 2: Significance (P value) among time of exposures and interleukin-10 expressions within control groups and calcium hydroxide groups.

Groups & Time of Exposures	Control 24 hours	Control 72 hours	Control 168 hours	Calcium hydroxide 24 hours	Calcium hydroxide 72 hours	Calcium hydroxide 168 hours
Control 24 hours	-	0.000*	0.000*	0.000*	-	-
Control 72 hours	0.000*	-	0.000*	-	0.000*	-
Control 168 hours	0.000*	0.000*	-	-	-	0.000*
Calcium hydroxide 24 hours	0.000*	-	-	-	0.000*	0.000*
Calcium hydroxide 72 hours	-	0.000*	-	0.000*	-	0.000*
Calcium hydroxide 168 hours	-	-	0.000*	0.000*	0.000*	-

*Significant (P<0.05)

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