Calcium hydroxide increases the attachment and the mineralization gene expression of dental pulp stem cells on the dentin treated by sodium hypochlorite

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The Doctoral Dissertation
submitted to the Department of Dentistry,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Dental Science

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June 2014

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June 2014

ACKNOWLEDGEMENTS

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2014 년 6 월
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Abstract

Calcium hydroxide increases the attachment and the mineralization gene expression of dental pulp stem cells on the dentin treated by sodium hypochlorite

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(Directed by Professor Il Young Jung)

Sodium hypochlorite (NaOCl) is an excellent bactericidal agent, but it has a profoundly detrimental effect on stem cell survival, attachment and differentiation. Intracanal medicaments can also affect the survival and differentiation of the dental pulp stem cells (DPSCs). Recent studies found that $Ca(OH)_2$ promoted greater survival and proliferation of stem cells from apical papilla (SCAP). The purpose of this study is to evaluate the effect of the sequential use of NaOCl and $Ca(OH)_2$ on the attachment and differentiation of DPSCs. Addtionally, to further investigate the optimal protocols to reduce the cytotoxicity of NaOCl on DPSCs.

Human DPSCs were obtained from human third molars. Dentin specimens were sterilized by ethylene oxide gas sterilization and an EDTA

treatment to produce specimens without a smear layer produced during the preparation of the dentin specimen. Group 1 was treated by NaOCl. Groups 2, 3, 4, 5, which underwent other treatment processes after the NaOCl and $Ca(OH)_2$ treatment. Group 2, for which NaOCl and $Ca(OH)_2$ treatments were followed by PBS washing. Group 3, for which NaOCl and $Ca(OH)_2$ treatments were followed by EDTA. Group 4, for which NaOCl and $Ca(OH)_2$ treatments were followed by EDTA and culture media for 24 hours. Group 5, for which NaOCl and $Ca(OH)_2$ treatments were followed by instrumentation and EDTA. DPSCs morphology was observed by SEM after 7 days of culture. A MTT assay was performed to assess the cell survival rate by group after the treatment. After 4 days of culture, gene expression level of cell adhesion was measured. After 4 weeks of culture, gene expression level of odontogenic differentiation by quantitative real-time polymerase chain reaction was investigated.

The DPSCs in the Group 1 were not attached, but the cells in the Groups 2, 3, 4, 5 were attached to the dentin surface. The cell viability in the Groups 2, 3, 4, 5 was lower than control group which cells were grown in plates treated with PBS alone. The Fibronectin-1 (FN-1) and Secreted phosphoprotein-1 (SPP-1) gene expression level was significantly higher in Groups 3, 4, 5 than Group 2. The gene expression level of Dentin matrix protein-1 (DMP-1) was significantly higher in the Groups 2, 3, 4, 5 than control. The Dentin sialophosphoprotein (DSPP) level was significantly higher in the Groups 3, 4, 5 than control. But, the DMP-1 and DSPP level was not significantly different between Group 4 and Group 5.

In conclusion, application of $Ca(OH)_2$ promoted the attachment and differentiation of DPSCs. After treatment of $Ca(OH)_2$, additional treatment such as EDTA or instrumentation enhanced the attachment and differentiation of DPSCs.

Keywords: regenerative endodontics, dental pulp stem cells, sodium hypochlorite, calcium hydroxide, ethylenediaminetetraacetic acid, cell attachment, cell differentiation

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I. Introduction

Conventional calcium hydroxide apexification has been performed for treating immature permanent teeth with necrotic pulp. Even though successful results have been reported, there are several drawbacks of the calcium hydroxide ($Ca(OH)_2$) such as incomplete root development, root fracture, and long application period[1]. To overcome such shortcomings,

mineral trioxide aggregate (MTA) apexification has been introduced recently, but this treatment also could not increase root development, close the apex and increase thickness of dentinal walls[1]. Thus, the $Ca(OH)_2$ or MTA apexification may be less ideal treatment for many patients, and there is a great need for alternative treatment option for treating immature permanent necrotic teeth.

Recently, revascularization has been proposed as a new alternative treatment option to treat immature permanent teeth with necrosis pulp. Many clinical case reports have shown that the revascularization promotes absence of clinical symptoms, healing of periapical lesions and increases of dentinal wall thickness and root length as seen on radiographic evidences[2-4].

The ideal and final objective of the regenerative endodontic treatment is to complete the dental pulp regeneration. In other words, the new vital pulp tissue can regenerate in empty but infected root canal spaces, reaching the coronal pulp chamber. Therefore, the pulp regeneration is considered as an ideal treatment to keep the tooth homeostasis, protect from reinfections and fractures, and preserve tooth longevities.

To result in the successful regenerative endodontic treatments, disinfection of the infected root canal is essential. In order to disinfect the

entire root canal, proper irrigants should be selected on the basis of consideration of both antimicrobial properties and proliferative capacity of the stem cells. Sodium hypochlorite (NaOCl) is the most commonly used irrigants in the regenerative endodontic treatments[2,5,6]. NaOCl is an excellent bactericidal agent, but it has also been shown to be cytotoxic to human periodontal ligament stem cells, cultured fibroblasts, stem cells from human exfoliated deciduous teeth (SHEDs), stem cells from apical papilla (SCAP), dental pulp stem cells (DPSCs) that it has a profoundly detrimental effect on stem cell survival, attachment and differentiation[7-11]. Ring et al reported that NaOCl is the most toxic treatment to DPSCs, and Martin et al demonstrated that 6% NaOCl greatly decreased the SCAP survival and completely abolished DSPP expression[10]. Also, Galler et al found that in the dentin treated with NaOCl, resorption of lacunae was found at the cell-dentin interface created by multinucleated cells with clastic activity[12]. Both in vitro and in vivo study show that NaOCl definitely has a negative effect on DPSCs.

Chlorhexidine (CHX) is widely used as an endodontic irrigant for a substitute of NaOCl. CHX has shown a wide range of activity against gram positive bacteria, gram negative bacteria, bacterial spores, lipophilic viruses, yeasts[13]. CHX has demonstrated its cytotoxic potential such as

inhibition of protein synthesis, induction of apoptosis, and inhibition of DNA synthesis[14]. Lessa et al reported that all concentrations of CHX had a high direct cytotoxic effect to cultured odontoblast-like cells (MDPC-23)[15]. Trevino et al also demonstrated that EDTA combination with CHX groups showed no viable cells[11]. These results showed that CHX's potential cytotoxicity on the stem cells.

Currently, there is not any irrigant, which is as effective as NaOCl but less cytotoxic than NaOCl at the same time. Therefore, we cannot avoid using NaOCl. Previous studies have trials to neutralize the NaOCl cytotoxicity used by various irrigation protocols with different concentrations of NaOCl and neutralizing agents[6,10,11].

During the regenerative endodontic treatment, after the primary disinfection of the canals with irrigant, appropriate use of intracanal medicaments induces removal of any residual bacteria in the canals[16]. Common intracanal medicaments used in the regenerative endodontic treatments are triple antibiotic paste (TAP), double antibiotic paste (DAP), $Ca(OH)_2$, formocresol, and Augmentin[17-19]. Other than the irrigants such as NaOCl, these intracanal medicaments can also affect the survival and differentiation of the DPSCs. Recent studies investigated the effect of the intracanal medicaments on stem cells. They found that $Ca(OH)_2$ promoted

greater SCAP survival and proliferation than TAP and DAP[20,21].

Until now, there is not a known way to decrease the cytotoxicity of NaOCl. Moreover, the effect of sequential application of NaOCl and $Ca(OH)_2$ on DPSCs survival has never been investigated previously.

Therefore, the purpose of this study is to evaluate the effect of the sequential use of NaOCl and $Ca(OH)_2$ on the attachment and differentiation of DPSCs. Addtionally, to further investigate the optimal protocols to reduce the cytotoxicity of NaOCl on DPSCs.

II. Materials and Methods

1. Primary human dental pulp stem cells culture

Protocol of primary human DPSCs culture was directly imported from an earlier study[22]. Human third molars were collected from young adults (16-22 years of age) at the Department of Advanced General Dentistry, Yonsei University Dental Hospital. The pulp tissue was separated from the apex of the extracted third molars with a barbed broach and was then cut into 1㎟ blocks and placed in 60㎜ culture dishes (BD Falcon, Franklin Lakes, NJ, USA) with a counting chamber cover glass (Marienfeld-Superior, Lauda-Königshofen, Germany) to allow for the outgrowth of cells. The tissues were cultured in α-modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM/L glutamine, 100 μM/L ascorbic acid-2-phosphate (WAKO, Tokyo, Japan), 100 U/㎖ penicillin, and 100 ㎍/㎖ streptomycin (Biofluids, Rockville, MD, USA) at 37℃ in 5% CO_2. The outgrowth cells were transferred to 5 X 10㎝ culture flasks (passage 1) and grown to confluence. The obtained cells were harvested and kept frozen in liquid N_2. Cells from the passage 3 were used in this experiment.

A. B.

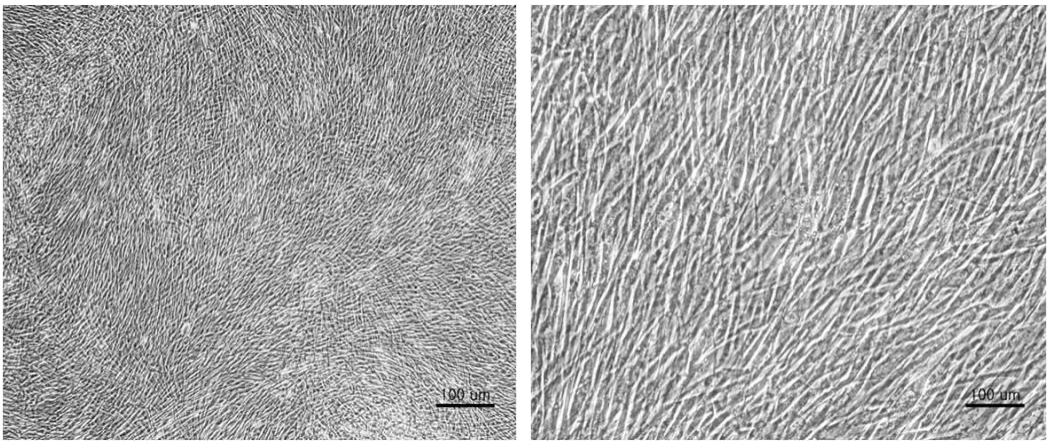

Figure 1. Morphology of DPSCs obtained from human third molars. The DPSCs showed elongated, spindle shaped and had typical fibroblast-like cell morphology. (A) magnification x100, (B) magnification x200

2. Preparation of dentin slices

The dentin slices were prepared from the human third molars. The coronal dentin was cut into a disc shape approximately 1㎜ thick by low speed diamond cutter RB205 Metsaw-LS™ (R&B Inc., Daejeon, Korea) under sterile phosphate buffered saline irrigation (Mediatech Inc., Manassas, VA, USA). The dentin slices were sterilized by ethylene oxide gas sterilization.

3. Cell morphology analysis by Scanning Electron Microscope

After 7 days of culture, one dentin slice from each group was selected randomly. And then they were washed 3 times with PBS followed by fixed in 2% glutaraldehyde for 5 minutes. The slices were dehydrated in a graded series of ethanol and dried with hexamethyldisilane. After gold coating, cell morphology was assessed in each group with a scanning electron microscope (JEOL JSM-820; JEOL, Tokyo, Japan).

4. Adhesion analysis

A. MTT assay (Tetrazolium-based colorimetric assay)

The dentin slices were randomly assigned to the following groups (Figure 2). All dentin slices were 5 times wash-out with PBS and placed in the 100㎜ x 15㎜ sterile Nunclon® cell culture dishes (NUNC™, Roskilde, Denmark) in single layer. Harvested cells were seeded onto the dentin specimens ($1x10^6$ cells/plate) and cultured in normal growth medium for 7 days. At the end of the culture period, the dentin slices were removed and placed in new Nunclon cell culture dishes. Cells were detached with 0.25% trypsin/EDTA, and 2 minutes incubation in Thermo® Steri-Cycle CO_2 Incubators (Forma Scientific Inc., Mariotla, Ohio, USA) at 37℃ in 5% CO_2 for trypsin activation. After 5 minutes centrifugation was performed, the cells were placed in new plates and then MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay was performed for assessing cell viability. The absorbance at 570nm was measured using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

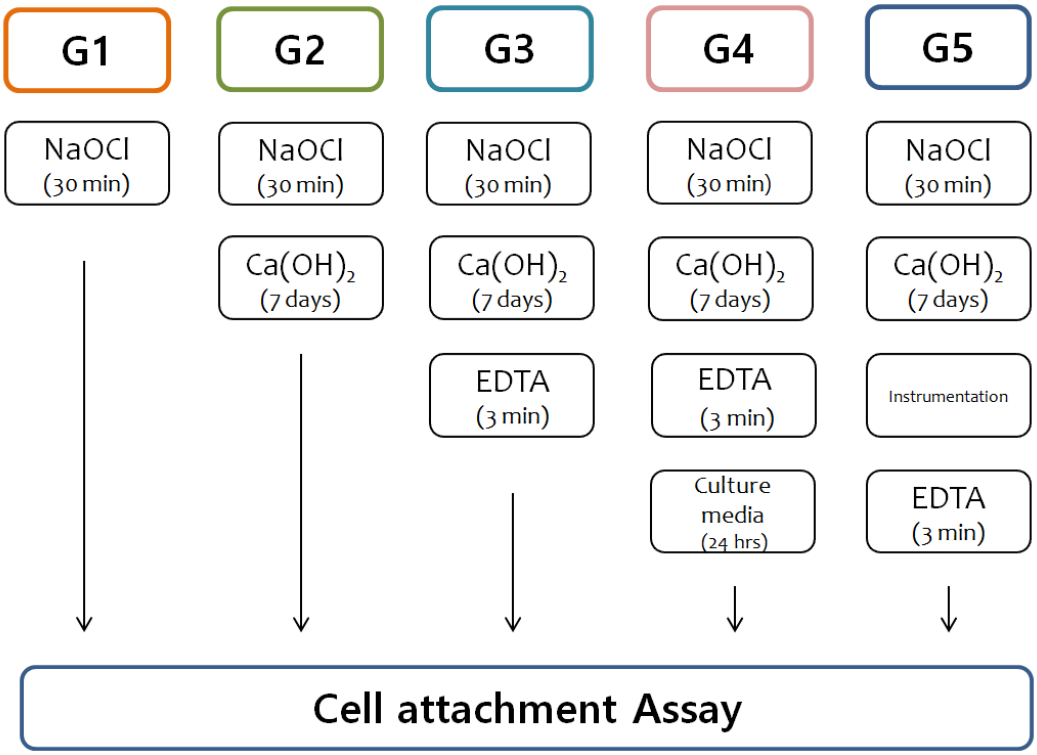

Figure 2. Experimental groups for attachment and differentiation of DPSCs.

A flowchart showing the steps for group categorization. Group 1 was treated by NaOCl. Group 2, for which NaOCl and Ca(OH)2 treatments were followed by PBS washing. Group 3, for which NaOCl and Ca(OH)2 treatments were followed by EDTA. Group 4, for which NaOCl and Ca(OH)2 treatments were followed by EDTA and culture media for 24 hours. Group 5, for which NaOCl and Ca(OH)2 treatments were followed by instrumentation and EDTA.

B. Cell attachment analysis by Quantitative Real-time Polymerase Chain Reaction

Cell attachment was evaluated based on the expression level of Fibronectin-1 (FN-1) and Secreted phosphoprotein-1 (SPP-1). Total cellular RNA was extracted from DPSCs in each group using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The RNA was treated with the RNase-free DNase-set (Qiagen) during the RNA extraction. The Complementary DNA samples were prepared from the isolated RNA using the RT First Strand Kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-qPCR) analysis was performed using ABI 7500 software (Applied Biosystems, Foster City, CA, USA) according to the standard protocol. The real-time polymerase chain reaction cycles included 40 cycles of general denaturation at 94℃ (30 seconds), annealing, and elongation at 60℃ (45 seconds), except for the first cycle with a 15-minute denaturation and the last cycle with a 7-minute elongation at 72℃. The primers used for assessing the FN-1, SPP-1 expression level are shown in Table 1.

Table 1. PCR Primer used for cell adhesion markers

Gene name	Direction	Primer Sequence
Fibronectin-1	Forward	TCACAGACAGTGGTGTGGTC
	Reverse	TCCTGCCCATTGTAGGTGAAT
Secreted phopsphoprotein-1	Forward	CAGCAACCGAAGTTTTCACT
	Reverse	GCATCAGGGTACTGGATGTC

5. Cell differentiation analysis by Quantitative Real-time Polymerase Chain Reaction

To compare differential gene expressions under various conditions, RT-qPCR analysis of the mineralization-related genes was performed. The primers for the differentiation markers of mineralization are given in Table 2. All DPSCs were cultured in vitro at 37℃ in a humidified atmosphere of 5% CO_2 for 4 weeks. The culture medium in all groups was replenished every 3 days. The procedures were the same as those for the cell attachment assay.

6. Statistical Analysis

Mann-Whitney U tests was used to determine the statistical differences between the experimental groups by using SPSS software version 21.0 (SPSS, Chicago, IL, USA). Adjusted p value less than 0.05 was considered to be statistically significant.

Table 2. PCR Primer used for odontogenic differentiation markers

Gene name	Direction	Primer Sequence
Dentin matrix protein-1	Forward	CCCAAGATACCACCAGTGAG
	Reverse	CACCCAGTGCTCTTCACTCT
Dentin sialophosphoprotein	Forward	TTAAATGCCAGTGGAACCAT
	Reverse	ATTCCCTTCTCCCTTGTGAC

III. Results

1. Cell morphology analysis by Scanning Electron Microscope

After 7 days of culture, the DPSCs in the Group 1 were not attached the dentin surfaces (Figure 3A). This result was consistent with preliminary studies. However, the cells in the Groups 2, 3, 4, 5 were attached to the dentin surface. Especially, Dentin surface was overlapped by proliferating cell layers in Group 5 (Figure 3E).

2. Adhesion analysis

A. MTT assay (Tetrazolium-based colorimetric assay)

For Group 1, the SEM images confirmed that there were few if any living cells. Thus, this group was exempt from the MTT assay (Figure 4). The cell viability of Groups 2, 3, 4, 5 was significantly lower than control group (p=0.037). The cell viability between the Groups 2, 3, 4, 5 was not significantly different (p>.05).

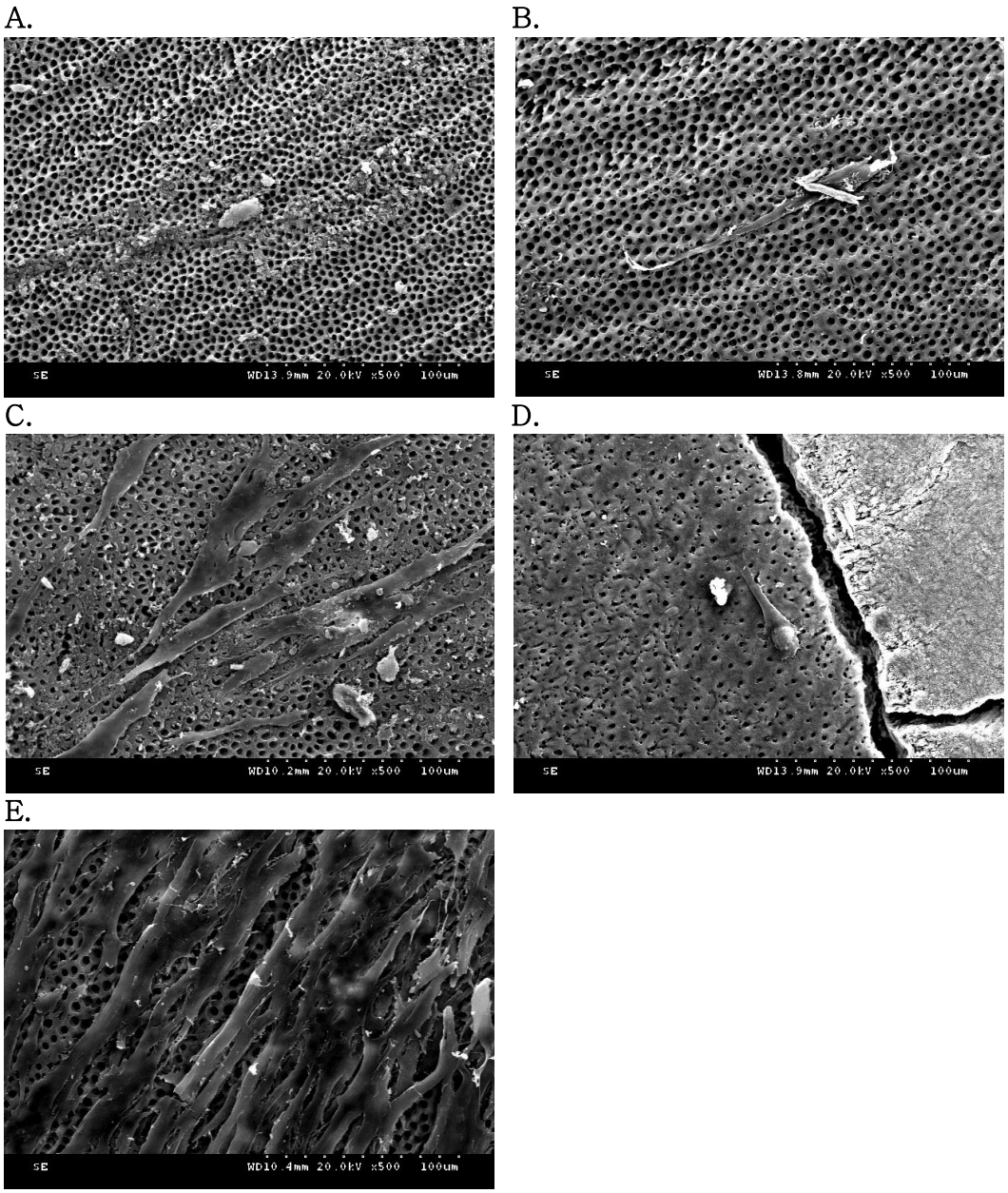

Figure 3. SEM views of DPSCs morphology in dentin surface. The denser cell attachment and proliferation was shown on the $Ca(OH)_2$ treated dentin. (A) Group 1, (B) Group 2, (C) Group 3, (D) Group 4, (E) Group 5 (magnification x500)

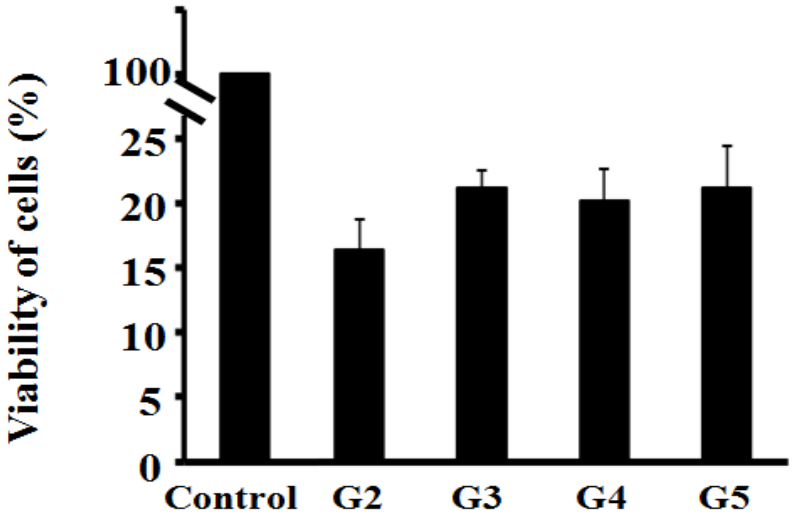

Figure 4. Cell viability in the experimental groups. $Ca(OH)_2$ showed positive effects on cell survival. Cells were cultured for 7 days after the treatment, and the number of attached and viable cells was quantified with MTT assay. Cells in the control were grown in plates.

B. Quantitative Real-time Polymerase Chain Reaction for cell attachment Analysis

For Group 1 was exempt from the cell attachment RT-qPCR process similar to MTT assay. The Fibronectin-1 (FN-1), Secreted phosphoprotein-1 (SPP-1) gene expression levels of Groups 2, 3, 4, 5 were compared after 4 days of culture (Figure 5). The FN-1 and SPP-1 gene expression level was significantly higher in Groups 3, 4, 5 than Group 2 (p=0.037).

3. Quantitative Real-time Polymerase Chain Reaction for cell differentiation Analysis

Group 1, which rarely had living cells, was not included. The Dentin matrix protein-1 (DMP-1) and Dentin sialophosphoprotein (DSPP) gene expression level was compared between the groups after 4 weeks of culture (Figure 6). The DMP-1 level was significantly higher in the Groups 2, 3, 4, 5 than control (p<.05). The DSPP level was significantly higher in the Groups 3, 4, 5 than control (p<.05). But, the DMP-1 and DSPP level was not significantly different between Group 4 and Group 5 (p=0.05)

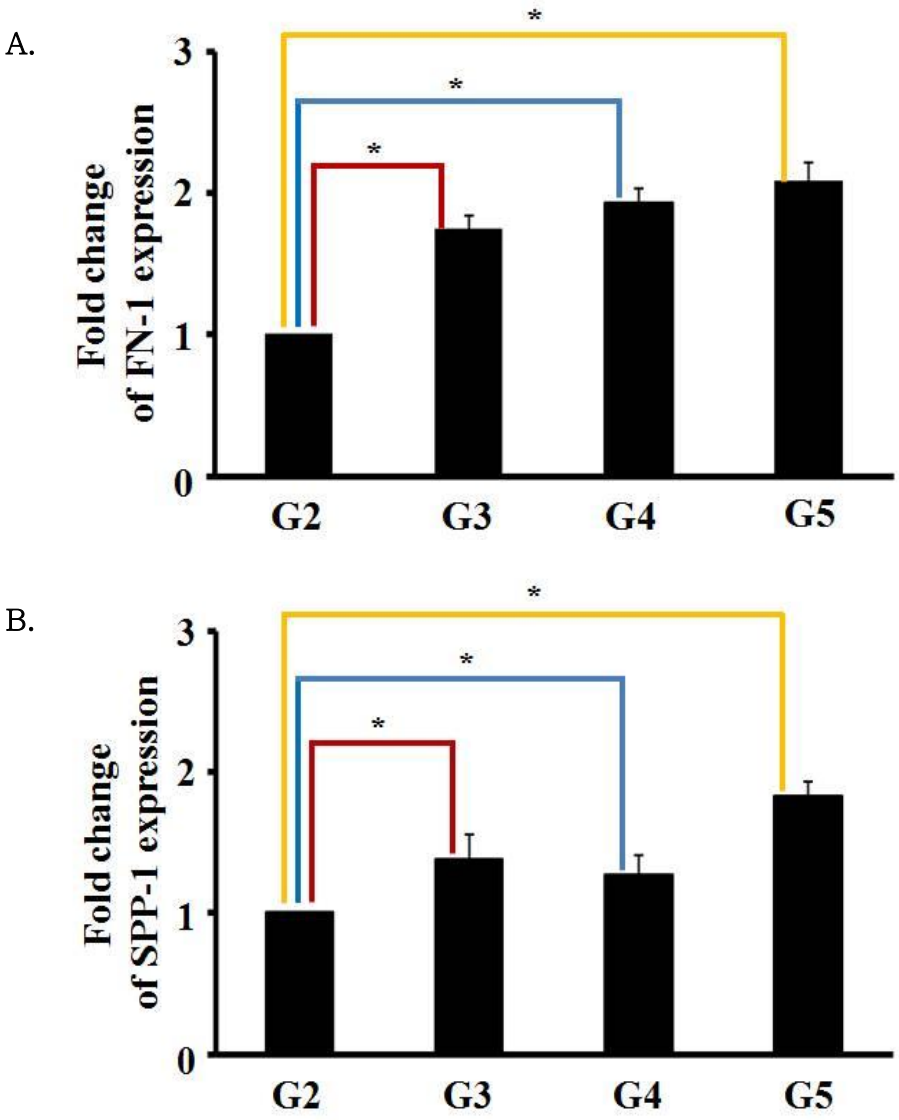

Figure 5. Expression levels of adhesion molecules. Relative expression patterns of (A) Fibronectin-1 (FN-1), (B) Secreted phosphoprotein-1 (SPP-1) after 4 days of culture. *Mann-Whitney U test, $p<.05$

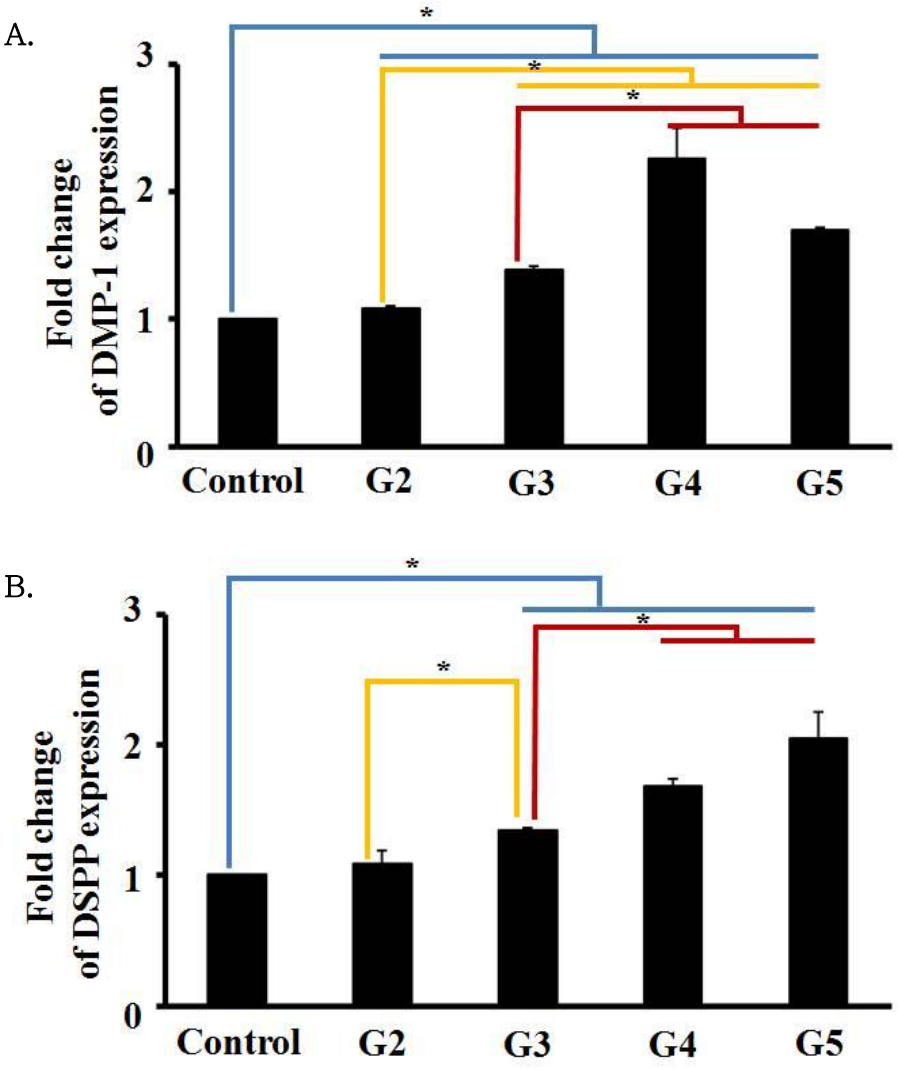

Figure 6. Expression levels of odontogenic differentiation molecules. Relative expression patterns of (A) Dentin matrix protein-1 (DMP-1), (B) Dentin sialophosphoprotein (DSPP) after 4 weeks of culture. Cells in the control were cultured in differentiation media. *Mann-Whitney U test, $p<.05$

IV. Discussion

This present study aimed to evaluate the effect of the sequential application of NaOCl and $Ca(OH)_2$ on the attachment and differentiation of DPSCs. For the purpose of this study, an experiment setting similar to a clinical trial was planned. Regenerative endodontics in clinical practice does not produce a smear layer, as it minimizes instrumentation within the root canal to protect the thin root dentin wall of an immature permanent tooth. The smear layer is 1-5㎛-thick denatured cutting debris produced on instrumented dentin surfaces. It is composed of dentin, odontoblastic processes, nonspecific inorganic contaminants and microorganisms[23-25]. The existence of a smear layer in regenerative endodontics is a critical factor which may be responsible for the failure of the surgery, as it interrupts the adhesion to the DPSCs[26]. Therefore, unlike previous research, this study involved the sterilization of dentin specimens with ethylene oxide gas and an EDTA treatment to produce specimens without a smear layer produced during the preparation of the dentin specimen[22]. The $Ca(OH)_2$ concentration was 1mg/㎖, a level at which the survival rate of SCAP was significantly higher according to Ruparel et al[20].

The first step in regeneration is to obtain proper stem cells. There were many previous studies to describe the characteristics of the DPSCs. It has been shown that DPSCs have a self-renewal potential and multilineage differentiation ability into odontoblasts, osteoblasts, adipocytes, chodrocytes, neurons[27-29]. Canine dental pulp stem cells from dog teeth (cDPSCs) showed superiority in the proliferation rate and multilineage differentiation over human bone marrow mesenchymal stem cells (hBMMSCs), but neurogenic differentiation ability was inferior than human dental pulp stem cells (hDPSCs). Also there is no evidence that cDPSCs could produce the uniform dentin[30]. The difference between SHEDs and adult DPSCs was that SHEDs were able to induce bone-like structure when implanted into immunocompromised mice subcutaneously, whereas DPSCs generated a dentin-like, pulp-like tissue[31]. Therefore, the cell sources used in this study were young adults' DPSCs that isolated from extracted pulps of human third molars.

First, The SEM images showed cells in Groups 2, 3, 4, 5 successfully adhering, as opposed to those in Group 1, which failed. In 7 days, it was confirmed that the cells had stretched and had long cytoplastic processes. Primarily, the $Ca(OH)_2$ treatment enhanced cell adhesion and proliferation. Secondarily, culture media, EDTA and instrumentation used during the

removal of $Ca(OH)_2$ changed the properties of the dentin surface, thereby creating an environment which facilitates better DPSCs adhesion.

A MTT assay was performed to assess the cell survival rate by group after the treatment. In the MTT assay, cell viability does not simply account for living cells in a plate. Rather, it evaluates living cells that are attached to the dentin specimens. Considering Groups 2, 3, 4, 5, which underwent other treatment processes after the $Ca(OH)_2$ treatment, which is believed to show positive effects of $Ca(OH)_2$ on cell survival. The interpretation here matched that of the SEM images. A high alkaline pH level of $Ca(OH)_2$ encourages the survival, migration and proliferation of pulp stem cells, helping the formation of reparative dentin[20,21,32]. Therefore, it is understood that $Ca(OH)_2$ plays a decisive role in boosting cell viability.

Next, cell attachment was assessed by means of the adhesion molecule RT-qPCR in response to Fibronectin-1 (FN-1) and Secreted phosphoprotein-1 (SPP-1). FN-1 is an extracellular matrix molecule important in cell adhesion[33]. SPP-1 is stored in bone matrix as one of the abundant noncollageous bone matrix proteins and plays a role in bone-cell attachment[34]. Group 2, for which NaOCl and $Ca(OH)_2$ treatments were followed by PBS washing, was chosen as a control for Groups 3, 4, 5. The results demonstrated a significant difference between the Group 2 and

Groups 3, 4, 5. The high expression of FN-1 and SPP-1 means that cell attachment has been also increased. The significant difference between Group 2 and 3 indicates that EDTA additionally decalcifies the dentin surface and increases the wettability. EDTA plays a role in cell adhesion and odontoblast differentiation by causing changes in the dentin surfaces of the specimens[22]. This is similar to a previous research which showed changes in the dentin surfaces caused by the chelating effect of EDTA[35]. The results for Groups 2 and 4 illustrate that EDTA initially created an environment for cells to adhere successfully, after which culture media including FBS neutralized the cell toxicity of NaOCl, thus increasing cell adhesion. Fetal bovine serum (Gibco, Tulsa, OK, USA) is a serum media rich in protein, electrolytes, inorganic substances, and nutrients. It dilutes or neutralizes the toxicity irrigants and helps protect living cells[36]. The use of instrumentation, as shown in Groups 2 and 5, can effectively remove any remaining $Ca(OH)_2$. Although $Ca(OH)_2$ is commonly used in various clinical situations as an antiseptic for root canal treatment, imperfectly removed $Ca(OH)_2$ can block the penetration of the sealer into dentinal tubules and increase apical leakage, resulting in a failed root canal treatment[37]. Therefore, $Ca(OH)_2$ applied to the root canal should be thoroughly removed to completely seal it off. An experiment by Kenee et

al. in which rotary and ultrasonic instrumentation techniques were more effective in removing $Ca(OH)_2$ than an instrument with an irrigant was considered. Thus, the instrumentation was standardized at the #40/.04 taper profile at 300rpm and scraping of the entire dentin surfaces was performed once[38]. The removal of $Ca(OH)_2$ by instrumentation was followed by the decalcification of the dentin surfaces using EDTA, which caused the exposure of the dentinal tubule and collagen fiber, creating an environment in which stem cells could easily adhere.

For the cell differentiation RT-qPCR analysis, the control group underwent a cell culture assay in differentiation media without dentin samples. Dentin matrix protein-1 (DMP-1), an essential noncollagenous and acidic phosphorylated extracellular matrix protein, is highly expressed in odontoblast. DMP-1 has been shown to play a prime role in dentin mineralization[39]. Dentin sialophosphoprotein (DSPP) is the only protein produced uniquely by odontoblasts. DSPP plays an important role in the regulation of mineral deposition[40]. As a result, DMP-1 was expressed at higher levels in Groups 2, 3, 4, 5 than the control group, while DSPP showed higher levels in Groups 3, 4, 5. The significantly higher DMP-1 and DSPP levels expressed as compared to the control group can be interpreted as showing that after the NaOCl treatment, the $Ca(OH)_2$

treatment triggered odontogenesis, leading to odontoblast differentiation. In particular, the lack of a significant difference between Group 4 and Group 5 demonstrates that there is no difference between the technique in which culture media is used for 24 hours as a neutralizing treatment agent for NaOCl and the technique in which instrumentation is performed followed by a NaOCl and $Ca(OH)_2$ treatment. In other words, creating an environment to facilitate better stem cell attachment and differentiation in the root canal through instrumentation and an EDTA treatment is more effective than the cumbersome process of using culture media for 24 hours as a neutralizing treatment agent for NaOCl.

In this study, a $Ca(OH)_2$ treatment was shown to improve the reduced survival and attachment of cells after a NaOCl treatment. The $Ca(OH)_2$ treatment is believed to have caused a favorable change in cell attachment and differentiation on the dentin surfaces. Possible mechanisms include that $Ca(OH)_2$ exposes the collagen fiber on the dentin surfaces, allowing better cell attachment[41], and $Ca(OH)_2$ releases a growth factor from dentin and enhances cell attachment and differentiation[42]. If this could lead to the improvement of biomechanical and biochemical environments, the survival and differentiation of stem cells are expected to increase.

Numerous studies of revascularization were clinical case reports, with animal studies showing that newly formed tissues into the root canal space have little similarity to normal pulp tissue but with more cementum-like, periodontal ligament-like, and bone-like tissue[43,44]. Recently, some interesting studies have tried to use tissue engineering technique in pulp regeneration. Huang et al reported that vascularized pulp-like tissues and dentin-like mineral structures were formed in the root canal space by containing synthetic scaffolds seeded with stem/progenitor cells from apical papilla and dental pulp[45]. Iohara et al demonstrated complete pulp regeneration in the canine model after pulpectomy by transplantation of $CD105^{+}$ with stromal cell-derived factor-1[46]. Wang et al achieved that autologous cDPSCs combined with Gelfoam transplanted into pulpless root canals led to generate pulp-like tissues containing blood vessels and dentin-like tissues and thickening of the root canal wall was also observed[47].

This present research is an in vitro study which takes place immediately before a transplantation experiment, a practice currently in full swing targeting large animals for the complete regeneration of pulp. It can be used as the basis for in vivo experiments.

Regenerative endodontics in clinical practice involves intracanal medication after gradually using an irrigant in an infected root canal. Previous studies have performed NaOCl treatments and $Ca(OH)_2$ treatments independently. This study is significant in that it integrated both treatments for use, creating an environment closer to that of actual clinical practice. However, due to the in vitro limitations, additional in vivo studies are necessary down the road. Also, a long-term study is required, as the RT-qPCR results for the attachment factor and for the differentiation factor were measured at specific points and therefore have limitations. In addition, studies of a counteragent to neutralize the toxicity of NaOCl and research on scaffolding and growth factors for enhanced tissue engineering are needed. If the technique elaborated in this study can be successfully performed in regenerative endodontics for immature permanent teeth in clinical practice, it will be proven as applicable to endodontics for mature permanent teeth as well. Ultimately, the goal of regenerative endodontics will be met through complete pulp-dentin complex regeneration.

V. Conclusion

Based on the results of this study, the following can be concluded.

1. Application of $Ca(OH)_2$ promotes dental pulp stem cells attachment and differentiation.

2. After treatment of $Ca(OH)_2$, additional treatment such as EDTA or instrumentation enhanced the attachment and differentiation of dental pulp stem cells.

Raw data

1. MTT assay

Group	Optical Density (O.D)
Control	0.82
	0.79
	0.82
G2	0.20
	0.17
	0.17
G3	0.21
	0.22
	0.20
G4	0.23
	0.20
	0.19
G5	0.23
	0.21
	0.19

2. Adhesion molecule by RT-qPCR

Group	FN-1	SPP-1
G2	1	1
	1	1
	1	1
G3	1.83	1.06
	1.75	1.30
	1.66	1.39
G4	2.07	1.09
	1.93	1.27
	1.87	1.38
G5	2.21	1.67
	2.09	1.84
	1.95	1.84

3. Differentiation molecule by RT-qPCR

Group	DMP-1	DSPP
Control	1	1
	1	1
	1	1
G2	1.09	1
	1.09	1
	1.05	1
G3	1.35	1.34
	1.39	1.34
	1.39	1.31
G4	2	1.6
	2.26	1.71
	2.48	1.69
G5	1.71	2.06
	1.72	2.11
	1.68	1.74

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Abstract (In Korean)

NaOCl 로 처리한 상아질에서 수산화칼슘 처치로 인한 치수줄기세포의 부착과 경조직 형성 유전자의 발현증가

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박 민 정

Sodium hypocholorite (NaOCl)는 재생근관치료에서 근관 내 소독을 위해 사용하는 세척용액으로 우수한 항균 효과를 갖고 있지만 줄기세포의 생존을 방해한다. 세척제와 더불어 근관 내 첩약제도 재생근관치료에 사용되는데, 특히 Calcium hydroxide ($Ca(OH)_2$)는 줄기세포의 생존과 증식에 도움이 되는 것으로 알려져 있다. 아직까지 NaOCl 의 부정적인 효과를 줄이는 방법은 아직 알려지지 않았고 NaOCl 과 $Ca(OH)_2$ 를 연이어 사용한 후 평가한 연구는 없다. 따라서 본 연구에서는 임상환경과 유사하게 상아질 시편에 NaOCl 과 $Ca(OH)_2$ 를 순차적으로 사용한 후 NaOCl 의 치수줄기세포에 대한 부정적인 효과를 줄이는 방법을 찾아보고자 한다.

발치된 제 3 대구치에서 치수조직을 채취해 일차배양을 시행하여 줄기세포를 준비하였다. 발치된 대구치의 치관부를 1mm 두께로 절삭하여 상아질 시편을 만들고, 임의로 5 개 군으로 나눈 후 다른 처치를 하였다. 이전 연구와는 달리 Ethylene oxide gas 로 상아질 시편을 멸균하고 EDTA 3 분 처리를 하여 시편제작과정 중에 발생한 도말층을 제거하였다. 먼저 모든

그룹은 NaOCl 30 분 처리하였다. 그룹 1 은 NaOCl 처리만 하였고 그룹 2, 3, 4, 5 는 추가로 $Ca(OH)_2$ 를 일주일 동안 처치하였다. 그룹 2 는 $Ca(OH)_2$ 처치까지 한 군이고 그룹 3 은 추가로 EDTA 처리한 군, 그룹 4 는 EDTA 처리하고 culture media 24 시간 처리한 군, 그룹 5 는 기구조작하고 EDTA 처리한 군이다. DPSCs 의 생존과 부착은 MTT assay 로 정량화하였고 상아질 시편에 부착되어 있는 세포의 모습은 전자현미경을 통해 관찰하였다. 그리고 4 일, 4 주간 배양하여 세포부착과 경조직 형성 세포로의 분화와 관련된 유전자의 발현양상을 정량적 실시간 중합효소 연쇄반응으로 분석하였다.

전자현미경 사진에서는 cell 이 부착하지 못한 그룹 1 에 비해 그룹 2, 3, 4, 5 는 7 일 뒤 cell 이 붙었고 성장해 나간 것을 확인할 수 있었다. MTT 실험 결과 그룹 2, 3, 4, 5 는 대조군에 비해 세포생존력이 낮았으나, 그룹 간의 유의차는 없었다. 세포부착 유전자의 발현결과 Fibronectin-1 (FN-1) 과 Secreted phosphoprotein-1 (SPP-1)은 그룹 2 에 비해 그룹 3, 4, 5 가 모두 유의하게 증가하였다. 세포분화 유전자의 발현결과 Dentin matrix protein-1 (DMP-1)은 대조군보다 그룹 2, 3, 4, 5 가 높게 발현되었고 Dentin sialophosphoprotein (DSPP)은 그룹 3, 4, 5 가 높게 발현되었다. DMP-1 과 DSPP 는 그룹 4, 5 간의 유의차는 없었다.

결론적으로 $Ca(OH)_2$ 처치과정은 NaOCl 로 처리한 상아질 시편에서 치수줄기세포의 부착과 분화가 일어날 수 있도록 도움을 준다. 또한 $Ca(OH)_2$ 처치 후 추가적으로 EDTA 처리나 기구조작을 통해 상아질 시편을 처리하면 치수줄기세포의 부착과 분화가 증진된다.

핵심되는 말: 재생근관치료, 치수줄기세포, sodium hypochlorite, calcium hydroxide, 세포부착, 세포분화