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ORIGINAL ARTICLE

# Effects of chlorhexidine on stem cells from exfoliated deciduous teeth

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**KEYWORDS**
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stem cells from human exfoliated deciduous teeth (SHED)

*Background/Purpose:* Chlorhexidine (CHX) is a type of chemical antiseptic that is widely used in dental practice. Stem cells from human exfoliated deciduous teeth (SHED) are multipotent cells. However, there is little knowledge about the effects of chlorhexidine on SHED cells. The purpose of this study is to investigate the effects of CHX on SHED.
*Methods:* SHED cells were treated with 0.1%, 0.01%, 0.001%, and 0.0001% CHX for 10 seconds to test the effects of different concentrations of CHX on SHED cells. The cells were also treated with 0.01% CHX for 10 seconds, 1 minute, and 5 minutes to test the time effects of CHX on SHED cells. Cell proliferation was investigated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and an autonomously replicating sequence (ARS) assay was used for the evaluation of the mineralization potential.
*Results:* This study demonstrated that different concentrations of CHX had cytotoxic effects on SHED cells in a dose- and time-dependent manner. The proliferation of SHED cells was inhibited by approximately 50% by the use of 0.01% CHX. It was also found that the cell proliferation and mineralization potential of SHED cells were inhibited to some degree by different concentrations of CHX.
*Conclusion:* Different concentrations of CHX can inhibit SHED cell proliferation in a dose- and time-dependent manner. In addition, the mineralization potential of SHED cells is inhibited to some degree by different concentrations of CHX.


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## Introduction

Chlorhexidine (CHX) is a chemical antiseptic. It is a synthetic cationic bis-biguanide that consists of two symmetric 4-cholorophenyl rings and two biguanide groups connected by a central hexamethylene chain.[1] CHX is a biguanide antiseptic active against Gram-positive and Gram-negative bacteria, facultative anaerobes and aerobes, and fungi.[2] It is a positively charged hydrophobic and lipophilic molecule, and interacts with phospholipids and lipopolysaccharides on the cell membrane of bacteria.[3] The effect of CHX is due to the interaction between positively charged molecules and negatively charged phosphate groups on the microbial cell walls.[4] CHX has been found to be an effective antimicrobial agent and is widely used for topical preoperative skin disinfection, skin wound treatment, general skin cleansing, and as a surgical hand scrub. The most common oral preparation, CHX gluconate is water soluble and at physiological pH, it readily dissociates and releases the positively charged CHX component.[1] In dental practice, CHX is commonly used as a root canal irrigant for pulp therapy of primary teeth and permanent teeth. CHX is also found to have a broad-spectrum matrix metalloproteinase (MMP)-inhibitory effect, and it can significantly improve the resin—dentine bond stability.[5] While performing pulp revascularization therapy, the use of 2% CHX as an irrigant is recommended.[6]

Stem cells from exfoliated deciduous teeth (SHED) are a type of dental mesenchymal stem cell. SHED were initially isolated from normal exfoliated human deciduous incisors pulp tissue by Miura et al.[7] in 2003. SHED were found to express the cell surface molecules STRO-1 and CD146, two early mesenchymal stem-cell markers.[7] After *in-vitro* induction, SHED showed osteogenesis, adipogenesis, myogenesis, and chondrogenesis.[7,8] SHED appear to synthesize and secrete dentin matrix similar to the odontoblast cells they replace.[9]

Previous studies indicated that CHX was cytotoxic to rat fibroblast cell lines,[10,11] human dermal fibroblasts,[12] human gingival fibroblasts,[13,14] human periodontal ligament cells,[15] human alveolar bone cells,[16] and human osteoblast cell lines.[10] However, there is little known about the effects of CHX on SHED. The purpose of this study is to investigate the effects of CHX on stem cells.

## Materials and methods

### Isolation and culture of SHED

In this study, all teeth collection and experiments were under IRB regulation permission from the College of Medicine, National Taiwan University and signed by patient consent. Normal exfoliated human deciduous incisors were collected from 6—8-year-old children. Pulp tissues were separated from the tooth and were then minced into small pieces (about $1 \times 1 \times 1$ mm$^3$), and placed into 6-cm culture dishes. They were cultured by an explant technique in Minimum Essential Medium alpha (MEM alpha) containing 10% fetal bovine serum (FBS), 1% penicillin, and 1% glutamate at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. When the growth of explant pulp cells reached approximate confluence, they were subcultured at a ratio of 1:3. SHED cultured for five to eight passages were used for these studies. Generally, the cultured SHED are spindle-shaped in appearance with extended cellular processes.

### Identification of SHED

The characterization of SHED was performed with flow cytometric analysis of surface markers STRO-1 and CD146 expression in the third to seventh passage. Cells were incubated with primary antibody mouse antihuman antibodies STRO-1 (1:10; Invitrogen) for 30 minutes, and then incubated for 30 minutes with a secondary antibody goat antimouse immunoglobulin M—FITC antibody (1:50) and R-phycoerythrin conjugated monoclonal antihuman antibodies CD146 (1:50; Biolegend). Cells treated without primary antibody were used as control. Cells in both the experimental and control groups were then washed twice with 2%FBS in PBS before flow cytometry analysis.

### Chlorhexidine preparation

Chlorhexidine digluconate solution (CHX, Sigma Chemical Co, USA; C9394) was directly diluted using distilled water. The final concentrations of CHX were 0.1%, 0.01%, 0.001%, and 0.0001%, respectively.

### Research design

There were $2.5 \times 10^4$ cells/well in 24-well plates, and these were used for further experiment.

Experiment I: In the experimental groups, SHED cells were treated with 0.1% CHX (Group 1, $n = 6$), 0.01% CHX (Group 2, $n = 6$), 0.001% CHX (Group 3, $n = 6$), or 0.0001% CHX (Group 4, $n = 6$) for 10 seconds. SHED cells without CHX treatment were used as the control group ($n = 6$).

Experiment II: In the experimental groups, SHED cells were treated with 0.01% CHX for 10 seconds (Group 1, $n = 6$), for 1 minute (Group 2, $n = 6$) or for 5 minutes (Group 3, $n = 6$). SHED cells without CHX treatment were used as the control group ($n = 6$).

Cell proliferation was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and an autonomously replicating sequence (ARS) assay was used for the evaluation of mineralization.

### Cell proliferation assay

After treatment with CHX (Experiments I and II), SHED were cultivated in DMEM supplemented with 10% FBS and 1% penicillin for an additional 3 and 7 days. The MTT assay was measured at Day 0, Day 3, and Day 7 after the treatment. In this assay, MTT performed a formazan salt by the mitochondrial dehydrogenase of viable cells. Briefly, $2.5 \times 10^4$ cells/well in 24-well plates were used to perform the MTT assay. The optical density (OD) of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK,) at 540 nm (OD 540). Results were expressed as a percentage of the control (as 100%).

### ARS assay

After the SHED were treated with CHX (Experiments I and II), the cells with a density of $2.5 \times 10^4$ cells/well in 24-well plates were then cultivated in a mineralization medium for an additional 21 days. ARS assays were performed on the 21st day. In these assays, cells were washed twice with PBS, and 200-μL 2% Alizarin Red S (Sigma Chemical Company, St. Louis, MO, USA) (pH = 4.2) solution was then added into each well for 30 minutes. After washing the cells twice with PBS, 200-μL 1% cetylpyridinium chloride was added. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK,) at 562 nm (OD 562). Results were expressed as a percentage of the control (as 100%).

## Results

SHED expressed surface markers STRO-1 (13.25 ± 1.7%) ($n$ = 3) and CD146 (16.02 ± 3.31%), which were presented positively as analyzed by flow cytometry (Fig. 1).

Experiment I: After treatment with different concentrations of CHX for 10 seconds, the cell proliferation of SHED was found to be affected, and about 50% of cell proliferation was inhibited by CHX with 0.01% concentration. In the group 1 of CHX with 0.1% in concentration, about 90% cell proliferation of SHED was inhibited (Fig. 2).

In the experimental group, after the SHED were treated with 0.1% CHX for 3 days, more than 90% cell proliferation of the SHED was inhibited. It was also found that more than 30% cell proliferation of SHED was inhibited after the treatment with 0.01% CHX for 3 days (Fig. 3).

In the experimental group, after SHED were treated with 0.1% CHX for 7 days, more than 90% cell proliferation of SHED was inhibited. It was also found that more than 10% cell proliferation of SHED was inhibited after treatment with 0.01% CHX for 7 days (Fig. 4).

After treatment with CHX for 21 days, the mineralization potential of cells in the experimental groups was found to decrease. In the group treated with 0.1% CHX, the mineralization potential was about 5% of that in the control group with a significant difference, and in the group treated with 0.01% CHX, the mineralization potential was found to be about 80% of that in the control group with a significant difference (Fig. 5).

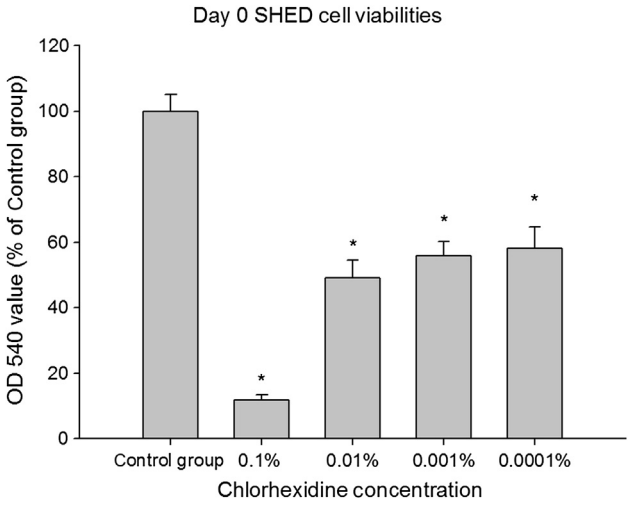


**Figure 2** Day 0 of MTT assay. The effect of various concentrations of CHX on cell proliferation of SHED is shown. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

Experiment II: After treatment with 0.01% CHX for different periods, we performed the MTT assay immediately. The cell proliferation of SHED in the group treated with CHX for 5 minutes was found to decrease to about 60% of that in the control group with a significant difference. In the group treated with CHX for 1 minute, the cell proliferation was found to decrease to about 55% of that in the control group with a significant difference. In the group treated with CHX for 10 seconds, the cell proliferation was found to decrease to about 50% of that in the control group with a significant difference (Fig. 6).

Three days after being treated with 0.01% CHX for different time periods, the cell proliferation of SHED treated with CHX for 5 minute was found to decrease to about 40% of that in the control group with a significant difference. In the group treated with CHX for 1 minute, the cell

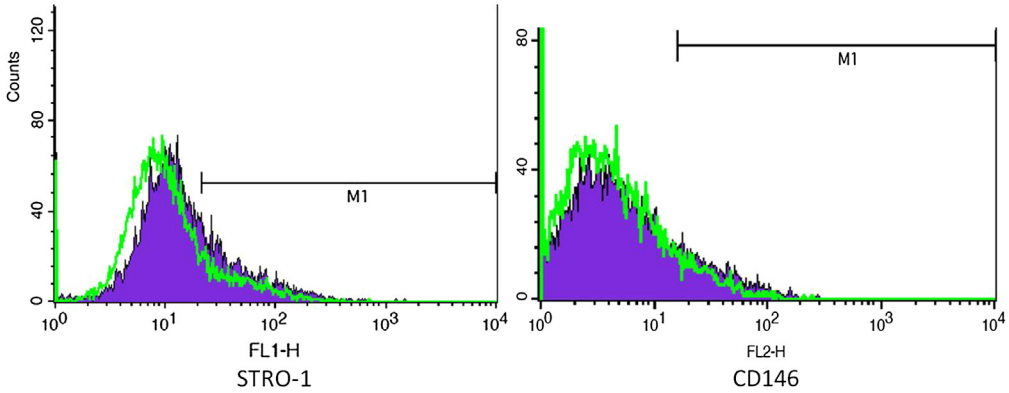


**Figure 1** Surface marker of STRO-1 and CD146 expression of SHED were analyzed by flow cytometric. For all graphs, isotype control staining was indicated by the purple histogram and specific markers staining were indicated by the green curve. M1: region chosen for initial sorting of fluorescent cells and the percentages (STRO-1: 13.25 ± 17%, and CD146 16.02 ± 3.31%) indicated the part of the total cell populations in the M1 regions.

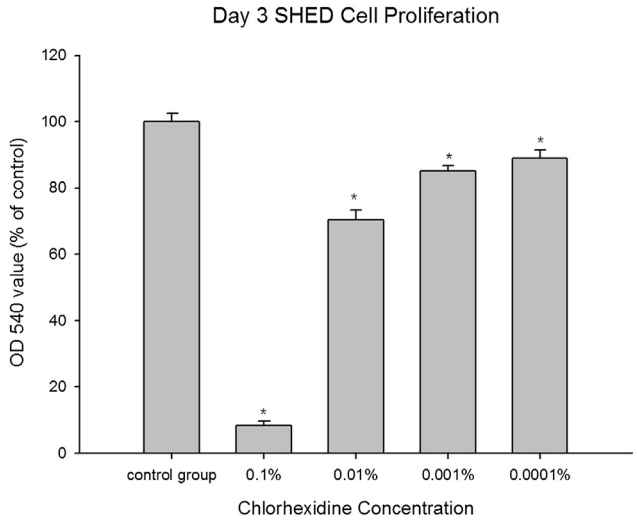


**Figure 3** Day 3 of MTT assay. The effect of various concentrations of CHX on cell proliferation of SHED is shown. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

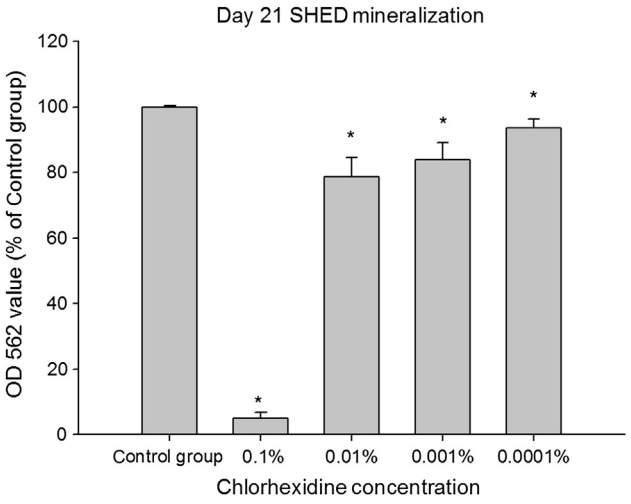


**Figure 5** Day 21 of ARS assay. The effect of various concentrations of CHX on cell mineralization of SHED is shown. Results are expressed as a percentage of OD 562 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

proliferation was found to decrease to about 35% of that in the control group with a significant difference (Fig. 7).

Seven days after the treatment with 0.01% CHX for different time periods, the cell proliferation of SHED treated with CHX for 5 minutes was found to decrease to about 30% of that in the control group with a significant difference. In the group treated with CHX for 1 minute, the cell proliferation was found to decrease to about 25% of that in the control group with a significant difference (Fig. 8).

After 21 days, our results demonstrated that the mineralization potential of SHED in the experimental groups decreased and in the group treated with 0.01% CHX for 5 minutes, the mineralization potential was about 65% of that in the control group with a significant difference. It was also found that in the group treated with CHX for 1 minute, the mineralization potential of cells was about 70% of that in the control group with a significant difference (Fig. 9).

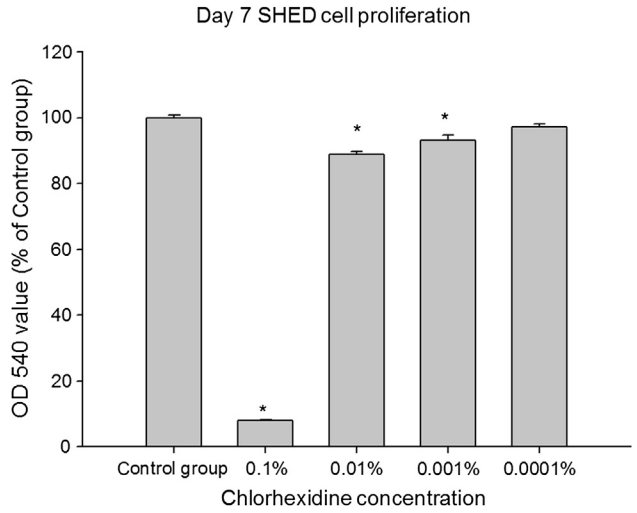


**Figure 4** Day 7 of MTT assay. The effect of various concentrations of CHX on cell proliferation of SHED. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

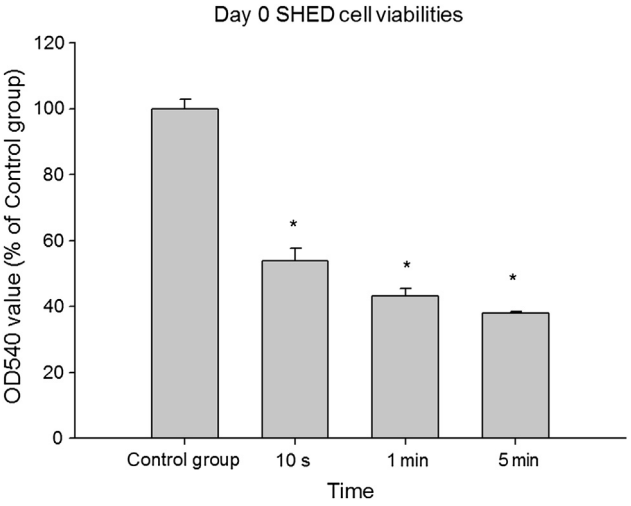


**Figure 6** Day 0 of MTT assay. The effect of various time periods taken to treat CHX on cell proliferation of SHED is shown. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

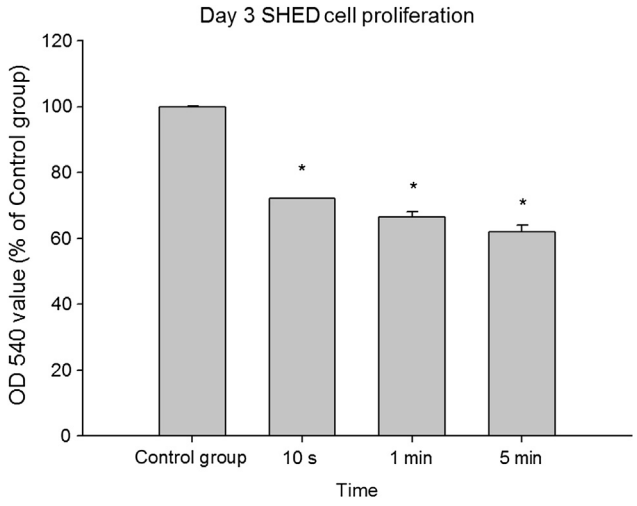


**Figure 7** Day 3 of MTT assay. The effect of various time periods taken to treat CHX on cell proliferation of SHED is shown. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

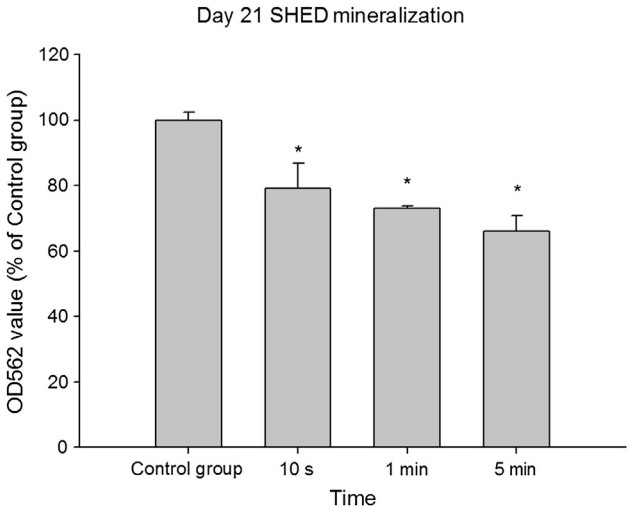


**Figure 9** Day 21 of ARS assay. The effect of various time periods taken to treat CHX on cell mineralization of SHED is shown. Results are expressed as a percentage of OD 562 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

## Discussion

SHED are a type of mesenchymal stem cell. A previous study had shown that SHED expressed the cell surface molecules STRO-1 and CD146, two early mesenchymal stem-cell markers that were found to be present in bone marrow mesenchymal stem cells (BMMSCs) and dental pulp stem cells (DPSCs).[7] In this study, we also found that SHED expressed STRO-1 and CD146, which were similar to the previous study.

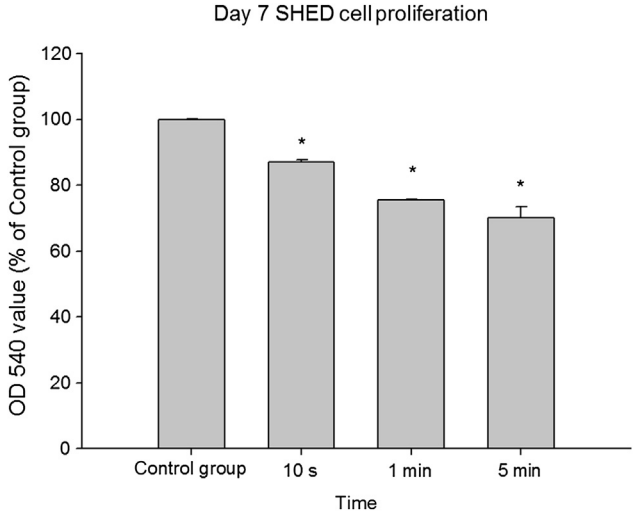


**Figure 8** Day 7 of MTT assay. The effect of various time periods taken to treat CHX on cell proliferation of SHED is shown. Results are expressed as a percentage of OD 540 relative to untreated control. Data are shown as mean ± SD. * Significant differences from control values: $p < 0.05$. CHX = chlorhexidine; OD = optical density; SD = standard deviation; SHED = stem cells from human exfoliated deciduous teeth.

CHX is a cationic bis-biguanide with excellent antimicrobial action. According to the previous studies, CHX was applied as an irrigant for the treatment of infected root canals; there is no significant difference between the antibacterial effects of 2.5% NaOCl and 0.12% CHX.[16,17] Therefore, using 0.12% CHX as an irrigant for primary dental pulp therapy is feasible.

In Experiment I, the effects of CHX application with different concentration for 10 seconds were investigated. We performed the MTT assay for checking cell viability immediately after the treatment with CHX. We found that the concentration of CHX as used in dental clinics (about 0.1%) showed high cytotoxicity of SHED, and about 90% of cell viability was inhibited. It was found that after treatment with 0.01% CHX, about 50% cell viability was inhibited, and this result was the same as that obtained in previous report on the cytotoxicity of CHX on human osteoblastic cells.[2] Cell proliferation in the group treated with 0.1% CHX was found to be 8% of that in the control group, after culturing for seven days. Our results indicated that CHX with a concentration higher than 0.001% could inhibit cell proliferation significantly ($p < 0.05$). This result was similar to that obtained in a previous study.[2] We also found that the mineralization potential of SHED was affected after the treatment with CHX. The effect of inhibition of proliferation or mineralization potential of SHED was dose dependent ($p < 0.05$).

In Experiment II, we treated SHED with the same concentration of CHX for different periods. We found that the inhibition of proliferation or mineralization potential of SHED was found to be dose dependent ($p < 0.05$). A previous study had also demonstrated that the toxicity of CHX to human gingival cells is dependent on the time period of exposure,[18] which was similar to our result.

Our study showed that CHX inhibited the mineralization potential of SHED. Previous studies have shown that CHX

inhibited protein synthesis and collagen synthesis, which may also impair collagen protein synthesis and mineralization potential.[2,13,15]

Previous studies have shown that CHX is a cytotoxic agent irrelevant to the cell type.[2,11–19] It was found that CHX rapidly disrupts the cell membrane of both crevicular and peripheral blood neutrophils at concentrations above 0.005% within 5 minutes, indicating that its inhibitory effect on neutrophil function is mostly because of its lytic properties.[19] Glutathione (GSH) depletion might be one of the mechanisms underlying the cytotoxicity of CHX on human osteoblastic cells.[2] A previous study also showed that irrigation protocols that contained 2% CHX appeared detrimental to stem cells from the apical papilla in this model, yielding no viable cells.[20] Further study is needed for identifying the level of cytotoxicity of CHX on SHED. CHX can inhibit the proliferation and mineralization potential of SHED cells in a dose- and time-dependent manner.

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