Pulpal response of three calcium silicate - based cements in dog's pulpotomy model

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Pulpal response of three calcium silicate - based cements in dog's pulpotomy model

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A Dissertation Thesis

Submitted to the Department of Dentistry

and the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Dental Science

Hwang, Ji-Won

December 2017

연세대학교
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감사의 글

먼저 이 논문이 나올 때까지 도와주신 모든 분들께 감사 드립니다.

애정을 갖고 열성으로 지도해주신 송제선 교수님, 신유석 교수님께 진심으로 감사 드리며 실험 및 데이터 정리에 도움을 주신 강정민 선생님, 전미정 선생님께도 감사의 말씀 드립니다.

특히 논문을 심사해주시고 많은 조언을 해주신 최형준 교수님, 이제호 교수님 항상 감사 드립니다.

또 지금은 은퇴하신 저의 전 지도교수님이신 손흥규 교수님께 감사 드리며 학교에 갈 때마다 많은 격려를 해주신 최병재 교수님과 김성오 교수님께도 깊은 감사 드립니다.

논문 작성에 많은 도움을 주신 후배 이혜원 선생님과 김별이라 선생님께도 감사의 인사를 전하며, 따뜻한 격려와 조언을 해주신 원광대 안소연 교수님께도 감사 드립니다. 하나님과 저희 부모님, 시어머님, 남편과 아들을 비롯한 가족 여러분께도 감사 드리며, 백미슬 선생님을 비롯한 병원 식구들께도 감사의 말씀을 전합니다.

개원의와 주부, 엄마로서 바쁜 삶을 살며 자칫 포기할까 했던 박사 과정을 많은 분들의 도움과 격려로 포기하지 않고 9년만에 무사히 마치게 되어 더욱 더 감격스럽습니다.

논문을 마치고 나니 속이 시원하긴 하지만 한편으로는 좀 더 일찍 시간을 투자하여 잘 쓰고 빨리 끝낼 걸 하는 아쉬움만이 가득합니다.

게으른 저를 채찍질하여 이끌고 와주신 송제선 교수님, 신유석 교수님께 다시 한번 감사 드리며 이 논문을 제가 이 학위를 받기까지 도움을 주신 많은 분들께 바칩니다.

저 자 씀

Table of Contents

List of Figures

List of Tables

Abstract

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(Directed by Professor Je Seon Song, D.D.S., M.S., Ph.D.)

This study was conducted to compare histologic responses to different calcium silicate based cements, ProRoot MTA®, Ortho MTA® and Endocem MTA® in beagle dog's pulpotomy models. Full pulpotomies were performed on beagle dog's 44 teeth. The exposed pulp tissues were randomly covered with ProRoot MTA® (n=15), Ortho MTA® (n=18) or Endocem MTA® (n=11). The teeth were extracted and processed for histological and immunohistochemical examinations using osteocalcin and dentin sialoprotein. Calcific barrier formation, inflammatory reaction, and the odontoblastic layer were evaluated and scored in a blind manner.

The areas of newly formed calcific barriers were measured for each group. In most of the ProRoot MTA® and Ortho MTA® specimens, continuous calcific barriers were formed and the pulps contained palisading patterns in the odontoblastic layer that were free of inflammation. However, the Endocem MTA® specimens had lower quality calcific barrier formation, higher inflammation, and less favorable odontoblastic layer formation. Ortho MTA® could provide an alternative to ProRoot MTA® . Both materials produced favorable pulpal responses that were similar, whereas Endocem MTA® produced less favorable pulpal responses.

Key words: Calcium silicate based cements; ProRoot MTA; Ortho MTA; Endocem MTA; pulpotomy, pulpal response; calcific barrier; inflammation; odontoblastic layer

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I. Introduction

Vital pulp therapy (VPT) is a biological and conservative therapy to maintain pulp vitality and function of the remaining crown and root in permanent vital teeth that still have vitality. In VPT, an external local source of inflammation is removed and a pulp protecting agent is placed directly or indirectly on top of the pulp (Hargreaves et al., 2011). This therapy requires a dental restoration that closely seals so that no germ can penetrate the interface between the dentin and the restoration. Calcium hydroxide-based materials have been widely used as a pulp protecting agent for VPT because they induce hard tissues on the upper part of the pulp and generate the reparative dentin. However, calcium hydroxide has a relatively low success rate because of creating a thin calcific barrier and many

tunnels to use as a VPT material. Currently, the mineral trioxide aggregate (MTA) is frequently used because it causes cellular differentiation in odontoblast-like cells, and thus results in molecular and cellular healing that is more appropriate for healing in hard tissues (Tziafas et al., 2002). Accordingly, MTA is gaining attention as a substitute for various calcium hydroxide-based materials, and better clinical and experimental outcomes have been reported using MTA (Queiroz et al., 2005).

The MTA, which is a Portland cement-derived calcium silicate based cement, was developed at Loma Linda University in the 1990s, and was approved by the US Food and Drug Administration (FDA) in 1998 (Torabinejad et al., 1993). MTA is used widely in clinical practice for root canal filling, pulp capping, pulpotomy, restoration of root perforation, formation of the root apex, apical retrofilling, and external resorption therapy (Torabinejad and Chivian, 1999). According to many clinical trials, the success rate of short-term and long-term VPT using MTA is reported to exceed 90% and 85%, respectively (Fuks, 2008; Witherspoon, 2008). Furthermore, MTA has been known as a biocompatible substance that induces the formation of the dentin both *in vivo* and *in vitro* more effectively than calcium hydroxide (Akhlaghi and khademi, 2015).

ProRoot MTA® (Dentsply, Tulsa Dental, Tulsa, OK, USA) (PMTA), which was first commercialized in 1998, contains 75% Portland cement, 20% bismuth oxide,

and 5% calcium sulfate dehydrate (gypsum). Its major components include tricalcium silicate ($3CaO \cdot SiO_2$), dicalcium silicate ($2CaO \cdot SiO_2$), and tricalcium aluminate ($3CaO \cdot Al_2O_3$) (Storm et al., 2008). Moreover, PMTA is better than other filling materials because of its great marginal sealing (O'Connor et al., 1995, Maltezos et al., 2006), bioactivity (Gandolfi et al., 2010), and antimicrobial action (Aeinehchi et al., 2003). However, PMTA also has its drawbacks. For example, it contains heavy metals (Chang et al., 2011), has a long setting time (Ber et al., 2007; AlAnezi et al., 2011), and causes tooth discoloration (Belobrov and Parashos, 2011). More importantly, problems such as changes in physical properties due to long setting time and microleakage have been reported as well (Kim and Kim, 2012). MTA based substances with improved physical properties have been developed to compensate for these drawbacks (Ber et al., 2007).

Since the development of MTA, various similar products have been developed and available in the market, and they are all collectively referred to as calcium silicate-based cements (CSC). The products developed and distributed from overseas include MTA-angelus® (Angelus, Londrina, Brasil), Bioaggregate® (Innovative Bioceramics, Canada), Micromega MTA® (Micromega, Besanchon, France), and Biodentine® (Septodont, Saint-Maur-des-fosses, France). In Korea, many companies have also created and sold a variety of MTA products, the most noticeable of which include Ortho MTA® (OMTA; BioMTA, Seoul, Korea),

developed in 2010, and Endocem MTA® (EMTA; Maruchi, Wonju, Korea), developed in 2011.

The OMTA is a bioceramic materials produced by a reagent manufacturing method and its powder is consisted of 2-μm hydrophilic particles. Its major components include tricalcium silicate, dicalcium silicate, and bismuth oxide (Bi_2O_3). Moreover, OMTA forms set colloidal gel within 5 hours in a condition where there is water or humidity. In addition, OMTA has good sealing ability, biocompatibility, odontoblastic potential (Chang et al., 2014), antimicrobial action (Kim, 2012), OMTA has almost no heavy metals hazardous to the human body (Chang et al., 2011). Nevertheless, OMTA also has a weakness, which is its long setting time (Kang, 2011).

The EMTA contains fine particles of pozzolan, which is a silicate-based substance that generates cement materials. According to the manufacturer, EMTA is a next generation MTA that uses a pozzolanic reaction, has a short setting time, operation convenience based on adequate consistency, and washout resistance (Choi et al., 2013; Jang et al., 2013), and is a product that features outstanding sealing ability and biocompatibility. Indeed, EMTA is reported to have a biocompatibility similar to that of the existing MTA, form the tertiary dentin *in vivo*, and cause almost no inflammation (Park et al., 2014). It is also reported to have improved washout resistance, and cause almost no tooth discoloration (Kang et al., 2015).

Despite the fact that various CSCs have been used increasingly in VPT, there has been no study that compared OMTA and EMTA with PMTA and examined the pulp's inflammation reaction and hard tissue formation ability histologically in an *in vivo* full pulpotomy model. In addition, most of those studies that have been conducted have a short duration for about 4 weeks; only a few long-term studies have been reported. Accordingly, the present study was conducted to evaluate and compare the levels of calcific barrier formation, inflammation reaction, and hard tissue barrier formation histologically following the application of PMTA, OMTA, and EMTA for the mid-term of 8 weeks in a dog's permanent tooth full pulpotomy model.

II. Materials and methods

1. Animal model

The present study used two male beagles, which were 18 to 24 months old, and weighed approximately 12 kg. They had non-damaged dentition and healthy periodontium. Among the teeth of the two dogs, 60 teeth (incisors, canines, and the first and second premolar teeth in the maxilla and mandible) were randomly selected for this study. Animal selection, control, and surgical operation and preparation were performed as per the procedure approved by the Yonsei University Health System's Institutional Animal Care and Use Committee (certification # 2013-0317-4).

The 60 teeth were randomly and equally distributed into three groups, each consisting of 20 teeth, based on the MTA. Table 1 summarizes the key features of each MTA group : PMTA group: positive control, OMTA group, EMTA group.

Table 1. Chemical compositions of the calcium silicate-based cements tested in this study

Materials	Manufacturer	Composition	(MW %)	Setting time
PMTA	Dentsply Tulsa, OK, USA	$3CaO \cdot SiO_2$ (Tricalcium silicate)	66.1%	Initial setting time: 70 to 74 minutes Final setting time: 210 to 320 minutes
		$2CaO \cdot SiO_2$ (Dicalcium silicate)	6.7%	
		$3CaO \cdot Al_2O_3$ (Tricalcium aluminate)	19.9%	
		Bismuth oxide		
		Calcium sulfate dihydrate (Gypsum)		
		Liquid : distilled water		
OMTA	BioMTA, Seoul, Korea	$3CaO \cdot SiO_2$ (Tricalcium silicate)	76.3%	324 ± 2.1 minutes
		$2CaO \cdot SiO_2$ (Dicalcium silicate)	11.8%	
		$3CaO \cdot Al_2O_3$ (Tricalcium aluminate)	8.0%	
		$4CaO \cdot Al_2O_3 \cdot Fe_2O_3$ (Tetracalcium aluminoferrite)	0.8%	
		Free CaO (Calcium oxide)	0.7%	
		Bismuth oxide		
EMTA	Maruchi, Wonju, Korea	CaO (calcium oxide)	46.7%	4.5 to 15 minutes
		SiO_2 (silicon dioxide)	12.8%	
		Al_2O_3 (aluminum oxide)	5.43%	
		MgO (magnesium oxide)	3.03%	
		Fe_2O_3(ferrous oxide)	2.32%	
		SO_3(sulphur trioxide)	2.36%	
		TiO_2(titanium dioxide)	0.2%	
		H_2O/CO_2	4.5%	
		Bi_2O_3 (bismuth oxide)	11%	

PMTA, ProRoot MTA®; OMTA, Ortho MTA®; EMTA, Endocem MTA®

2. Surgical procedure

All operations were performed in a clean sterilized room. Intravascular injections of Zoletile® (5 mg/kg, Virbac Korea, Seoul, Korea) and xylazine® (0.2 mg/kg, Rompun®, Bayer Korea, Seoul, Korea) were administered to the animals, and the inhalational anesthetic isoflurane® (Gerolan®, choongwae Pharmaceutical, Seoul, Korea) was used to put them under general anesthesia. To prevent infection, enfloxacin® (5 mg/kg) was injected subcutaneously right before and after the operation. For 5 to 7 days after the operation, amoxicillin clavulanate (12.5 mg/kg) was administered orally.

3. Full pulpotomy procedure

Lidocaine hydrochloride (2%) with 1:100,000 epinephrine (Kwangmyung Pharmaceutical, Seoul, Korea) was used for local anesthesia. After forming a cavity on the occlusal surface using the high-speed carbide bur 330 (H7 314 008, Brasseler, Germany), the pulp was exposed mechanically. The crown of the pulp was removed from the level of the cementoenamel junction, and bleeding was stopped by injecting sterile saline and applying slight pressure with sterile cotton

pellets. A total of 60 teeth were randomly divided into three groups, each consisting of 20 teeth, and the MTA in each group was applied to the top of the cut pulp as per the manufacturer's guidelines. When the MTA was applied to the pulp wound area, cotton balls soaked in saline were used. The final cavity restoration was performed using the self-curing glass ionomer cement Ketac-Molar (3M ESPE St Paul, MN). Eight weeks after the operation, the two dogs were euthanized by over-sedation.

4. Histological analysis: Hematoxylin and Eosin (HE) staining

The teeth were pulled out using forceps, and one third of the root was removed using the high-speed bur. The specimens were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours, and demineralized in ethylene diamine tetra acetic acid (EDTA; pH 7.4; Fisher Scientific, Houston, TX, USA) for 6 weeks before they were embedded in paraffin. For each specimen, 3-μm continuous sections were created in the buccolingual direction and were subsequently stained with HE. The specimens were observed using the Olympus BX40 optical microscope (Olympus Optical, Tokyo, Japan), and images were acquired using the Infinity 2.0 charge coupled device digital camera (Lumenera

Co., Ottawa, Ontario, Canada). The InnerView 2.0 image analyzer software (InnerView Co, Seongnam-si, Gyeonggi-do, Korea) was used for image analysis. Among the 60 teeth, five in the PMTA group, two in the OMTA group, and nine in the EMTA group were excluded during tooth removal or specimen production. A total of 44 specimens were evaluated in the final analysis: 15 in the PMTA group, 18 in the OMTA group, and 11 in the EMTA group. The produced specimens were examined by five observers (Hwang, Song, Shin, Kang, and Lee) who were blinded for the group treatments. The items of histological evaluations included calcific barrier formation (continuity, morphology, tubule formation, and thickness), dental pulp inflammation (extensity, intensity, type, and dental pulp congestion), and odontoblastic cell layer. The specimens were evaluated for inflammation reaction and hard tissue formation with the scoring system reported in Nowicka et al. (Nowicka et al., 2013) and revised by Lee et al. (Lee et al., 2015) (Table 2). The score agreed by at least three out of the five observers was adopted. In addition, the area of the newly formed hard tissue was measured using Image J (version 1.48, National Institute of Health, Bethesda, MD, USA).

Table 2. Scores used during the histological analysis of calcific barriers and dental pulp

Scores	Calcific barrier continuity
1	Complete dentin bridge formation
2	Partial/incomplete dentin bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site
3	Initial dentin bridge formation extending to no more than one-half of the exposure site
4	No dentin bridge formation
Scores	**Calcific barrier morphology**
1	Dentin or dentin-associated with irregular hard tissue
2	Only irregular hard tissue deposition
3	Only a thin layer of hard tissue deposition
4	No hard tissue deposition
Scores	**Tubules in calcific barrier**
1	No tubules present
2	Mild (tubules present in less than 30% of the calcific barrier)
3	Moderate to severe (tubules present in more than 30% of the calcific barrier)
4	No hard tissue deposition
Scores	**Inflammation intensity**
1	Absent or very few inflammatory cells
2	Mild (an average of < 10 inflammatory cells)
3	Moderate (an average of 10–25 inflammatory cells)
4	Severe (an average > 25 inflammatory cells)
Scores	**Inflammation extent**
1	Absent
2	Mild (inflammatory cells next to the dentin bridge or area of pulp exposure only)
3	Moderate (inflammatory cells observed in one-third or more of the coronal pulp or in the mid pulp)
4	Severe (all of the coronal pulp is infiltrated or necrotic)
Scores	**Inflammation type**
1	No inflammation
2	Chronic inflammation
3	Acute and chronic inflammation
4	Acute inflammation
Scores	**Dental pulp congestion**
1	No congestion
2	Mild (enlarged blood vessels next to the dentin bridge or area of pulp exposure only)
3	Moderate (enlarged blood vessels observed in one-third or more of the coronal pulp or in the mid pulp)
4	Severe (all of the coronal pulp is infiltrated with blood cells)
Scores	**Odontoblastic cell layer**
1	Palisade pattern of cells
2	Presence of odontoblast cells and odontoblast-like cells
3	Presence of odontoblast-like cells only
4	Absent

This scoring system was excerpted from Lee *et al.*'s study (Lee et al., 2017).

5. Immunohistochemistry (IHC)

For IHC, 3-μm cross-sections were deparaffinized with xylene, rehydrated, and rinsed with distilled water. For antigen retrieval, protease K (Dako, Carpinteria, CA, USA) was used for osteocalcin (OC) and dentin sialoprotein (DSP) staining. To activate endogenous peroxidase, 3% hydrogen peroxide was added, while non-specific binding was prevented by incubating sections in 5% bovine serum albumin (Sigma-Aldrich). Subsequently, sections were incubated overnight with the following primary antibodies: anti-OC antibody (rabbit polyclonal, Ab109112, 1:10,000; Abcam, Cambridge, UK) or anti-DSP antibody (rabbit polyclonal, sc-33586, 1: 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, EnVision + System-Horseradish peroxidase (HRP)-Labeled Polymer anti-rabbit (K4003, Dako North America Inc., CA, USA) was applied for 20 minutes. After developing color using the labeled streptavidin biotin kit (Dako) as per the manufacturer's guidelines, the sections were counterstained with Gill's hematoxylin (Sigma-Aldrich).

6. Statistical Analysis

Statistical analyses were performed using the SPSS version 23 software (SPSS, Chicago, IL, USA). To analyze the area of the newly formed calcific barrier, one-way analysis of variance (ANOVA) (significance at $p < .05$) and the *post-hoc* Scheffé test (Bonferroni correction; $p < .017$) were applied.

III. Results

Among the 60 teeth, five in the PMTA group, two in the OMTA group, and nine in the EMTA group failed and were excluded during tooth removal or specimen production; only 44 specimens were evaluated in the final analysis. Eventually, the PMTA (n = 15), OMTA (n = 18), and EMTA (n = 11) specimens were analyzed histologically. Based on the HE staining, the hard tissue barrier was formed in all three groups. In most of the PMTA and OTMA specimens, continuous calcific barriers were formed, and the pulps contained palisading pattern in the odontoblastic layer that were free of inflammation. However, the EMTA specimens had relatively lower quality calcific barrier formation, extensive inflammation, and less favorable odontoblastic layer formation (Fig. 1). The area of the newly formed calcific barrier in each group was compared, and a statistical analysis revealed that the calcific barrier in the PMTA group was most widely formed, followed by the OMTA group, and the EMTA group. Among them, there was a statistically significant difference between the PMTA and EMTA groups ($P < .05$; Figure 2). Furthermore, the DSP and OC staining also indicated the formation of hard tissue in all three groups. The DSP was highly expressed in all three groups. Although OC was also expressed in all three groups, its expression

was relatively less in the EMTA group than in the PMTA group (Fig. 3). Tables 3, 4, and 5 summarize the scores related to hard tissue formation, pulp inflammation reaction, and odontoblastic cell layer patterns in each group, respectively. In summary, the EMTA group produced overall less favorable results than the PMTA and OMTA groups. The EMTA group showed relatively less complete calcific barriers and poorer inflammation reaction.

1. Histological analysis

Calcific barrier formation

When the layer's continuity of the formed hard tissue was observed, PMTA showed the greatest results, followed by OMTA and EMTA. All specimens in the PMTA group formed a complete calcific barrier, while some in the OMTA and EMTA groups produced a partially discontinued calcific barrier (Figure 1). No calcific barrier formation was observed in 9% of the teeth in the EMTA group. For the shape of the formed calcific barrier, the PMTA group produced the hard tissue most similar to the dentin, while a partially irregular or thinly formed calcific layer was observed in the OMTA and EMTA groups. The examination of

the tubule formation in the formed hard tissue indicated that almost no tubule was observed or relatively well-formed hard tissues were observed in all three groups (Table 3). The area of the formed hard tissue layer in each group was compared, and a statistical analysis revealed that the calcific barrier in the PMTA group was most widely formed, followed by the OMTA group, and the EMTA group. Among them, there was a statistically significant difference between the PMTA and EMTA groups ($P < .05$; Figure 2).

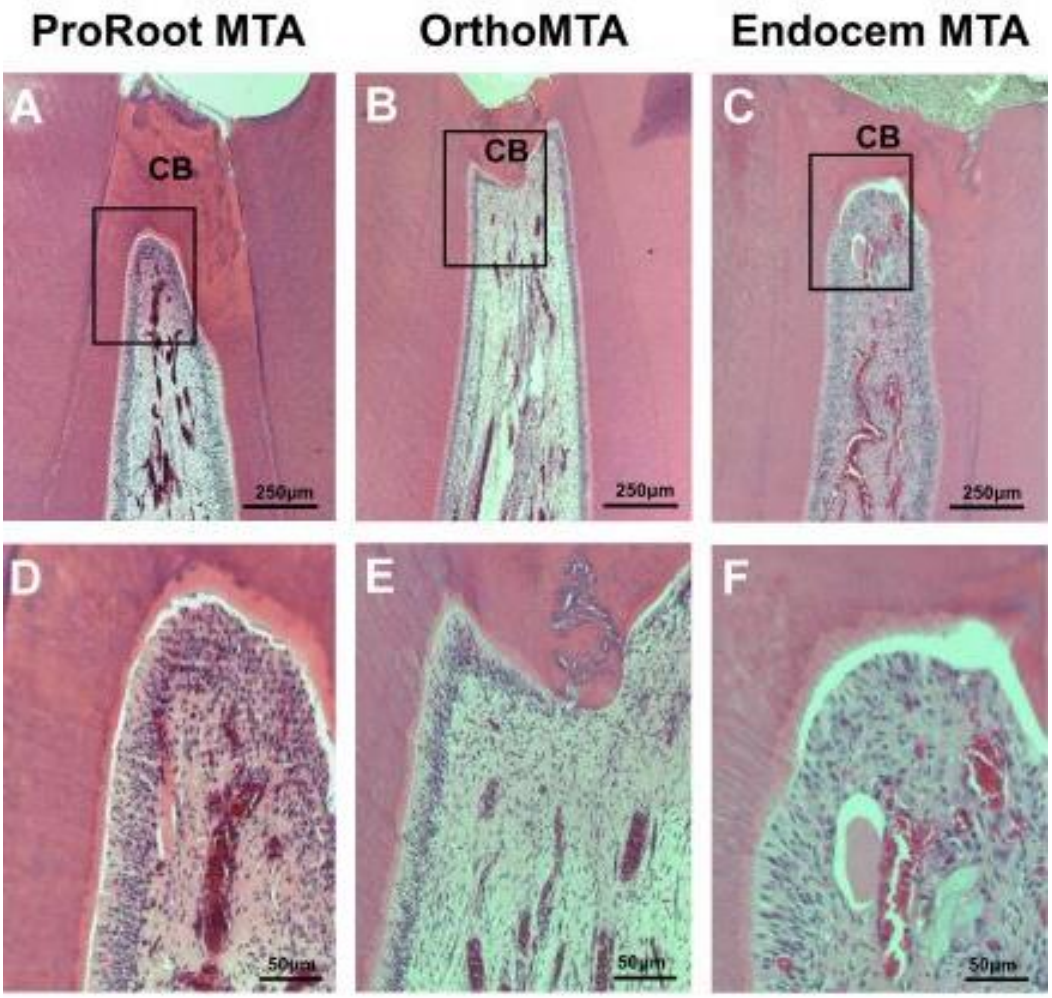

Figure 1. Hematoxylin-eosin (HE) staining for the evaluation of the histomorphologic characteristics of the newly formed calcific barrier (CB) after 8 weeks. A–C shows the characteristics of the CB for each test material. Dentinal tubules can be seen in higher-magnification views in D-F. (A-C: scale bars=250㎛, D-F: scale bars=50㎛)

Table 3. Score percentages for calcific barriers

Groups	Calcific barrier continuity (%)			
	1	**2**	**3**	**4**
PMTA	100 (15/15)	-	-	-
OMTA	66.67 (12/18)	16.67 (3/18)	16.67 (3/18)	-
EMTA	45.45 (5/11)	18.18 (2/11)	27.27 (3/11)	9.09 (1/11)

Table 3. Continued

Groups	Calcific barrier morphology (%)			
	1	**2**	**3**	**4**
PMTA	86.67 (13/15)	13.33 (2/15)	-	-
OMTA	38.89 (7/18)	27.78 (5/18)	33.33 (6/18)	-
EMTA	45.45 (5/11)	18.18 (2/11)	27.27 (3/11)	9.09 (1/11)

Table 3. Continued

Groups	Tubules in calcific barrier (%)			
	1	**2**	**3**	**4**
PMTA	60 (9/15)	33.33 (5/15)	6.67 (1/15)	-
OMTA	61.11 (11/18)	27.78 (5/18)	11.11 (2/18)	-
EMTA	63.64 (7/11)	18.18 (2/11)	9.09 (1/11)	9.09 (1/11)

*(number of teeth receiving the score/total number of teeth evaluated)

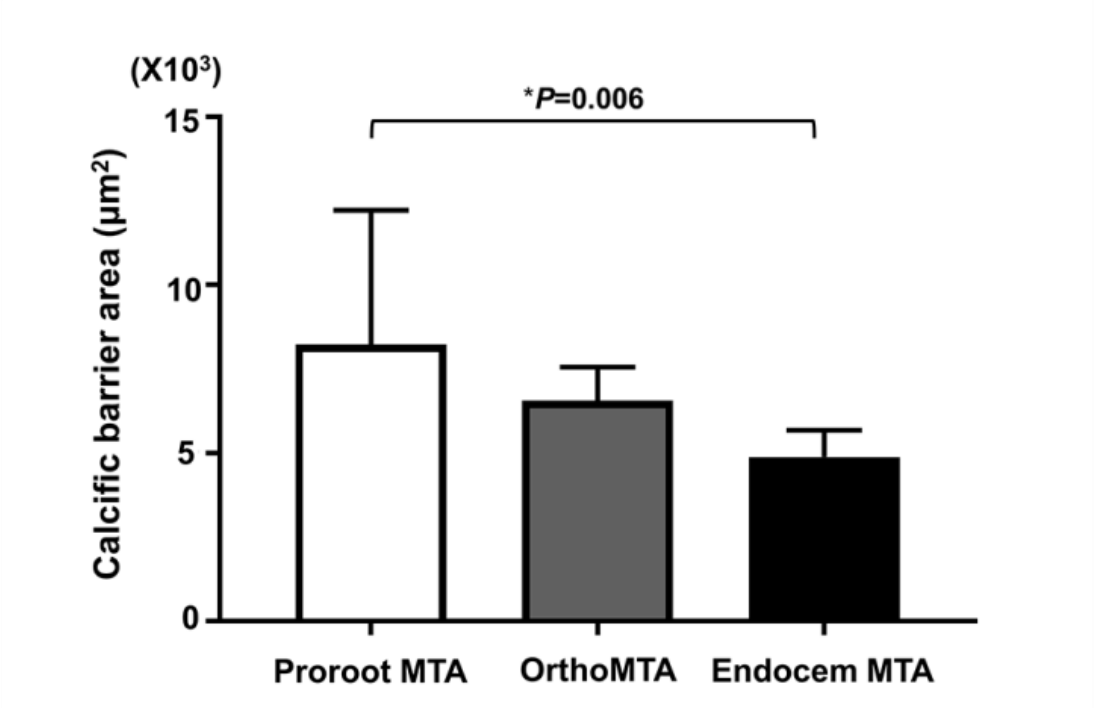

Figure 2. The area of newly formed calcific barrier for each material after 8 weeks. The y-axis represents the area of calcific barrier (1 X 10^3 μm^2). The bars represents the mean ± standard deviation. HE staining for evaluation of the histomorphologic characteristics of the newly formed CB after 8 weeks. Staticstical analysis was performed with SPSS (Version 23.0). One-way ANOVA (P< .05) and the post hoc Scheffé test (Bonferroni correction, P< .017) were applied to analyze the area of newly formed calcific barrier. The number of specimens was n = 15 in PMTA, n = 18 in OMTA, and n = 11 in EMTA group

Pulpal reaction

Inflammation was not observed in 73.33% of the specimens of the PMTA group, 55.56% in the OMTA group, and 36.36% in the EMTA group. The PMTA group showed the least inflammation reaction, while inflammation intensified in the order of OMTA and EMTA. However, all three groups showed mild inflammation. The extent of inflammation was comparable among the three groups. While inflammation was almost non-existent or mild in the PMTA and OMTA groups, it was observed in 9% of the EMTA group, where inflammation was moderately high up to the middle of the pulp. In addition, the type of inflammation was also comparable among the three groups. While there was almost no inflammation in any of the group, the few cases of inflammation had exclusively chronic inflammation. Inflammation was found more severe in the order of OMTA and EMTA, and was least in the PMTA group. The pulp's congestion reaction was least in PMTA, and most severe in EMTA. No severe inflammation above the moderate level was observed in any of the three groups (Table 4).

Table 4. Score percentages for inflammatory responses

Groups	Inflammation intensity (%)				Inflammation extensity (%)			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
PMTA	73.33 (11/15)	26.67 (4/15)	-	-	73.33 (11/15)	26.67 (4/15)	-	-
OMTA	55.56 (10/18)	44.44 (8/18)	-	-	55.56 (10/18	44.44 (8/18)	-	-
EMTA	36.36 (4/11)	63.64 (7/11)	-	-	36.36 (4/11)	54.55 (6/11)	9.09 (1/11)	-

Table 4. (continued)

Groups	Inflammation type (%)				Dental pulp congestion (%)			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
PMTA	73.33 (11/15)	26.67 (4/15)	-	-	40 (6/15)	53.33 (8/15)	6.67 (1/15)	-
OMTA	55.56 (10/18)	44.44 (8/18)	-	-	27.78 (5/18)	61.11 (11/18)	11.11 (2/18)	-
EMTA	36.36 (4/11)	63.64 (7/11)	-	-	18.18 (2/11)	63.64 (7/11)	18.18 (2/11)	-

*(number of teeth receiving the score/total number of teeth evaluated)

Odontoblastic cell layer

When the shape of the formed odontoblast cell layer was evaluated, it was most tightly arranged in the PMTA group, and mostly consisted of odontoblast or odontoblast-like cells. OMTA and EMTA group showed less favorable result comparing with PMTA group: All OMTA specimens showed odontoblastic cell layer. On the other hand, about 9% of EMTA group showed no odontoblastic cell layer (Table 5).

Table 5. Score percentages for the odontoblastic cell layer

Groups	Odontoblastic cell layer (%)			
	1	**2**	**3**	**4**
PMTA	60(9/15)	26.67(4/15)	13.33(4/15)	-
OMTA	33.33(6/18)	50(9/18)	16.67(3/18)	-
EMTA	45.45(5/11)	18.18(2/11)	27.27(3/11)	9.09(1/11)

*(number of teeth receiving the score/total number of teeth evaluated)

2. Immunohistochemistry

DSP and OC staining indicated the formation of hard tissue in all three groups. The DSP was highly expressed in all three groups (Figure 3 A-F). Therefore, odontoblasts and tertiary dentin formation could be inferred from this result. OC was also expressed in all three groups; although its expression was relatively less in the EMTA group than in both PMTA and OMTA group. So the result meant that EMTA's odontoblastic differentiation inducing ability was less than those of PMTA and OMTA (Figure 3 G-L).

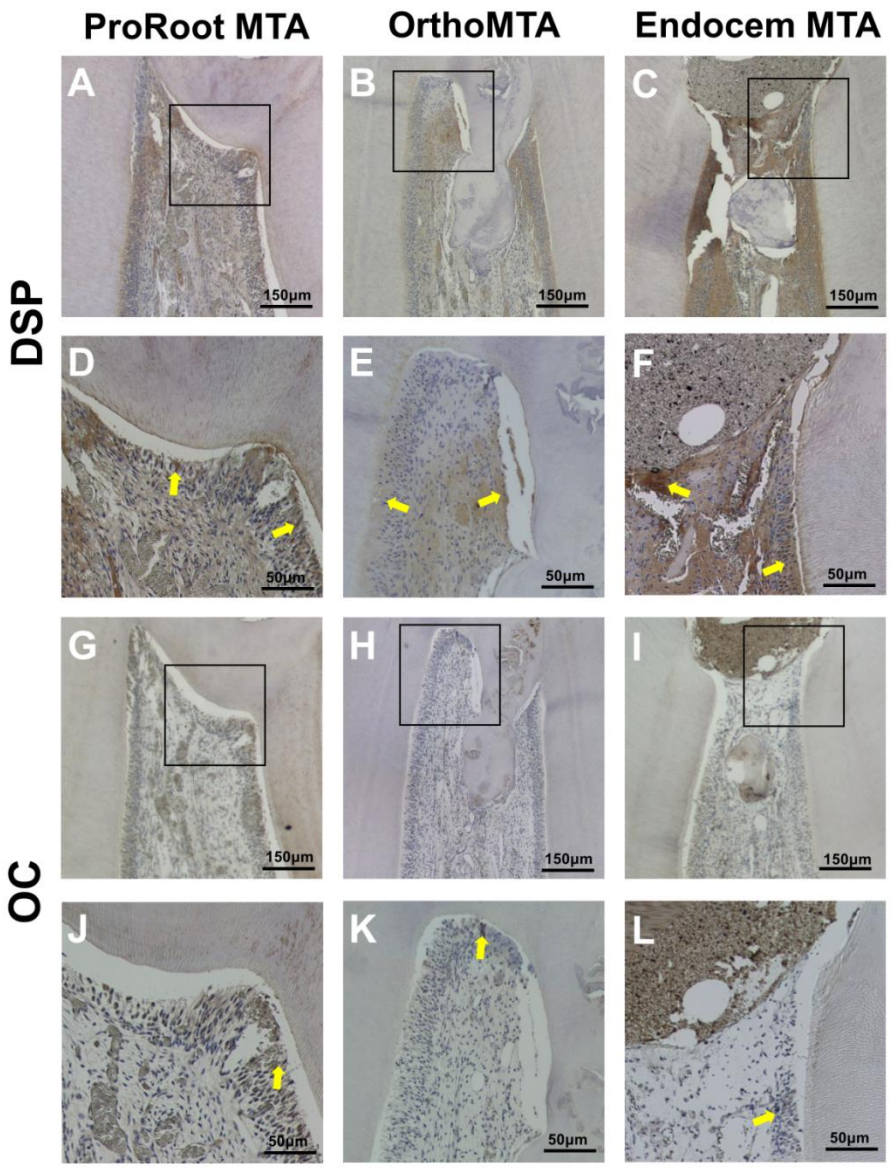

Fig 3. Immunohistochemical staining of dentin sialoprotein (DSP) and osteocalcin (OC). Yellow arrows indicate cells with a positive signal. (A-C: scale bars=150㎛, D-F: scale bars=50㎛, G-I: scale bars=150㎛, J-L: scale bars=50㎛)

IV. Discussion

The purpose of this study was to compare and evaluate pulp reaction associated with the three MTA products over 8 weeks in a beagle dog's pulpotomy model. We used three calcium silicate derived cements—PMTA, OMTA, and EMTA. The PMTA was used as a positive control, and has been proven as a gold standard in many studies on calcium silicate cements for its extraordinary biocompatibility and ability to induce dentin and bone formation. The OMTA showed inflammation and cellular reaction relatively comparable to PMTA, formed an almost complete calcific barrier in the upper part of the cut pulp tissue, and produced almost no inflammatory reaction. The EMTA showed a significantly stronger inflammatory reaction than the other two MTAs, had a lower level of calcific barrier formation, produced many thinly and incompletely formed calcific layers, and had more tubular defects.

It is still controversial to say that calcific barrier formation on the interface between the pulp and the material indicates the success of VPT. Indeed, calcific barrier formation does not necessarily indicate the healing of the pulp tissues. Calcific barrier formation could be interpreted as a healing process or a reaction to stimulation (Schroder, 1985; Dominguez et al., 2003; Al-Hezaimi et al., 2011). The pulp forms the tertiary dentin to protect itself as a reaction to hazardous

stimulation such as cavity, trauma, and iatrogenic damage. If the environment is good, hazardous stimulation is applied to the tooth, and subsequently dentin formation ensues as a protective mechanism. Accordingly, dentin formation can be regarded as an indication of healing or a reaction to stimulation. Based on this, this study interpreted dentinal bridge formation as a sign of healing and a positive reaction to stimulation.

Calcific barrier formation does not mean the pulp is fully sealed from the external environment because formed bridges are penetrable. According to previous studies, the initial tertiary dentin created after pulpotomy was formed in a disorderly structure. However, over time, it became less penetrable and was sealed more solidly between the pulp and the cavity as it was mineralized (Dominguez et al., 2003). Thus, calcific barrier formation after VPT does not necessarily indicate the success of the procedure. The thickness, continuity, structure, and tubule formation of the formed calcific barrier should be evaluated depending on the used material, not simply quantitatively but qualitatively. Likewise, it is necessary to not only simply evaluate whether there is inflammation but also specifically analyze the type of inflammation, the level of pulp congestion, whether the dentin cell is the real odontoblast cell or a similar cell, and how tightly the formed odontoblast cell layer was arranged in a fence

structure to assess truly the material's ability of hard tissue formation. In this regard, this study performed quantitative and qualitative evaluations on each of these items.

The DSP is a non-collagen protein existing in the extra cellular matrix and a type of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family (Fisher et al., 2001). It is the first protein accumulated in the odontoblast, and a special chemical marker to evaluate the function of an odontoblast (D'Souza et al., 1995). On the other hand, OC is a relatively late-stage marker in odontoblastic differentiation, and an important protein that controls mineralization in the dentin and bone (Malaval et al., 1994). Accordingly, this experiment used two specific markers and evaluated mineralization and odontogenicity using immunofluorescence. Our results indicated that both DSP and OC were expressed in all three materials, and CSCs in all three groups induced hard tissue formation. However, the PMTA and OMTA groups showed a comparable higher level of expression than the EMTA group.

Several previous *in vitro* studies demonstrated that PMTA induces odontogenic differentiation in the dental pulp stem cell (Seo et al., 2013; Hakki et al., 2009; Min et al., 2009). In addition, CSC, including MTA, facilitates the differentiation of neighboring pulp cells into odontoblasts, and inhibits the action of osteoclast

macrophages (Maeno et al., 2005; Sun et al., 2009; Chen et al., 2010; Hung et al., 2013). Although the exact mechanism for odontoblastic differentiation induced by PMTA is still unknown, it is well established that calcium originating from PMTA plays an important role in odontoblastic differentiation (Woo et al., 2013). Furthermore, PMTA generates calcium ions through hydration (Camilleri, 2008), and these flow in turn into the cell and affect odontoblastic and osteoblastic differentiation (Matsumoto et al., 2013; Woo et al., 2013). The setting of PMTA results in the formation of calcium oxide, which reacts with the tissue fluid and generates calcium hydroxide (Koh et al., 1998). Calcium hydroxide reduces plasma outflow and affects microvasculature, and induces the mineralization of neighboring pulp tissues (Heithersay, 1975). The MTA frees calcium hydroxide and raises the pH, which increases the alkaline phosphatase activity and calcium-dependent pyrophosphatase activity, and liberates growth factors (TGF-β1) from the dentin matrix (Pradhan et al., 2006). According to Seux et al., after contacting the pulp tissue, MTA forms a structure similar to that of calcite crystals like calcium hydroxide (Seux et al., 1991). Calcium ions facilitate the pulp cells of the cut part and generate fibronectin in a dose-dependent manner, while calcite crystals are mediated by fibronectin and mineralized (Mizuno and Banzai, 2008). Fibronectin plays an important role in cellular adhesion and differentiation, and induces the differentiation of odontoblast-like cells and calcific barrier formation

(Yoshiba et al., 1996). Accordingly, the mechanism of action of MTA is deemed similar to that of calcium hydroxide (Faraco et al., 2001). Many recent studies reported that MTA up-regulated odontogenic markers in human pulp cells (hDPCS) (Min et al., 2009; Zhao et al., 2012; Woo et al. 2013), and increased the expression of DSP mRNA even in Portland cement (Wang et al., 2015). Calcium originating from set PMTA forms the dentinal bridge, but a high pH causes the coagulation of neighboring pulp tissues and the necrosis of the pulp (Soares, 1996). It has been shown that 3 hours after the mixture of PMTA, pH rose up to 12.5 (Parirokh and Torabinejad, 2010). This alkaline pH has been known to remain high in an environment with moisture for at least 8 weeks after setting (Fridland and Rosado, 2005). This high pH induces an environment conducive to cell division, matrix formation, and antimicrobial action, and can form a hard tissue barrier. However, it has also been shown to produce a hazardous inflammatory reaction in the pulp (Fridland and Rosado, 2005; Maria de Lourdes et al., 2008). As PMTA sets relatively late, it can be washed out when it comes in contact with blood or tissue fluid, which can lead to a failure of the therapy (Tingey et al., 2008; Nekoofar et al., 2010; Kang et al., 2012). Furthermore, PMTA has its downsides because it is difficult to control, relatively expensive, and causes tooth discoloration (Ber et al., 2007). These shortcomings can be minimized if the cement sets quickly before it is exposed to blood or tissue fluid.

Thus, several studies have been conducted to shorten the setting time of PMTA by adding setting accelerators such as calcium chloride, Na_2HPO_4, and calcium lactate (Kogan et al., 2006; Hsieh et al., 2009; Hung et al., 2013). There have been many attempts to develop new calcium silicate-based (MTA-modified) substances that overcome the weaknesses of PMTA (Camilleri, 2008; Gandolfi et al., 2009; Gandolfi et al., 2012). Although these attempts have successfully shortened the setting time, it was still too long in clinical application. Furthermore, the added substances may exert adverse effects on the biological and physical properties of MTA (Kang et al., 2013; Lee et al., 2011; Camilleri et al., 2005).

Recently, two calcium silicate-based materials were developed in Korea (Kang et al., 2013). According to the manufacturer, their advantages include their easy use, shorter setting time, low heavy metals content, weak tooth discoloration effect, and relatively low cost compared with the existing MTA. The first material is OMTA, which was developed for VPT and apical filling, and is now available commercially (Yoo et al., 2014; Kim et al., 2015). Intratubular mineralization was observed on the interface between the dentin and the filled OMTA in a scanning electron microscope *in vitro* study (Yoo et al., 2014) (Kim et al., 2015). In a clinical trial, OMTA showed similar results to those of PMTA in pulpotomy on both intact teeth and teeth with dental caries (Azimi et al., 2014; Kang et al.,

2017). However, another *in vitro* study reported that OMTA was less biologically compatible than PMTA or glass ionomers. (Lee et al., 2012)

The second material is EMTA, which includes fine particles of pozzolan, amorphous or glassy silica. Pozzolan is a siliceous or silico-aluminous material that contains SiO_2 and Al_2O_3, chemically reacts with calcium hydroxide under the presence of moisture, and forms a substance that has the same properties as cement (Jo et al., 2007) The advantage of EMTA is its short setting time of 4.5 minutes. It is reported that EMTA is dissolved less in the tissue fluid than PMTA, and has the ability to induce biocompatibility and mineralization similarly to PMTA (Choi et al., 2013; Song et al., 2014). Furthermore, EMTA has been reported to cause less tooth discoloration than PMTA (Jang et al., 2013), and its sealing ability is as good as that of PMTA (Choi et al., 2013; Song et al., 2014). Park et al. evaluated the odontogenic effect of EMTA on human dental pulp cells (hDPS) after 4 weeks of pulp capping. They reported that the continuous tertiary dentin was formed right below the material in all specimens in the PMTA and EMTA groups. In both groups, the continuous tertiary dentin was created in the lower part of the capping agent and in the upper part of the exposed pulp. Furthermore, both materials had comparable biocompatibility and hard tissue formation effects (Park et al., 2014). On the other hand, EMTA showed only a minor pulp inflammatory reaction, which could be ignored (Park et al., 2014).

Similar to PMTA, EMTA contains approximately 40 wt% calcium (Park et al., 2014). Liberated calcium has been reported to induce the expression of DSP and OC and cause mineralization in the cells. In a study on the release of calcium ions, PMTA liberated more calcium than EMTA, and caused more sediments of calcium and phosphorus, which are the major components of hydroxyapatite (Han et al., 2015). The shorter setting time of EMTA compared with PMTA is that it contains small particles of pozzolan, and provides a wider area of contact when it is mixed with water (Choi et al., 2013). In addition, an increase in compressive strength and durability originates from a pozzolanic reaction because, in this reaction, calcium hydroxide is consumed to generate additional calcium silicate hydrate (CSH) and calcium aluminate hydrate reaction byproducts. Such byproducts fill up pores, contribute to rearranging tightly pore structures or sizes, and reduce penetrability (Chappex and Scrivener, 2013). A randomized clinical trial reported success rates of 95.5% and 90.5% when using PMTA and EMTA, respectively, 12 weeks after pulp capping, with no statistically significant differences (Song et al., 2015).

Kim et al. compared the biological properties of PMTA, OMTA, and EMTA, and reported a significantly shorter setting time in EMTA (15 ± 0.5 min) than PMTA (318 ± 56.0 min) or OMTA (324 ± 2.1 min). Furthermore, on the 7th day after mixture with water, the pH was 11.9 for PMTA, 11.42 for OMTA, and 11.33

for EMTA; EMTA showed the lowest level of acidity. In addition, OMTA showed a significantly higher level of cytotoxicity than PMTA or EMTA ($P < .05$), and PMTA was better in hard tissue formation or biological properties than OMTA or EMTA (Kim et al., 2014). A study the *in vitro* cytotoxicity of EMTA in human periodontal ligaments reported that an initially lower cell viability than PMTA and Angelus MTA, which recovered to the same level of the other materials with passing time (Song et al., 2014).

In this study, the absence of complete barrier formation in the EMTA group suggested that this material had a relatively lower level of biocompatibility than PMTA or OMTA; therefore, it was associated with a higher level of inflammation. In addition, the PMTA and OMTA groups formed palisading patterns in the odontoblast cell, which demonstrates that these two materials had stronger odontogenic differentiation potential than EMTA. Several reasons for the low biocompatibility of EMTA in this study could be suggested. First, EMTA has been reported to have a lower level of cell viability than PMTA immediately after mixing (Song et al., 2014), thus contributing to its low level of biocompatibility. Second, as demonstrated by Chung et al., the high concentration of aluminum in EMTA could be associated with the temporary cytotoxicity in the initial stage (Chung et al., 2016). Third, the relatively lower initial pH of EMTA might not be

suitable for inducing mineralization, and results in less antimicrobial action compared to the other MTAs. Fourth, the reduced inducement of hard tissue formation could be related to the low quantity of calcium ions liberated by EMTA. Despite the comparable results between EMTA and PMTA in short-term studies, the failure rate increased and there were poorer outcomes in our mid-term study of 8 weeks when using EMTA.

Fifth, a pozzolanic reaction arising from pozzolan added to EMTA to facilitate setting might have interfered with hard tissue formation, and caused an inflammatory reaction; this notion requires more research for confirmation.

OMTA showed results similar to those of PMTA in this study because its components are almost the same as those included in PMTA. In contrast, the composition of EMTA is quite different from that of PMTA. Accordingly, we believe that EMTA induces a different chemical reaction when mixed with water, induces tissue setting differently than other MTAs. When hydrated, pozzolan contained in EMTA sets, and the calcium hydroxide is consumed when a pozzolanic reaction occurs, thus forming CSH. The amount of calcium hydroxide eluted from the resulting set pozzolan in EMTA is smaller than that from other MTAs. As a result, the pH decreases, which explains the better outcome in the *in vitro* cytotoxicity experiment. The major components of OMTA or PMTA are

tricalcium silicate, dicalcium silicate, and tricalcium aluminate, which, when mixed with water, they become eluted, CSH is formed, and calcium hydroxide precipitates and binds to the CSH surface. As a result, the pH becomes high in the eluent even after setting, and bad cytotoxicity outcomes are observed in the initial mixture. Subsequently, the tissue fluid, *in vivo* neutralization, or dilution by blood flow during setting decreases cytotoxicity, and calcium hydroxide induces hydroxyapatite formation, thus leading to the formation of hard tissues. Therefore, whether it is washed out or not, the setting time determines the inflammatory reaction in an *in vivo* experiment. Furthermore, alkalis are neutralized by body fluids and diluted by blood flow, which is believed to contribute to different results than those in *in vitro* conditions. Not only inflammation or hard tissue formation but also bacterial sealing and setting without washout are equally important when using MTA for VPT. Thus, our present study conjectures that further studies using these three materials must be conducted and viewed from various angles.

Several *in vitro* and *in vivo* histological studies demonstrated the successful formation of calcific barriers and odontogenic healing when PMTA was used for VPT. However, there have been few studies on OMTA or EMTA. This study examined the effects of the three types of MTAs on pulp tissues for 8 weeks.

There have been many short-term studies spanning over a period of 4 weeks on the pulp's reaction to VPT materials. However, the present study is particularly significant because it compared the three materials using newly developed MTA derived cements (MDC) under *in vivo* conditions over a period of 8 weeks; this period is to our knowledge the longest to date.

There were several limitations in the present study: There was no negative control in this study and the sample size was relatively small. Furthermore, the results of the animal model do not necessarily reflect the results of human teeth for example, the time until the formation of hard tissue barriers varies significantly; a complete hard tissue barrier appeared in animals 1 week after therapy (Parirokh et al., 2005; Liu et al., 2015), whereas no hard tissue barrier was formed in humans within 2 weeks (Swarup et al., 2014; AlShwaimi, et al., 2016). Most previous studies reported that it took 30 to 42 days to form a hard tissue barrier in humans (Eskandarizadeh et al., 2011; Shahravan et al., 2011; Yoshiba et al., 2012). Accordingly, in the further study, many clinical trials on human teeth should support the CSC materials used in this study. When statistically comparing the area of newly formed calcific barrier in this study, the size of pulp would be different according to the kinds of teeth. Therefore comparing simply the area of newly formed calcific barrier in the different kinds of teeth would be controversy. Healthy

pulp tissue was observed in this animal experiment. Thus, the findings may not reflect the conditions in clinical situations where there is typically slight inflammation or chronic reaction due to dental caries. Moreover, previous studies showed that the histological results of VPT were not necessarily consistent with clinical symptoms (Iwamoto et al., 2006; Caicedo et al., 2006). Therefore, when applying the findings of this study to clinical practice, they should comprehensively consider various aspects, including the presence of inflammatory reaction, radiographic examinations, and clinical findings. In the future, it is also required to evaluate the mineralization rate of each material. Studies assessing long-term success are also needed.

V. Conclusion

Ortho MTA® can be used as a substitute for ProRoot MTA® because of its similar effects on inducing mineralization and anti-inflammatory reaction. Based on our results, Endocem MTA® formed a lower-quality calcific barrier, and showed a higher level of pulp inflammatory reaction.

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국문 요약

개의 치수절단술 모델에서 3가지 종류의 치수절단용 칼슘 규산염 시멘트들에 의한 치수의 반응

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본 연구는 비글개의 치수 절단술 모델에서 3가지 칼슘 규산 기반 시멘트인 ProRoot MTA®, Ortho MTA®, Endocem MTA®에 대한 치수의 반응을 평가하고 조직비교를 하기 위해 수행되었다. 치수절단술은 비글개의 44개 치아에서(원래 각 군 당 20개의 치아를 실험하였으나 시편 제작 과정에서 일부가 탈락되고, 최종적으로 44개의 치아 시편을 평가함)수행되었다. 노출 된 치수 조직을 ProRoot MTA®, Ortho MTA® 및 Endocem MTA®으로 무작위로 선정하여 치수 복조하고 8주 후, 치아를 발치하여 조직학적 검사를 시행하였는데, osteocalcin (OC)과 dentinsialoprotein (DSP)을 이용한 면역 조직 화학 검사 (IHC)를 시행하였다. 최종적으로 44개의 시편이 제작되었으며, ProRoot MTA® (n =

15), Ortho MTA® (n =18)및 Endocem MTA® (n =11) 각 시편들의 석회화 장벽 형성, 염증 반응 및 상아질 층을 평가하여 눈가림방식으로 점수를 매겼고, 새로 형성된 석회 장벽의 면적을 각 그룹별로 측정 하였다. ProRoot MTA®와 Ortho MTA® 시편의 대부분에서 지속적인 석회화 장벽이 형성되었으며 치수에는 palisading 패턴이 포함되어 있었다. 그러나 Endocem MTA®표본은 낮은 수준의 석회화 장벽 형성과 염증반응 및 덜 바람직한 상아질층 형성을 나타내었다. 본 연구결과를 토대로 Ortho MTA®는 ProRoot MTA®에 대한 대안을 제공 할 수 있을 것으로 사료된다. 두 재료 모두 우수한 치수 반응을 나타냈지만 Endocem MTA®은 덜 바람직한 치수 반응을 나타내었다.

핵심되는말: 칼슘규산염계시멘트, Proroot MTA®, Ortho MTA®, Endocem MTA®, 치수 절단술, 치수 반응, 석회화 장벽, 염증, 조상아세포층