Effect of Root Canal Irrigants on Attachment and Differentiation of Human Dental Pulp Cells

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The Graduate School

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Effect of Root Canal Irrigants on Attachment and Differentiation of Human Dental Pulp Cells

(Directed by Prof. Il Young Jung, D.D.S., M.S.D., Ph.D.)

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This certifies that the dissertation thesis
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December 2012

감사의 글

먼 길이지만 지치지 않았던 것은 그 끝에 바라보아야 할 목표를 주시고 힘을 북돋아주시며 격려와 조언을 아끼지 않고 함께 그 길을 동행해주신 많은 분들이 계시기 때문인 것 같습니다.

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그리고 제 맘속에 또 한 분의 큰 스승님이신 김진 교수님과 힐링 멘토이신 강정완 교수님, 언제 어디서나 전폭적인 지원과 격려로 지치지 않게 힘을 주셨던 김기덕 교수님, 친언니보다 더 가족 같은 정복영 교수님, 정말 닮고 싶은 선배 박원서 교수님. 정말 감사합니다.

마지막으로 지금까지 언제나 한결 같은 믿음으로 든든한 지원군이 되어주신 사랑하는 가족들과 이 모든 과정을 주관하시고 섭리로 이끌어내신 하나님께 이 논문을 바칩니다.

2012 년 12 월

방난심 드림

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Abstract

Effect of Root Canal Irrigants on Attachment and Differentiation of Human Dental Pulp Cells

Nan-Sim Pang D.D.S., M.S.D.

(Directed by Prof. Il Young Jung, D.D.S., M.S.D., Ph.D.)

Purpose: Sodium hypochloride (NaOCl) and ethylenediaminetetraacetic acid (EDTA) are commonly used for each antibacterial and cleansing effect in conventional root canal treatment, but they are questionable candidates for cytotoxicity in regenerative endodontic treatment. In clinical practice for regenerative endodontics, NaOCl is obliged to be used for the canal disinfection despite the damage to the living cells. EDTA has been suggested as a chelating agent to release growth factor form dentin matrix, without scientific evidence analog to actual clinical situation. The aim of this study was to evaluate the effect of dentin application with NaOCl and EDTA on dental pulp cells (DPCs) attachment and differentiation on the dentin surface. I investigated whether EDTA could release any biochemical components from dentin and change cell behaviors.

Materials & Methods: Human dental pulp cells were obtained from 11 normal human third molars and investigated the stemness by flow cytometry analysis. Dentin specimens were cut into disc shape and applied with 5.25% NaOCl (30 minutes) and 17% EDTA (1

minute). After procedure to minimize the residual toxicity, pulp cells were seeding on the conditioning dentin slices. To investigate the growth factor releasing effect, we included a group that dental pulp cells were cultured without dentin contact under a cell culture insert, in which EDTA-treated dentin slices were placed. After 3 day culture, cell attachment of each group was measured. After 21 day culture, I investigated differentiation gene expression level by quantitative real-time PCR. Then we observed calcification level by Alizarin Red S staining and scanning electron microscopy (SEM).

Results: After residual irrigants were removed after application of NaOCl and EDTA, pulp stem cell attached significantly denser on EDTA-treated dentin surface. The pulp stem cells on EDTA or NaOCl-treated dentin surface showed higher gene expression of DSPP and DMP-1 than that on untreated dentin or no dentin after 3weeks culture. In this study, dental pulp cells under a cell culture insert, in which EDTA-treated dentin slices were placed, did not show any differentiation gene expression.

Conclusion: The physical property or topography of dentin surface might be changed by EDTA or NaOCl, which is considered to induce cell differentiation. How to treat the substrate (dentin) can be a crucial factor to regulate pulp stem cell behaviors (migration, attachment, proliferation, and differentiation) in regenerative endodontic treatment.

Key words: regenerative endodontics, pulp stem cell, cell attachment, cell differentiation, Sodium hypochloride (NaOCl), ethylenediaminetetraacetic acid (EDTA), cell-substrate intereaction

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I. Introduction

Conventional root canal therapy involves the removal of inflamed or necrotic pulp tissue and the insertion of a synthetic material into the root canal system, which results in the loss of physiological form and function of the dental pulp. Complications can occur, especially in teeth with incomplete root formation, which are typically characterized by thin, funnel-shaped dentin walls that make

obturation difficult and root fractures likely. An apexification protocol with calcium hydroxide can be used to establish a calcified barrier. However, treatment periods are variable, the risk of fracture increases through repeated manipulation, and a continuation of root development cannot be achieved in most cases[1]. True regeneration of the pulpodentin complex is especially desirable in those cases[2].

Recent clinical reports describe a revascularization procedure that includes asymptomatic teeth, continued root formation, and healing, even in cases with preoperative periapical lesions[3,4]. The protocol includes disinfection of the root canal system, provocation of bleeding into the canal to provide a guide rail for tissue in-growth, and coverage with mineral trioxide aggregate[2]. The following processes are then required for the ideal regeneration of a functional pulpodentin complex in a root canal space: (i) revascularization from the apical end, (ii) cell attachment and differentiation into new odontoblasts on the existing dentin of the root canal surface, and (iii) new dentin production by the new odontoblasts and continued root formation[5].

The essential prerequisite throughout this process is intracanal disinfection[6]. Various irrigants and protocols have been proposed for achieving canal cleansing and disinfection. The irrigants include sodium hypochloride (NaOCl) and chlorhexidine, which are commonly used for their bactericidal and bacteriostatic effects in conventional root canal treatment; 17% ethylenediaminetetraacetic acid (EDTA) is also often used for removal of the smear layer and exposure of dentinal

tubules to allow access for other bactericidal irrigants or medicaments in conventional root canal treatment[7]. The availability of each irrigant should be considered carefully in regenerative endodontic treatment.

Previous studies have found that irrigants can influence the attachment of dental pulp stem cells (DPSCs) to the root canal dentine[8]. The authors use NaOCl for irrigation, analogous to conventional root canal preparations, with concentrations ranging from 0.5% to 6%[9]. Because of its potent antimicrobial and proteolytic activity, NaOCl dissolves organic debris and aids in both the elimination of microorganisms and the removal of necrotic tissue. Higher concentrations increase this effect, but there is a trade-off for this enhanced effectiveness with increased toxicity, because NaOCl causes an inflammatory response and severe damage when in contact with vital tissue[10]. Furthermore, NaOCl is unable to remove the smear layer on the dentin surface after root canal preparation. Since the success of any regenerative procedure may be dependent upon the migration of the resident stem cells of the apical papilla to recolonize the canal space, the presence of a toxic agent and the smear layer might create unfavorable conditions for cell survival, migration, adhesion, and proliferation. This renders NaOCl a questionable candidate for irrigation in this context[2].

For conventional root canal preparation, the use of additional irrigants to create a clean and smear-layer-free surface before obturation has frequently been advocated[7]. Chelating agents such as EDTA are able to withdraw calcium from

the inorganic calcium phosphate crystal lattice, which results in demineralization of the superficial dentin layer. With regard to a cell-friendly environment, decalcification of the dentin leads to the exposure of collagen fibrils of the organic matrix; these present adhesion motifs that enable cell binding via integrin receptors[11,12]. Furthermore, EDTA treatment exposes growth factors entrapped in the dentin matrix, including transforming growth factor-beta (TGF-β)[13,14], bone morphogenetic protein 2 (BMP-2)[14], and angiogenic factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor 2[15]. It has been shown that EDTA-soluble factors stimulate matrix secretion, odontoblast differentiation, and tertiary dentin formation[2,14]. However, *in vitro* EDTA treatment impairs cell attachment, regardless of removal the smear layer[8,16]. Moreover, *in vivo* clinical treatment, EDTA has been used for only 1~2 minutes as a final canal cleanser, unlike more than the 10-day soaking period described in previous studies showing growth factor release[13–15,17–19]. Hence, further clinical studies are required to establish the neutralization of the remaining cytotoxicity and the true effectiveness of these irrigants in better simulating clinical conditions in regenerative endodontic treatment.

The purpose of this study was to characterize the effects of dentin application of NaOCl and EDTA on the attachment and differentiation of human dental pulp cells (DPCs) on the dentin surface under conditions of minimum potential

cytotoxicity. The relationship between changes in the behavior of DPCs and dentin matrix component release was also investigated.

II. Materials and Methods

1. Establishment of cell lines

A. Primary cell culture

Normal human third molars were collected from young adults (16–22 years of age) at the Department of Oral and Maxillofacial Surgery and the Department of Advanced General Dentistry, Yonsei University Dental Hospital (*n*=11). The pulp tissue was gently separated from the apex of the extracted third molars with a barbed broach and then cut into 1-mm^3-sized blocks and placed in 60-mm culture dishes (BD Falcon, Lincoln Park, NJ, USA) with a counting chamber cover glass (Marienfeld Superior, Lauda-Königshofen**,** Germany) for the outgrowth of cells. The tissue samples were cultured in alpha-modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mmol/l glutamine, 100 μmol/l ascorbic acid-2-phosphate (WAKO, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Rockville, MD, USA) at 37°C in 5% CO_2. The outgrown cells were transferred to 5×10 cm culture flasks (passage 1) and grown to confluence. The obtained cells were harvested and kept frozen in liquid N_2. All primary cells used in this study were at passage 3.

B. Flow cytometry analysis for surface-marker expression

Mesenchymal stem-cell surface antigen expression was analyzed by flow cytometry. The DPCs (1×10^6) were harvested in phosphate-buffered saline (PBS), washed, and resuspended in the flow cytometry staining buffer (eBiosciences, San Diego, CA, USA). They were incubated with fluorescently [fluorescein isothiocyanate (FITC)] labeled mouse antihuman antibodies (CD146-FITC, CD90-FITC, CD105-PE, CD34-FITC, and CD45-PE; all supplied by eBioSciences) or antihuman Stro-1 (DyLight 488, BD Biosciences, San Jose, CA, USA) for 1 h at 4°C. The expression profiles were examined using an LSR II flow cytometer system (BD Biosciences). At least 50,000 total events for fluorescence-activated cell-sorting analysis.

C. Immunocytochemical staining for surface marker expression

In brief, cover glasses were sterilized under UV irradiation for 30 min. DPCs (3×10^4 cells/well) were seeded into six-well plates containing the cover glass. After 3 days of culture, the cover glasses were removed and fixed with 95% ethanol for 30 min. Preincubation with 5% bovine serum albumin for 1 h was followed by incubation with a primary antibody: CD146 (Novus Biologicals, Littleton, CO, USA) or Stro-1 (BD Biosciences). The cell nuclei were stained with 4, 6-diamidino-2-phenylindole for 5 min. Samples were examined with the

aid of a scanning confocal microscope (LSM 510 Meta laser, Carl Zeiss Microimaging, Gottingen, Germany).

2. Preparation of dentin slices

Dentin specimens were prepared from the collected human third molars after the pulp tissue had been removed to obtain pulp cells. The coronal dentin was cut into a disc shape (approximately 1-mm thick) under cooling with sterile PBS (Mediatech, Manassas, VA, USA). The dentin slices were sterilized by soaking alternately with 10% povidone-iodine and 70% ethanol.

3. Measurements

A. Cell-attachment assay

The dentin slices were randomly assigned to one of the following experimental conditions: (1) 5.25% NaOCl for 30 min, (2) 17% EDTA (pH 7.2) for 1 min, and (3) untreated control dentin slices. All dentin slices were equilibrated with their culture media for 24 h after 5× washout with PBS. After being counted, the harvested cells were seeded onto six-well plates (at 3×10^4 cells/well) containing different dentin samples in a single layer. The influence of each dentin treatment on the adhesion of DPCs was estimated by dividing the experimental groups as listed in Table 1.

All groups were cultured in normal growth medium for 3 days. At the end of the culture period, the dentin slices were removed and placed in new six-well plates. They were then washed twice with PBS solution, detached with trypsin/EDTA, stained with trypan blue, and then counted using hematocytometer. The area of dentin surface was calculated with the aid of Image J software.

Table 1. Experimental groups for attachment of DPCs.

Group		Dentin treatment	Medium	Culture duration
1	Control	Untreated dentin	Proliferation	3 days
2	NaOCl-treated dentin	5.25% NaOCl	Proliferation	3 days
3	EDTA-treated dentin	17% EDTA	Proliferation	3 days

B. Gene expression analysis using quantitative real-time PCR; mineralization

For differentiation of DPCs, the experimental groups were subdivided as listed in Table 2 and figure 1. The medium for cellular differentiation was supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone (Sigma, St. Louis, MO, USA) to induce mineralization. In group 5, for differentiation of DPCs, EDTA-treated dentin slices were placed into a cell culture insert (BD Falcon, Franklin Lakes, NJ, USA) over the DPCs (so that they were not in direct contact with each other). All DPCs were cultured *in vitro* at 37°C in a humidified atmosphere of 5% CO_2 for 21 days; the culture medium was replenished every 3 days.

Total cellular RNA was extracted from DPC cells grown on dentin slices using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, after 3 weeks of culture. The RNA was treated with the RNase-free DNase set (Qiagen) during RNA extraction. The cDNA samples were prepared from the isolated RNA using the reverse transcriptase first-strand kit (Qiagen) according to the manufacturer's protocols.

Quantitative real-time PCR (qPCR) was performed using ABI 7500 software (Applied Biosystems, Foster City, CA, USA) according to the standard procedure. The real-time PCR cycles included 40 cycles of general denaturation at 94°C (30 s), annealing, and elongation at 60°C (45 s), except for the first cycle, with a

15-min denaturation, and last cycle, with a 7-min elongation at 72°C. Real-time polymerase quantification of the signals was performed by normalizing the gene signals with a β-actin signal (i.e., *ACTB*). The primers used to detect the expression levels of differentiation markers are given in Table 3.

Table 2. Experimental groups for differentiation of DPCs.

Group		Dentin treatment	Medium	Culture duration
1	Negative control	No dentin (only cells)	Proliferation	3 weeks
2	Untreated dentin	Untreated	Proliferation	3 weeks
3	NaOCl-treated dentin	5.25%NaOCl	Proliferation	3 weeks
4	EDTA-treated dentin	17% EDTA	Proliferation	3 weeks
5	EDTA-treated dentin (not in contact)	17% EDTA, in cell culture insert (not in contact with cells)	Proliferation	3 weeks
6	Positive control	No dentin (only cells)	Differentiation	3 weeks

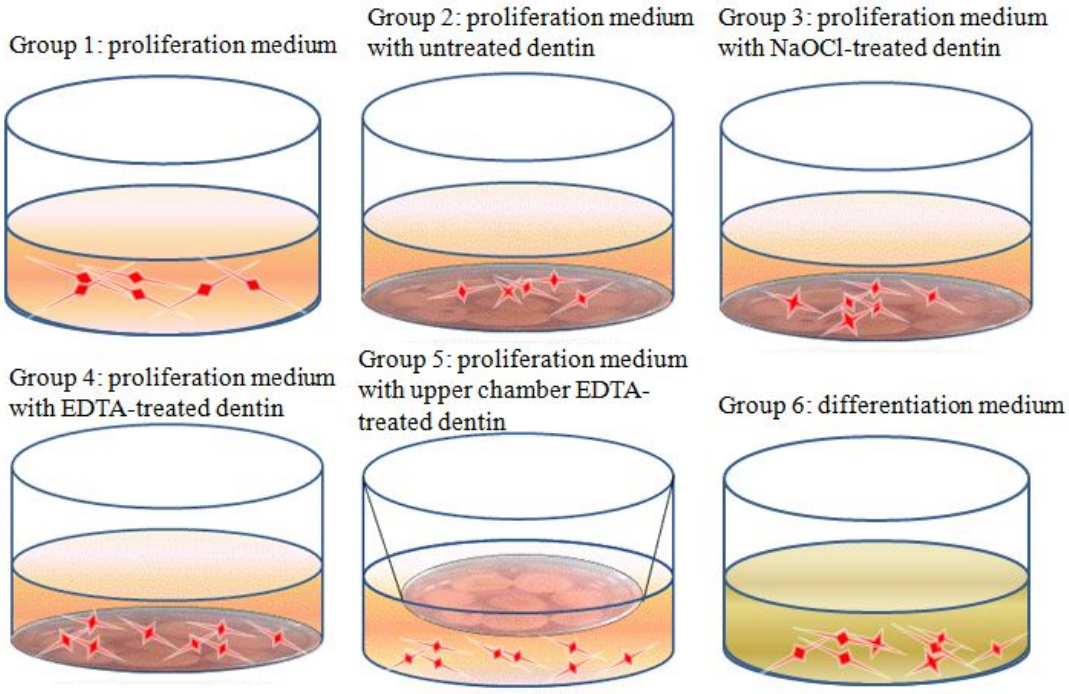


Figure 1. DPCs were cultured in six-well plates under various conditions. Group 1 was the control group for proliferation (DPCs grown on a culture dish in proliferation medium); group 2 consisted of DPCs grown on untreated dentin in proliferation medium; groups 3 and 4 included NaOCl- and EDTA-treated dentin, respectively; group 5 comprised EDTA-treated dentin placed in a cell-culture insert and placed over the DPCs; and group 6 was the control group for differentiation (differentiation-mineralization medium)

Table 3. Real-time PCR primer sequences.

	Primer set	Sequence 5'–3'
DSPP	Forward	TTAAATGCCAGTGGAACCAT
	Reverse	ATTCCCTTCTCCCTTGTGAC
DMP-1	Forward	CCCAAGATACCACCAGTGAG
	Reverse	CACCCAGTGCTCTTCACTCT
ALP	Forward	GGACCATTCCCACGTCTTCAC
	Reverse	CCTTGTAGCCAGGCCCATTG
Osteocalcin	Forward	CAAAGGTGCAGCCTTTGTGTC
	Reverse	TCACAGTCCGGATTGAGCTCA
ACTB	Forward	ATAGCACAGCCTGGATAGCAACGTAC
	Reverse	CACCTTCTACAATGAGCTGCGTGTG

C. Alizarin Red S staining

After 3 weeks, calcification of the extracellular matrix was visualized using Alizarin Red S staining. Briefly, cells were fixed for 30 min with 10% natural-buffered formalin at 4°C and stained with 2% Alizarin Red S (pH 4.2; Sigma, St Louis, MO) for 10 min at room temperature. The Alizarin Red S dye was extracted by adding 0.5 ml of 10% cetylpyridinium chloride (Sigma) to each well for 30 min. The absorbance at 570 nm was measured using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

D. Scanning-electron-microscope analysis of DPCs grown on dentin discs

After 3 days and 3 weeks of cell culture, one dentin specimen from each group was washed three times with PBS and then fixed in 2% glutaraldehyde for 5 minutes. They were dehydrated in a graded series of ethanol and dried in hexamethyldisilane. After gold coating, the preparations were observed with a scanning electron microscope (SEM; ×500 and ×1000 magnifications; JEOL JSEM-820, JEOL, Tokyo, Japan).

E. Statistical analysis

All of the experiments were repeated at least in triplicate under three independent conditions. All data are presented as mean±SD values. Descriptive analysis and Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni correction were performed using SPSS software (version 17.0; SPSS, Chicago, IL, USA). The level of statistical significance was set at $p<0.05$.

II. Results

1. Morphological and growth characteristics of DPCs

From a morphological point of view, DPC cultures were quite heterogeneous, containing fibroblast-like or spindle shaped cells with many cytoplasmic processes or filopodia (Fig. 2). The doubling time of cell growth was 28.8±2.2 h. All of the cell lines were passaged more than ten times and continued to grow in a monolayer (Table 4).

2. Flow cytometry analysis

CD146 and Stro-1 were frequently expressed in all of the cell lines. CD146 or Stro-1-positive cells were found in 85.6±5.7% and 13.4±2.7%, respectively, of all cells obtained from the pulp tissues of third molars (Fig. 3, Table 4). CD90, CD105, CD34, and CD45 staining was found in 93.7±2.3%, 92.3±3.9%, 1.2±0.5%, and 1.9±1.4% of the DPCs, respectively (Fig. 4, Table 4).

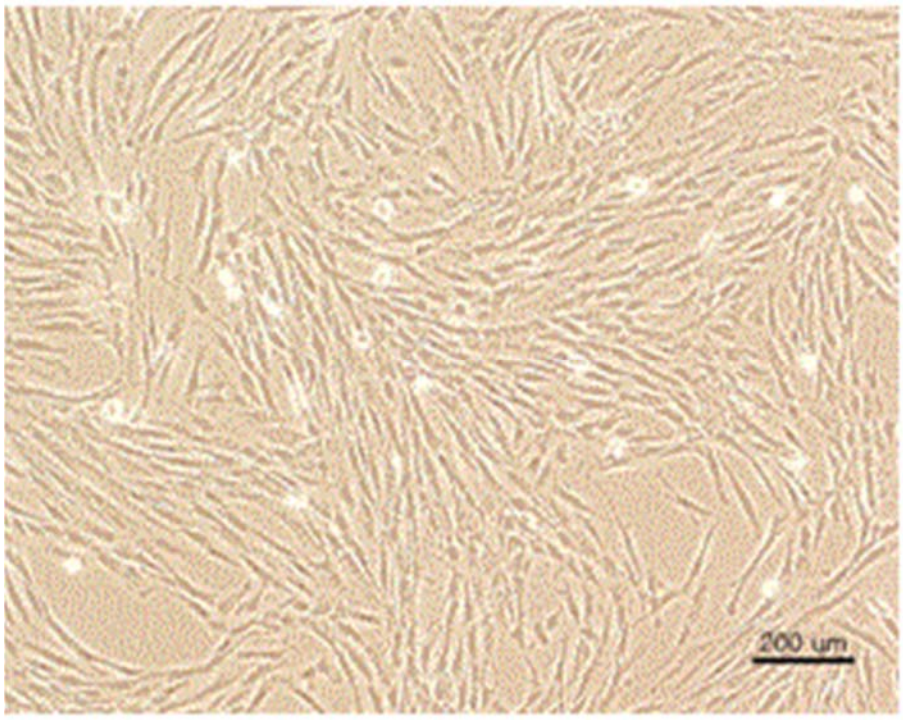


Figure 2. The DPCs exhibited a spindle-shaped and fibroblast-like appearance (passage 1).

Table 4. Growth characteristics and immunophenotypic characterization of established cell lines.

Cell lines	**Doubling time (h)**	**Growth characte-ristics**	**CD146 (%)**	**Stro-1 (%)**	**CD90 (%)**	**CD105 (%)**	**CD34 (%)**	**CD45 (%)**
1	28.2	Adherent	85.6	11.7	92.3	93.3	0.5	1.8
2	32.1	Adherent	78.2	15.3	96.2	92.2	0.9	2.0
3	25.3	Adherent	80.2	15.8	97.1	96.1	1.2	2.4
4	26.2	Adherent	89.5	14.9	91.8	94.7	1.5	1.5
5	29.3	Adherent	80.5	10.2	90.6	95.6	0.9	1.4
6	30.1	Adherent	95.1	11.9	93.2	89.3	1.3	1.9
7	28.5	Adherent	88.1	10.1	95.2	90.2	1.2	2.0
8	30.5	Adherent	87.2	17.2	95.1	95.5	1.4	2.1
9	29.4	Adherent	83.1	13.3	95.6	92.2	0.9	2.1
10	30.2	Adherent	77.1	17.1	97.2	93.1	1.0	1.8
11	27.6	Adherent	95.3	10.9	96.6	95.2	1.6	1.7
Total	28.8±2.2		85.6± 5.7	13.4± 2.7	93.7± 2.3	92.3± 3.9	1.2± 0.5	1.9± 1.4

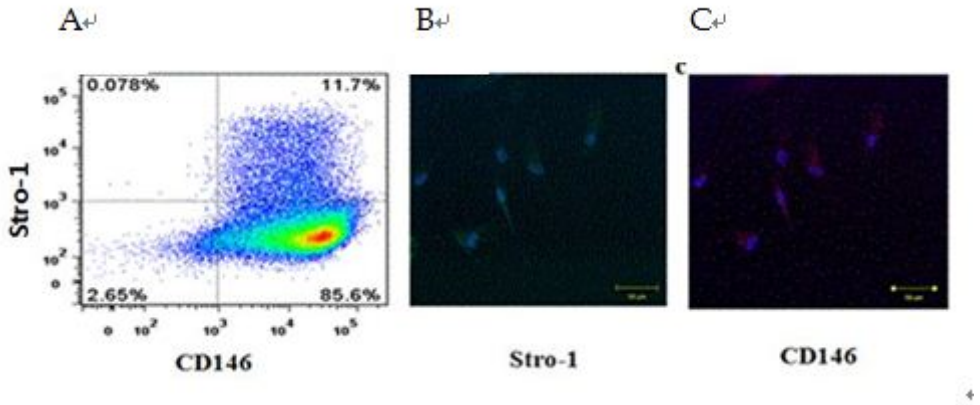


Figure 3. Flow cytometry analysis of mesenchymal stem cell markers (Stro-1 and CD146) in DPCs. (A) Forward vs. side scatter diagrams (Stro-1/CD146) of DPCs. (B, C) Fluorescence image of cells expressing Stro-1 and CD146, respectively.

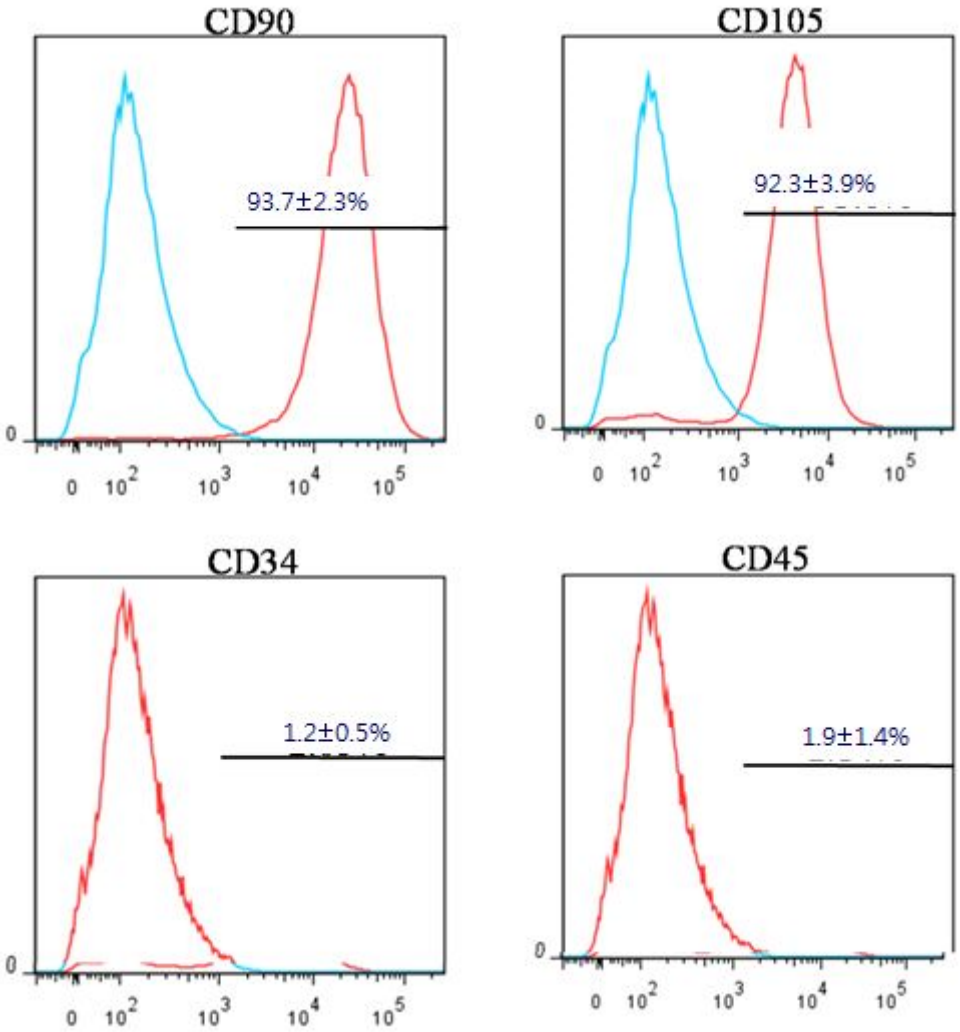


Figure 4. Flow cytometry analysis of mesenchymal stem cell markers (CD90, CD105, CD34, and CD45).

3. Cell attachment on the dentin surface after 3 days of culture

The cell density was significantly higher in the EDTA-treated group than in the other groups (Fig. 5). This finding was consistent with the SEM observations, which revealed that the cell density on the NaOCl-treated dentin was similar to that of the untreated dentin group, while the EDTA-treated group exhibited denser cell attachment and greater proliferation (Fig. 6).

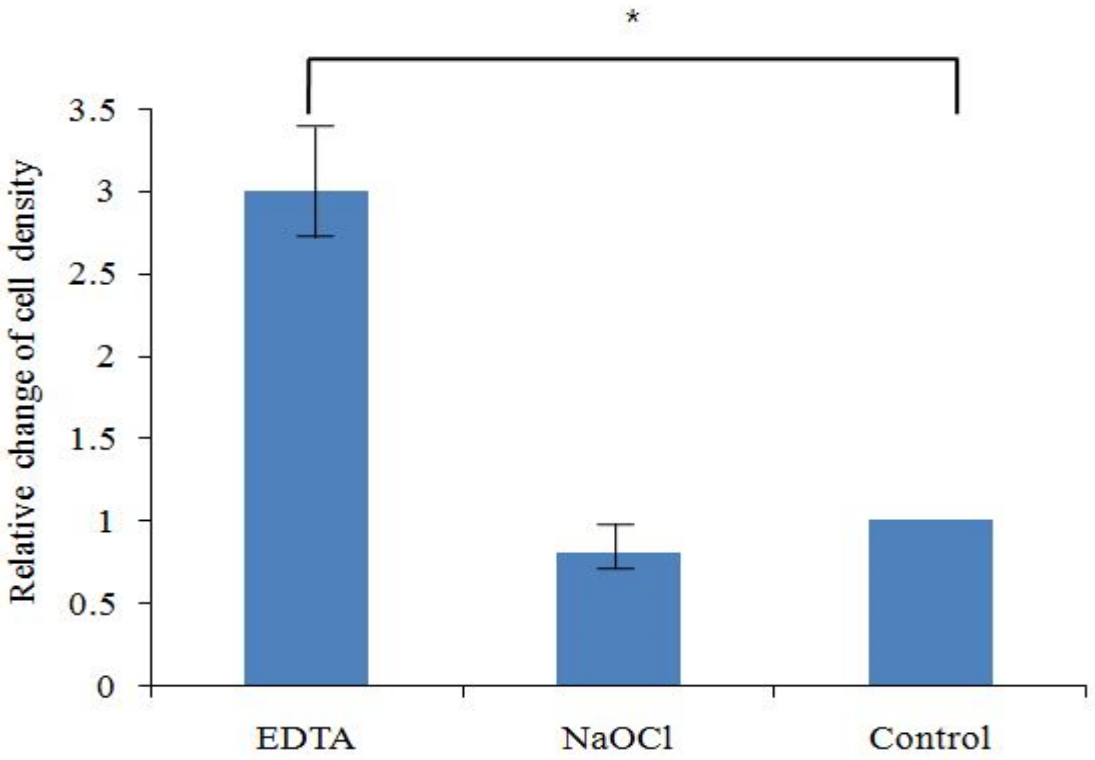


Figure 5. Relative change of cell density in the EDTA- and NaOCl-treated groups when normalized relative to control levels (*p<0.05, n=3).

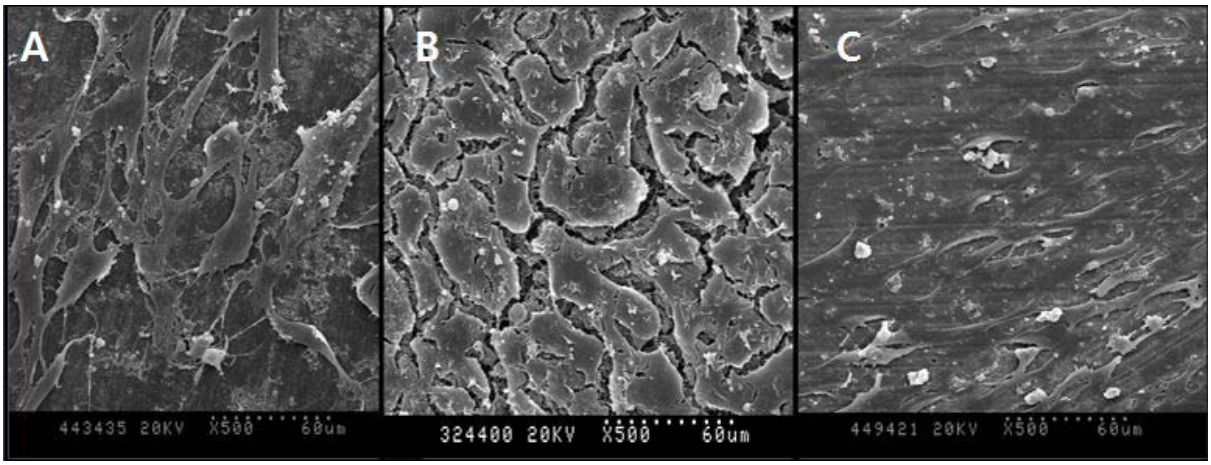
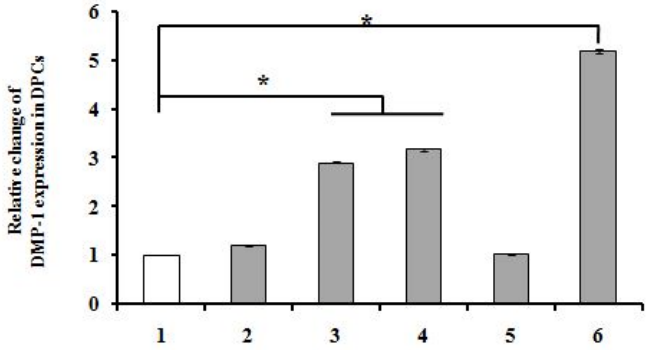


Figure 6. SEM views after 3 days of culture on NaOCl-treated (A), EDTA-treated (B) and control dentin (C). The cell attachment and proliferation were densest for the EDTA-treated dentin (magnification ×500).

4. Expression of differentiation molecules by qPCR

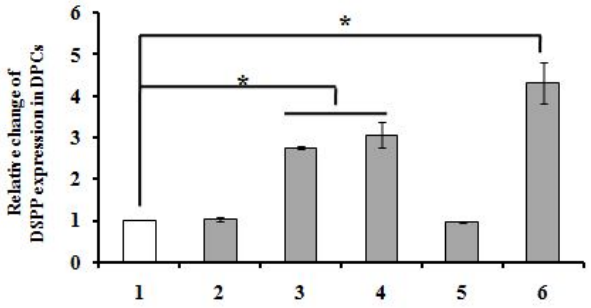
The gene expression levels for dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), alkaline phosphatase (ALP), and osteocalcin were compared in six groups, as listed in Table 2, after 21 days of culture (Fig. 7). The gene expression levels of *DSPP* and *DMP-1* were significantly higher in the NaOCl- and EDTA-treated dentin groups than the non-treated dentin and no-dentin groups. The level of *ALP*, which is an early-stage marker of osteogenesis, appeared to be higher in the EDTA group, but the change was not statistically significant. With respect to the levels of osteocalcin, which is known to be up-regulated at the end of the mineralization, the findings for all groups cultured with proliferation medium were remarkably similar.

A



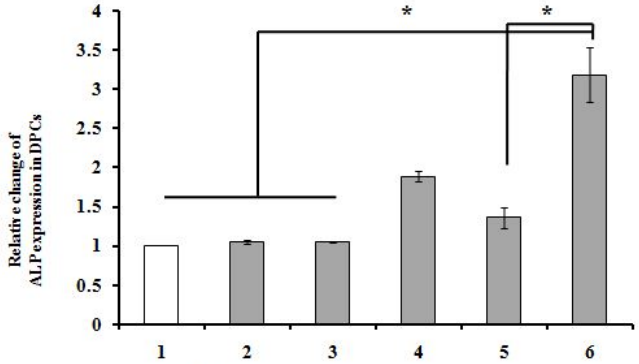
1: Proliferation medium; 2: PBS-treated dentin; 3: NaOCl-treated dentin; 4: EDTA-treated dentin; 5: upper chamber EDTA-treated dentin; 6: Differentiation medium

B

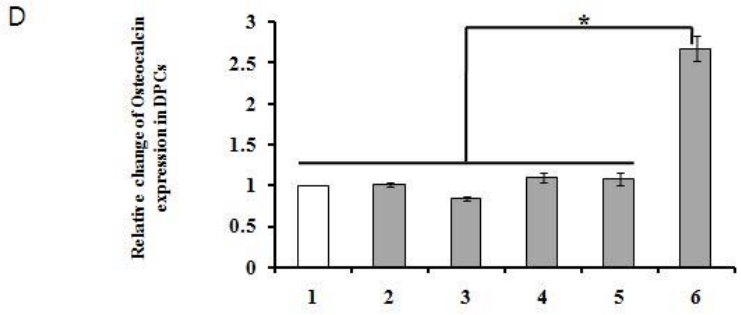


1: Proliferation medium; 2: PBS-treated dentin; 3: NaOCl-treated dentin; 4: EDTA-treated dentin; 5: upper chamber EDTA-treated dentin; 6: Differentiation medium

C



1: Proliferation medium; 2: PBS-treated dentin; 3: NaOCl-treated dentin; 4: EDTA-treated dentin; 5: upper chamber EDTA-treated dentin; 6: Differentiation medium



1: Proliferation medium; 2: PBS-treated dentin; 3: NaOCl-treated dentin; 4: EDTA-treated dentin; 5: upper chamber EDTA-treated dentin; 6: Differentiation medium

Figure 7. Gene expression patterns of *DSPP (A)*, *DMP-1 (B)*, *ALP (C)*, and Osteocalcin (D). The conditions for each group are given with each graph. Data were obtained from three separate experiments. Data are mean and SD values. *Kruskal-Wallis test, $p<0.05$.

5. Alizarin Red S staining

After 3 weeks culture, positive Alizarin Red S staining was seen in groups 3 (NaOCl-treated dentin), 4 (EDTA-treated dentin), and 6 (differentiation medium), but not in cells grown on untreated dentin and under the upper chamber containing EDTA-treated dentin. DPCs grown on EDTA-treated dentin surfaces displayed clear Alizarin Red S staining. The staining intensity was also much stronger for cells grown on EDTA-treated dentin surfaces than for those grown on other surfaces (Fig. 8) A subsequent quantitative odontogenic/osteogenesis assay was consistent with these results.

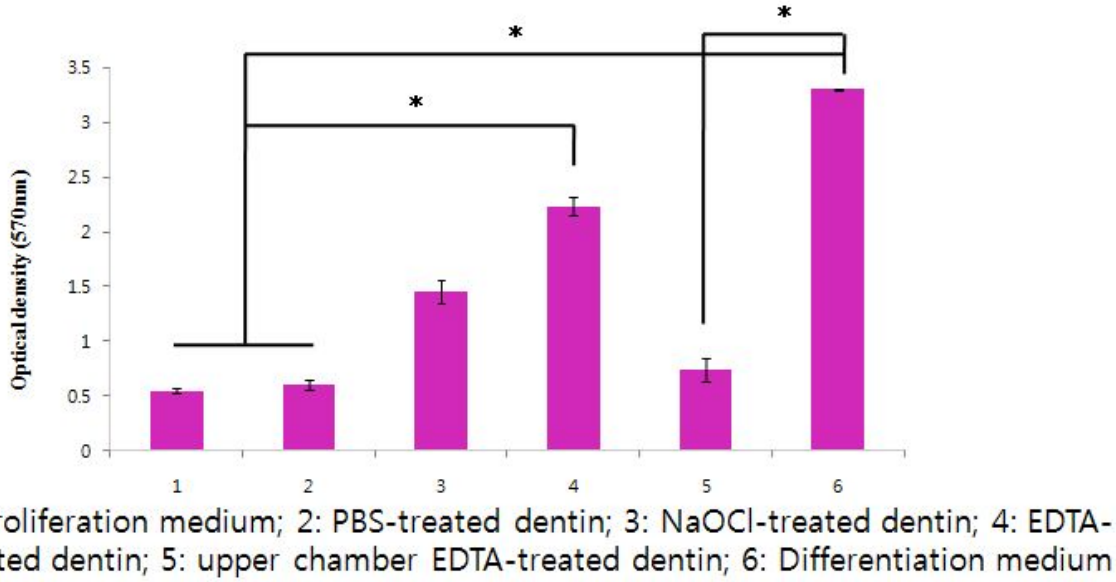


Figure 8. Alizarin Red S staining and its quantification. The staining intensity was much stronger for cells grown on EDTA-treated dentin surfaces than for those grown on other surfaces. *Kruskal-Wallis test, $p<0.05$.

5. SEM observations

After 3 weeks culture, DPCs were elongated and overlapped, forming bundles; sometimes the entire surface was rolled up after the cells had been detached in the untreated dentin group. Mineral nodules were seen on both EDTA- and NaOCl-treated dentin surfaces, even without differentiation medium.

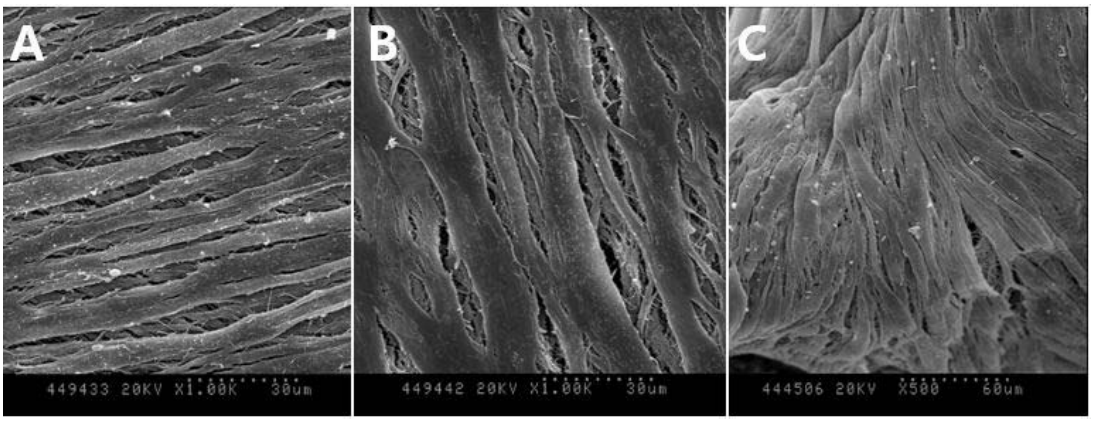


Figure 9. Scanning electron micrograph of DPCs grown on NaOCl (A) and EDTA (B, C) treated dentin surfaces (magnifications: ×1000, ×1000, and ×500, respectively).

IV. Discussion

The present study evaluated the effects of dentin application of NaOCl and EDTA on DPCs attachment and differentiation on the dentin surface. The first task was to characterize the pulp cells used in this study using growth assays and flow cytometry.

Flow cytometry analysis revealed that dental pulp stem cells isolated from third molar pulp tissue, expressed mesenchymal stem-cell surface markers such as Stro-1, CD146, CD90, and CD105, but not CD34 and CD45, which are known to be primitive hematopoietic progenitor and pan-leukocyte markers, respectively[20]. Stro-1 is a cell surface antigen that has been identified as an osteogenic precursor. CD146 is a pericyte marker, and was suggested to be a dental pulp progenitor that induces tooth repair by differentiating itself into an odontoblast in cases of dental trauma of adults[21,22]. The Stro-1 fraction was almost 6% of the total DPC population, 5–10% of DPSCs, and 18% of stem cells from the apical papilla (SCAP)[23]. Stro-1 was expressed in 13.4±2.7% of cells in this study, because the DPCs used comprised a mixture of DPSCs and SCAP.

To perform the pulp regeneration in clinical practice, stem cells should attach to the clean conditioning dentin surface, and then subsequently proliferate and differentiate into odontoblasts to produce new dentin[24]. Almost all of the previous *in vitro* studies of the management of the existing dentin surface found that cells

barely attached to chemically and mechanically treated dentin surfaces, let alone proliferated[25]. Indeed, our preliminary study revealed that following washing with sterile saline solution after application of 5.25% NaOCl or 17% EDTA, analogous to the situation in clinical practice, was not sufficient to minimize the presence of residual toxic agents. As a result, there were scarcely any living cells attached to the dentin surface. In reality, residual EDTA solution could interfere with cell attachment, not merely because of the remaining toxicity, but also because of its calcium-chelating properties[16]. In a preliminary study, the most effective method of diluting or neutralizing residual toxic irrigants was to place dentin slices in serum media for more than 24 h. The EDTA was thought to be self-limiting, and very reactive ionic groups. Once it forms a stable complex with the available ions, an equilibrium is reached and no further dissolution takes places[7]. The rich variety of proteins, electrolytes, inorganic substances, and nutrients in serum media such as FBS were considered to support the survival of cultured cells on conditioning dentin surfaces by dilution or neutralization of the chemical toxicity of irrigants and protection of living cells. Furthermore, soaking dentin slices in FBS for 24 h could keep the dentin surface and cell culture medium in equilibrium, adjusting the DPCs to their new environment. The same is true of NaOCl cytotoxicity. In clinical practice, the provocation of bleeding into the intracanal space could be considered to provide the opportunity for dilution and neutralization of toxic remnants by serum components.

The use of EDTA in regenerative endodontics as an intracanal irrigant have been supported by many previous studies showing that its chelating effect improves the release of dentin matrix components, including TGF-β, BMP-2, PDGF, and VEGF[13–15]. These growth factors are known to promote the proliferation and differentiation of stem cells[2,26]. In the present study, DPCs under a cell culture insert, in which EDTA-treated dentin slices were placed, did not show any differentiation gene expression. This finding is not consistent with the hypothesis that EDTA irrigation promotes the release of dentin-derived growth factors and cell differentiation.

However, once they are attached to the dentin surface, cells treated with NaOCl or EDTA become activated and differentiate into DPCs. In this condition, the gene expression levels of DSPP and DMP-1 were significantly higher than with nontreated dentin or where no dentin was provided. DSPP is strongly expressed in odontoblasts as mineralization marker for odontoblast/osteoblast differentiation[27]. DMP-1 is one of the dentin noncollagenous extracellular matrix proteins that is involved in the regulation of mineralization during development, and dentin repair[28,29]. DMP-1 and DSPP are expressed in functional odontoblasts at the early stage of odontogenesis[30]. Thus, this high level of expression of odontogenesis-related genes suggested that dentin treatment with EDTA or NaOCl could induce the regeneration of the pulpodentin complex by controlling the attached cell differentiation.

Stem-cell differentiation can be controlled by the biochemical environment, biomechanical environment, or likely a combination of both[31]. The biochemical environment surrounding stem cells (i.e., the characteristics of the extracellular substrate that guide cell differentiation and tissue regeneration) includes soluble growth factor, cytokines, and serum protein to induce the stem cells to proliferate and differentiate into a specific cell lineage. It was found recently that the cell–substrate interaction is crucial in controlling stem cell differentiation. The biomechanical property of the substrate is composed of substrate topography and matrix stiffness[32]. It is believed that the micro/nano structure of a substrate can alter cellular behaviors including cell orientation, adhesion, viability, morphology, motility proliferation, and even differentiation. For example, the nanograting structure can induce the up-regulation of neuronal differentiation, and the pillar/pit structure can be used for osteogenous differentiation[33,34]. The groove nanopattern can make stem cells align and elongate along the grooves, resulting in differentiation into an osteogenic lineage[35]. It is interesting that the typical topography for osteogenic differentiation is a tubular structure or a groove pattern, which is similar to the dentin structure with dentinal tubules. Meanwhile, the stiffness of the substrate can also modulate the differentiation of stem cells. The soft matrix could induce the neurogenic cell phenotype, while the stiff matrix leads to myogenic or osteogenic cell differentiation[36]. The biological mechanism underlying how stem cells can sense stiffness or topography from a substrate and

react to changes in cell phenotype is often presumed to be mechanotransduction via the integrin-focal adhesion-cytoskeleton pathway, but more research is needed to clarify this issue[34].

In the present study, NaOCl and EDTA were presumed to alter the physical properties of the dentin surface. NaOCl can remove some inorganic content as well as organic matrix, which can render the dentin surface weaker,[37,38] While EDTA can result in decalcification of the superficial dentin layer and alter the physical properties of dentin. Furthermore, removal the smear layer by EDTA changes the topography of the dentin surface, including dentinal tubule exposure.

More research is needed to determine the alteration in the properties of dentin related to cell differentiation, but findings presented here indicate that the control of PDSC differentiation by dentin conditioning will be an essential and valuable method for clinical regenerative endodontic treatment.

V. Conclusion

The aim of this study was to determine the effects of NaOCl- and EDTA-treated dentin on the attachment and differentiation of DPCs on that dentin surface. I investigated whether EDTA releases any biochemical components from dentin or changes the behavior of DPCs.

The following results were obtained:

1. When residual irrigants were removed after the dentin application of NaOCl and EDTA, DPCs attached significantly more densely onto EDTA-treated dentin surfaces.
2. The DPCs grown on EDTA- or NaOCl-treated dentin surfaces exhibited stronger expressions of DSPP and DMP-1 than those grown on untreated dentin or without dentin after 3 weeks of culture.
3. In the present study, DPCs placed under a cell culture insert, in which EDTA-treated dentin slices were placed, did not exhibit any differentiation gene expression.

Hence, the physical properties or topography of the dentin surface might be changed by either EDTA or NaOCl so as to induce cell differentiation. The treatment of the substrate (dentin) may thus be a crucial factor in the regulation of DPSC behaviors (i.e., migration, attachment, proliferation, and differentiation) in regenerative endodontic treatment.

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국문요약

상아질 표면의 근관세척제 처리가 치수세포의 부착과 분화에 미치는 영향

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목적: NaOCl 이나 EDTA 등의 근관세척제는 일반적인 근관 치료술식에서 멸균 및 세척의 효과 때문에 흔하게 사용된다. 그러나 이러한 효과 자체가 재생근관치료방법에 적용할 수 있는지에 대해서는 아직도 알려진 바가 적다. NaOCl 은 그 자체의 세포 독성 때문에 살아있는 세포나 조직에 유해하다는 보고도 있지만 재생근관치료시 선행되어야 하는 근관세정 및 소독효과 때문에 쓰지 않을 수 없는 입장이다. 반면, EDTA 는 근관내벽의 도말층을 제거할 뿐 아니라 상아질 표면의 탈회과정에서 상아질 기질 단백질 중 성장요소를 유출시킬 수도 있다는 점 때문에 사용을 권하고 있으나 구체적인 연구보고는 없다. 이번 연구는 NaOCl 과 EDTA 의 상아질 처치가 치수세포의 부착, 증식, 분화에 각각 어느 정도의 변화를 주는지 알아보고 EDTA 처치가 실제로 이런 생화학적 유도물을 통해 세포에 영향을 주는지 알아보고자 하였다.

방법: 발치된 제 3 대구치에서 치수조직을 채취해 일차배양하고 줄기세포여부를 유세포분석을 통해 확인하였다. 제 3 대구치의 치관부를 1mm 두께로 절삭하여 상아질 시편을 각각 NaOCl 30 분, EDTA 1 분 처리하고 PBS 세척, FBS 24 시간 세척 후 치수세포를 그 위에서 3 일, 3 주간 배양하여 세포부착정도와 세포분화여부를 정량적 역전사 중합효소 연쇄반응을 통해 알아보았고 경조직 형성정도를 Alizarin red 염색과 전자현미경으로 확인하였다.

결과: 세포부착정도가 EDTA 처리군에서 유의하게 증가하였으며 NaOCl 도 처리하지 않은 군만큼의 부착과 증식이 일어났다. 3 주간 증식 배양에서 세포를 배양한 결과 EDTA 와 NaOCl 처리 상아질 위에서 dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1) 유전자 발현이 유의하게 증가하였으며 이는 alizarin red 염색과 전자현미경을 통해서도 관찰되었다. 반면, EDTA 처리한 상아질 시편을 치수세포와 접촉하지 않고 배양한 군에서는 세포의 증식 외에 분화와 관련된 어떤 변화도 발견되지 않았다.

결론: EDTA 처리로 상아질 내부로부터 유출된 상아질 기질 물질이 치수세포의 분화를 유도하는 현상은 나타나지 않았다. 따라서 EDTA 나 NaOCl 의 상아질 처치가 상아질 표면에 야기한 형태, 기계적인 물성 변화가

상아질 표면의 기질 단백질과 관련된 생화학적 원인보다 치수줄기세포의 부착과 분화에 더 영향을 주는 것을 알 수 있었다. 이번 연구를 토대로 앞으로 세포부착 기질, 특히 상아질의 처치를 통해 세포의 분화를 유도하고 분화방향을 조절하는 방법에 관한 많은 연구가 이어진다면 재생근관치료영역의 발전에 도움을 줄 것으로 사료된다.

핵심되는 말: 재생근관치료, 치수줄기세포, 세포 부착, 세포 분화, 근관세척제, NaOCl, EDTA, 세포-기질 상호작용