Original Research Article

Evaluation of the cytotoxic effects of sodium hypochlorite on human dental stem cells

Shiqi Liu, Hanchao Zhai, Shaoping Fu, Chengyu Cui, Jian Xu, Jinjuan Jiang, Pengyu Pan, Baorong Zhang*
Department of Oral Therapy, The Oral Therapy Center Aviation General Hospital, Beijing, 100012, China

*For correspondence: **Email:** Shiqi65828@163.com; **Tel:** +86-010-59520180*

Abstract

Purpose: *To investigate the influence of sodium hypochlorite (NaOCl) on human dental stem cell proliferation and differentiation.*
Method: *Dental pulp stem cells (DPSCs), periodontal ligament stem cell (PDLSCs), and gingival mesenchymal stem cells (GMSCs) were treated with NaOCl. Cell viability was evaluated with cellular counting kit-8 (CCK8), and cellular adenosine triphosphate (ATP) levels were analyzed by bromodeoxyuridine (BrdU) incorporation and subsequent flow cytometry. Quantitative polymerase chain reaction (qPCR) and western blotting were performed to detect the expressions of differentiation markers.*
Results: *The viability and ATP levels of all three stem cells types were impaired by NaOCl in a concentration- and time-dependent manners. However, the decrease ATP in GMSCs was less than the other two stem cell population (p < 0.05). NaOCl treatment significantly suppressed the proliferation of dental stem cells (p < 0.05). With regard to differentiation marker expression levels, the decrease in Stro-1 was greater in treatment groups when compared to control on Day 7, while increase in levels of dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP), and osteocalcin (OC) was smaller (p < 0.05). The expressional changes of Stro-1, DSPP, BSP, and OC were more prominent in DPSMs and PDLSCs than in GMSCs.*
Conclusion: *NaOCl dose-dependently impairs the viability, proliferation and differentiation of dental stem cells. Thus, its toxicity to dental stem cells needs to be considered in clinical application.*

Keywords: *Dental stem cells, Sodium hypochlorite, Viability, Proliferation, Differentiation*

INTRODUCTION

Sodium hypochlorite (NaOCl), usually used at concentrations from 0.5 to 5.25 %, is one of the most frequently used irrigants in regenerative endodontic procedures because of its bactericidal ability and excellent tissue dissolution capacity [1]. Although it is effective and convenient, side effects have been extensively reported in both clinical cases and basic research studies [2]. Previous investigations demonstrated that NaOCl negatively impacts dental stem cell survival and differentiation [3]. Moreover, the viability of dental

pulp stem cells (DPSCs) was significantly reduced by NaOCl in a dose-dependent manner [4]. *In vivo* studies showed that DPSC differentiation into odontoblast cells was significantly impaired following exposure to 5.25 % NaOCl [5,6]. Furthermore, 3 % NaOCl reduced the expression of DSPP by 50 %.

Dental pulp stem cells, the first isolated dental stem cells from human permanent third molars, were found to differentiate into osteoblast, chondrocyte- and myoblast like cells [7,8]. Periodontal ligament stem cells (PDLSCs) were firstly isolated in 2004 and are capable of differentiating into cementoblast-like cells, adipocytes, fibroblasts, and even neuronal precursors [9,10]. Gingival mesenchymal stem cells (GMSCs) were firstly identified in 2010 and are capable of differentiating into [11]. osteoblasts, adipocytes, chondrocytes, endothelial cells, and neurocytes [11-15]. Both dental pulp stem cells and gingival mesenchymal stem cells possess immunomodulatory capabilities based on their interactions with immunocytes, and various toll-like receptors are expressed in GMSCs [16].

Although the effects of irrigants on APSCs have been extensively studied, the exact impacts of NaOCl on the proliferation and differentiation of DPSCs, PDLSCs, and GMSCs remains largely unknown. The aim of this study was to evaluate the effects of different NaOCl concentrations and treatment times on viability, proliferation, and differentiation of different dental stem cell types.

EXPERIMENTAL

Cell culture

Human dental stem cells (DPSCs, PDLSCs and GMSCs) were purchased from Hongxin Biology Co., Ltd. (China). All stem cells were cultured in 96-well plates at 1×10^5 / well following the manufacturer's instruction. Briefly, cells were cultured in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (Gibco, USA) in an incubator with a humidified atmosphere containing 5 % CO_2.

NaOCl treatment

After 24 h incubation, the cells were treated with different concentrations of NaOCl for various periods. The concentrations of NaOCl was set at 0, 0.005, 0.025, 0.05, and 0.1 mg/mL, and the time courses were 2, 4, 8, and 24 h. Each condition had three replicates.

Cell viability assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8) assays according to the manufacturer's protocol (Dojindo; Tokyo, Japan). 2×10^3 cells were seeded in 96-well plates and incubated at 37 °C for 2, 4, 8, or 24 h in a humidified chamber containing 5 % CO_2. Then, 10 µL CCK-8 solution was added to each well, and the plates were incubated for 1 h at 37 °C. The absorbance of cells at 450 nm was measured in a microplate reader (Bio-Rad, USA).

Adenosine triphosphate (ATP) assay

ATP assay kits (Beyotime, China) were used to measure ATP levels according to the manufacturer's instruction. Dental stem cells treated with NaOCl were lysed with cell lysis buffer (200 µL), and centrifuged for 5 min at 4° C. Then 100 µL working buffer was added to measure the ATP level via luminometer.

Flow cytometry

DPSCs, PDLSCs, and GMSCs were placed in 96-well plates at 1×10^5 /well. After 24 h, NaOCl was added to the cells at 0, 0.005, 0.01, 0.025, 0.05, or 0.1 mg/mL. Simultaneously, bromodeoxyuridine (BrdU; Sigma, USA) was added to the cells for 24 h, and flow cytometry was performed to evaluate proliferation. Cells were digested with 0.25 % EDTA-trypsin after three washes with phosphate-buffered saline (PBS), and then fixed with 4 % paraformaldehyde (Huanhai, China) overnight at 4 °C. Next, 0.1 % Triton X-100 (Sigma, USA) was used to permeabilise the cellular membrane at 4 °C for 10 min. After centrifugation, the cells were incubated with 300 µL DNase I at 37 °C for 30 min to denature the DNA. The cells were resuspended in 50 µL staining buffer and incubated with PE-Mouse anti-BrdU (Thermo Fisher Scientific, USA) and isotype control (Thermo Fisher Scientific) at 4 °C for 30 min in the dark. After washing out the staining buffer, the cells were resuspended in PBS and subjected to fluorescence detection.

Quantitative polymerase chain reaction (qPCR)

DPSCs, PDLSCs, and GMSCs were plated in 6-well plates at 1×10^5 /well. After 24 h, the cells were treated with 0.025 mg/mL NaOCl for different time points. After three washes with PBS, the medium was replaced with differentiation-induction medium: αMEM plus 10 % FBS supplemented with dexamethasone (10 nM) (Sigma-Aldrich). The untreated groups were

taken as control, and all conditions were tested in three replicates. Cells were collected in Trizol at day 0, 3, and 7. cDNA synthesis was performed with PrimeScriptRT reagent kit (Takara, Dalian, China) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Primer sequences were listed below:

Variable	Forward (5' – 3')	Reverse (5' – 3')
Osteocalcin	AAGCAGGAGGGCAATAAGGT	TGAGCCCAGCATAGTCGTCA
Forbone sialoprotein	CAACAGCACAGAGGCAGAAA	CCGTACTCMCCCGTCCTAATCCA
Dentin	ATATTGAGGGCTGGAATGGGGA	TTTGTGGCTCCAGCATTGTCA
GAPDH	GGAGCGAGATCCCTCCAAAAT	

Western blotting

Cells were washed three times with PBS and lysed in radioimmunoprecipitation assay buffer with protease inhibitors on ice for 10 min. After centrifugation at 12000 g for 10 min at 4 °C, the supernatant was electrophoresed on 12 % sodium dodecyl sulphate gels followed by transfer to polyvinylidene fluoride membranes. The membranes were blocked with 5 % skimmed milk for 2 h at room temperature, and then incubated with primary antibodies at 1:1000 for Stro-1 (Santa Cruz Biotechnology, USA), 1 DSPP (Abcam, UK) and OC (Sigma, USA). Mouse actin was used as the loading control. After three 5-min washes in PBS-Tween (PBST), the membranes were incubated with secondary antibodies at ilution of 1:2000 for 1 h at room temperature. Protein signals were visualized by chemiluminescence after three 5-min washes in PBST.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analyses comparing two groups were performed using Student's t-tests using SPSS 16.0 software (SPSS Inc., Chicago, IL). Comparisons between multiple groups were performed using one-way analysis of variance followed by least significant difference post hoc tests. Differences were considered statistically significant at $p < 0.05$.

RESULTS

NaOCl impairs dental stem cell viability

After NaOCl treatment, the viabilities of all the three dental stem cell types were significantly lower, especially those treated at high concentration for long incubation times. There was no significant change in DPSC viability at NaOCl concentration of 0.005 mg/mL when the cells were treated for 2 h, whereas viabilities decreased by about 20 and 40 % at 0.01 and 0.05 mg/mL NaOCl, respectively (Figure 1 A). At 24 h, DPSC viabilities decreased by 20, 30, 60, 70, and 80 % at concentrations of 0.005, 0.01, 0.025, 0.05, and 0.1 mg/mL, respectively. With regard to time, cell death rates of DPSCs after 2, 4, 8, and 24 h in 0.01 mg/mL NaOCl reached up to 20, 40, 60, and 70 %, respectively. Similar results were also seen for PDLSCs and GMSCs. These findings suggest that dental stem cell viability decreased with higher concentrations and longer times of NaOCl treatment.
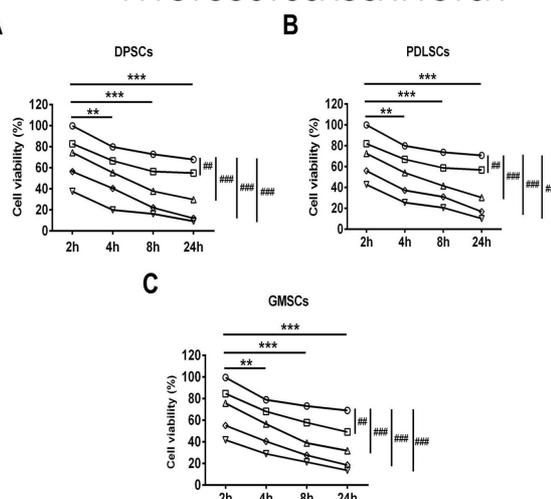
Figure 1: NaOCl treatment reduced the viability of DPSCs (A), PDLSCs (B), and GMSCs (C). Cells were collected at the indicated time points for viability analyses. Untreated group was taken as control and each condition was tested in triplicate (** or ## $p < 0.01$*** or ### $p < 0.001$). ∇: 0.1 mg/mL NaOCl; ◇: 0.05 mg/mL NaOCl; △: 0.025 mg/mL NaOCl; □: 0.01 mg/mL NaOCl; ○: 0.005 mg/mL NaOCl

ATP levels in dental stem cells decreased following NaOCl treatment

As shown in Figure 2, ATP level was also downregulated by NaOCl treatment in concentration- and time-dependent manners (20 % at 0.005 mg/mL vs 50 % at 0.01 mg/mL in 24 h group, $p < 0.05$; 20 % for 2 h vs 80 % for 24 h at 0.01 mg/mL NaOCl, $p < 0.05$). The ATP decrease in GMSCs was much smaller than in the other two cell lines, especially at lower NaOCl concentrations. As an example, ATP levels in DPSCs and PDLSCs decreased by nearly 50 % at 0.01 mg/mL of NaOCl at 24 h, when compared to 20 % in GMSCs ($p < 0.05$). This suggests that GMSCs are more resistant than DPSCs and PDLSCs to NaOCl-induced ATP loss.
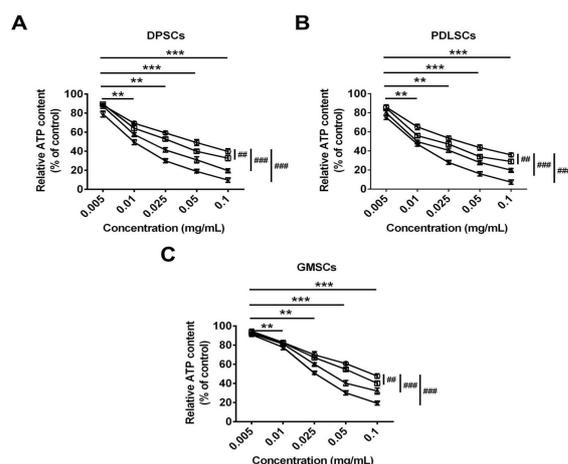
Figure 2: ATP levels in DPSCs (A), PDLSCs (B), and GMSCs (C) after NaOCl treatment. The ATP contents of the treatment groups were compared with the untreated group. The untreated group was taken as control and each condition was tested in triplicate (** or ##$p < 0.01$, *** or ###$p < 0.001$). : 24 h; △: 8 h; □: 4 h; ○: 2 h

NaOCl significantly affected dental stem cell proliferation

To further investigate whether NaOCl attenuate stem cell proliferation, cells were incubated with BrdU and performed flow cytometry after NaOCl treatment. As shown in Figure 3 A, the percentages of proliferative cells was significantly decreased upon 0.1 mg/mL NaOCl treatment after 24 h ($p < 0.05$). The proliferation of cells treated with other concentrations are shown in Figure 3B. Proliferation of DPSCs, PDLSCs, and GMSCs decreased in an NaOCl concentration-dependent manner. Importantly, NaOCl induced more prominent growth inhibition in DPSCs and PDLSCs than in GMSCs ($p < 0.05$), indicating that DPSCs and PDLSCs were more vulnerable to NaOCl treatment.

NaOCl impaired dental stem cell differentiation

Next, the expression of Stro-1 was measured, a pluripotent marker for dental stem cells, and the differentiation markers DSPP, BSP, and OC to verify whether NaOCl impacted cell differentiation. As shown in Figure 4, significantly less Stro-1 was expressed in the experimental groups relative to the untreated groups (25 % vs 10 % for DPSCs and PDLSCs at D0, $p < 0.05$). Similar to its effect on cell proliferation, NaOCl caused more prominent differentiation defect in DPSCs and PDLSCs than in GMSCs ($p < 0.05$). Similarly, DSPP, BSP, and OC levels were upregulated in a time-dependent manner. In DPSCs and PDLSCs, the downregulation in gene expression was much slower in the

experimental groups than the control groups (50 vs 90 % for DPSCs at D7 and 50 vs 90 % for PDLSCs, $p < 0.05$), the changes of these three markers were faster in GMSCs. Only DSPP significantly decreased in treated cells (70 vs 90 %, $p < 0.05$), while BSP levels remained unchanged (70 vs 70 %, $p > 0.05$) and OC levels were enhanced in the experimental groups (70 vs 80 %, $p < 0.05$), again suggesting less of an impact on GMSC proliferation.
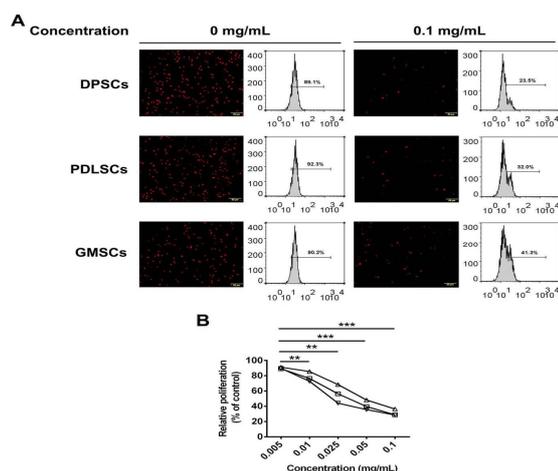
Figure 3: Flow cytometry data for DPSC, PDLSC, and GMSC proliferation. (A) The proliferation of three stem cell lines was assayed by fluorescence microscope (left) and flow cytometry (right). (B) Detained relative proliferation of the three stem cell lines treated with the 5 NaOCl concentrations in (A). **$p < 0.01$, *** $p < 0.001$; △: GMSCs; □: PDLSCs; : DPSCs

DISCUSSION

Dental pulp stem cells, gingival mesenchymal stem cells, and periodontal ligament stem cells are similar to bone marrow mesenchymal stem cells in that they possess powerful proliferation and differentiation abilities, making them valuable resources for both endodontic and periodontal regeneration [8]. NaOCl was recently recommended as an effective irrigant and is extensively used in dentistry due to its functions in microbial control and tissue dissolution [17].

NaOCl exert antibacterial function through multiple ways. For instance, NaOCl can reduce surface tension of solution by functioning as a fat solvent [18]. It can also degrade dentin collagen by breaking the bonds between carbon atoms, thus disorganizing the protein primary structure [2]. Moreover, NaOCl denatures bacterial enzymes by releasing chlorine, a strong oxidant that can bind to the amino groups to generate chloramines, thus interfering with bacterial metabolism [1,18].
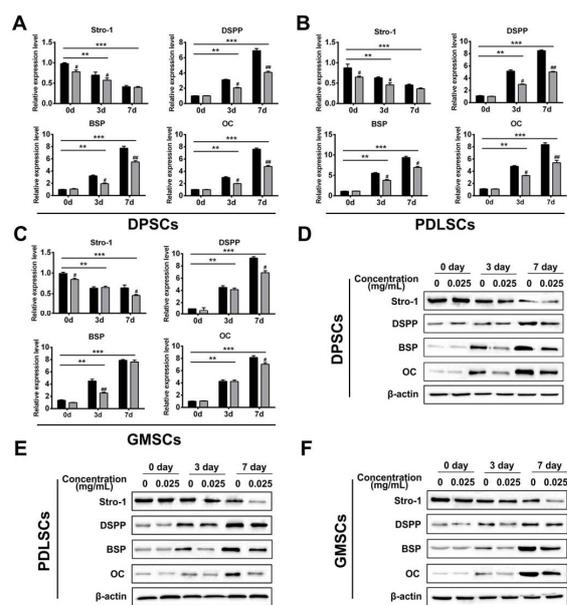
Figure 4: Differentiation marker expression levels in DPSCs, PDLSC, and GMSCs after NaOCl treatment on Days 0, 3, and 7. (A-C) qPCR analyses of Stro-1, DSPP, BSP, and OC in DPSCs (A), PDLSC (B), and GMSCs (C). The untreated group was taken as control, and results are expressed as fold change, (p < 0.05). (D-F) Stro-1, DSPP, BSP, and OC protein levels in the three dental stem cell lines in (A). Each condition was tested in triplicate (# $p < 0.05$, ** or ## $p < 0.01$, *** $p < 0.001$). ■: 0 mg/mL NaOCl; □:0.025 mg/mL NaOCl

Despite its antibacterial function, NaOCl can also induce some side effects such as canal tissue damage Furthermore, an additional chelating agent is needed to remove the smear layer [19]. Severe NaOCl-induced necrosis has been observed both in preclinical and clinical studies and is most likely to occur in maxillary teeth [20-22]. NaOCl was also reported to induce acute inflammation in vital tissues [23]. Although 6 % NaOCl conditioned with dentin was reported to prevent DPSC differentiation both *in vivo* and *in vitro* [5,24], there have been few reports on the effect of NaOCl on PDLSCs and GMSCs, especially with regard to differentiation.

In the present study, the impact of NaOCl on GMSC proliferation and differentiation was investigated. The results showed that NaOCl negatively affected dental mesenchymal stem cell survival and differentiation, and dental stem cell cytotoxicity was directly correlated with the concentration and treatment time. These results are consistent with previous studies, suggesting that cell viability and differentiation decrease along with a higher NaOCl concentration and longer treatment [3]. The present research firstly demonstrated that NaOCl affects the proliferation and differentiation of PDLSCs and GMSCs. It is noteworthy that the change in ATP level and

differentiation markers expression levels was less significant for GMSCs than the other two stem cells lines, indicating stronger tolerance of GMSCs to differentiation-induced stimuli. This might be attributable to their resistance to inflammatory stimuli.

A study demonstrated that GMSCs isolated from inflamed tissues displayed a normal phenotypic profile and developmental potential similar to cells obtained from healthy gingival tissues [25]. However, further investigation is needed to clarify the exact reasons for these observations. Taken together, a deteriorative effect of NaOCl on dental stem cell proliferation and differentiation was revealed in this study. It should therefore be used with caution during endodontic therapy.

CONCLUSION

NaOCl dose-dependently impairs the viability, proliferation and differentiation of dental stem cells. Thus, its toxicity to dental stem cells needs to be considered in clinical application.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shiqi Liu and Baorong Zhang designed all the experiments and revised the paper. Shaoping Fu, Chengyu Cui, Jinjuan Jiang, and Jian Xu performed the experiments, Pengyu Pan and Hanchao Zhai wrote the paper.

REFERENCES

1. Tan HK, Wheeler WB, Wei CI. Reaction of chlorine dioxide with amino acids and peptides: kinetics and mutagenicity studies. Mutat Res 1987; 188(4): 259-266.
2. Ishizuka T, Kataoka H, Yoshioka T, Suda H, Iwasaki N, Takahashi H, Nishimura F. Effect of NaClO treatment on bonding to root canal dentin using a new evaluation method. Dent Mater J 2001; 20(1): 24-33.
3. Alkahtani A, Alkahtany SM, Anil S. An in vitro evaluation of the cytotoxicity of varying concentrations of sodium hypochlorite on human mesenchymal stem cells. J Contemp Dent Pract 2014; 15(4): 473-481.

4. Park M, Pang NS, Jung IY. Effect of dentin treatment on proliferation and differentiation of human dental pulp stem cells. Restor Dent Endod 2015; 40(4): 290-298.

5. Galler KM, D'Souza RN, Federlin M, Cavender AC, Hartgerink JD, Hecker S, Schmalz G. Dentin conditioning codetermines cell fate in regenerative endodontics. J Endod 2011; 37(11): 1536-1541.

6. Martin DE, De Almeida JF, Henry MA, Khaing ZZ, Schmidt CE, Teixeira FB, Diogenes A. Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. J Endod 2014; 40(1): 51-55.

7. Verma K, Bains R, Bains VK, Rawtiya M, Loomba K, Srivastava SC. Therapeutic potential of dental pulp stem cells in regenerative medicine: An overview. Dent Res J (Isfahan) 2014; 11(3): 302-308.

8. Rosa V, Botero TM, Nor JE. Regenerative endodontics in light of the stem cell paradigm. Int Dent J 2011; 61 Suppl 1: 23-28.

9. Techawattanawisal W, Nakahama K, Komaki M, Abe M, Takagi Y, Morita I. Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. Biochem Biophys Res Commun 2007; 357(4): 917-923.

10. Gay IC, Chen S, MacDougall M. Isolation and characterization of multipotent human periodontal ligament stem cells. Orthod Craniofac Res 2007; 10(3): 149-160.

11. Fournier BP, Ferre FC, Couty L, Lataillade JJ, Gourven M, Naveau A, Coulomb B, Lafont A, Gogly B. Multipotent progenitor cells in gingival connective tissue. Tissue Eng Part A 2010; 16(9): 2891-2899.

12. Wang F, Yu M, Yan X, Wen Y, Zeng Q, Yue W, Yang P, Pei X. Gingiva-derived mesenchymal stem cell-mediated therapeutic approach for bone tissue regeneration. Stem Cells Dev 2011; 20(12): 2093-2102.

13. Ferre FC, Larjava H, Loison-Robert LS, Berbar T, Owen GR, Berdal A, Cherifi H, Gogly B, Hakkinen L, Fournier BP. Formation of cartilage and synovial tissue by human gingival stem cells. Stem Cells Dev 2014; 23(23): 2895-2907.

14. Gao Y, Zhao G, Li D, Chen X, Pang J, Ke J. Isolation and multiple differentiation potential assessment of human gingival mesenchymal stem cells. Int J Mol Sci 2014; 15(11):20982-20996.

15. Zhang Q, Shi S, Liu Y, Uyanne J, Shi Y, Shi S, Le AD. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009; 183(12): 7787-7798.

16. Fawzy El-Sayed KM, Klingebiel P, Dorfer CE. Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells. J Endod 2016; 42(3): 413-417.

17. Kandaswamy D, Venkateshbabu N. Root canal irrigants. J Conserv Dent 2010; 13(4):256-264.

18. Estrela C, Estrela CR, Barbin EL, Spano JC, Marchesan MA, Pecora JD. Mechanism of action of sodium hypochlorite. Braz Dent J 2002; 13(2): 113-117.

19. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. J Endod 1975; 1(7): 238-242.

20. Kleier DJ, Averbach RE, Mehdipour O. The sodium hypochlorite accident: experience of diplomates of the American Board of Endodontics. J Endod 2008; 34(11): 1346-1350.

21. Gernhardt CR, Eppendorf K, Kozlowski A, Brandt M. Toxicity of concentrated sodium hypochlorite used as an endodontic irrigant. Int Endod J 2004; 37(4): 272-280.

22. Hulsmann M, Hahn W. Complications during root canal irrigation--literature review and case reports. Int Endod J 2000; 33(3): 186-193.

23. Pashley EL, Birdsong NL, Bowman K, Pashley DH. Cytotoxic effects of NaOCl on vital tissue. J Endod 1985; 11(12): 525-528.

24. Casagrande L, Demarco FF, Zhang Z, Araujo FB, Shi S, Nor JE. Dentin-derived BMP-2 and odontoblast differentiation. J Dent Res 2010; 89(6): 603-608.

25. Ge S, Mrozik KM, Menicanin D, Gronthos S, Bartold PM. Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy. Regen Med 2012; 7(6): 819-832.