ABSTRACT

Objectives: In recent in vitro study, it was reported that osteostatin (OST) has an odontogenic effect and synergistic effect with mineral trioxide aggregate (MTA) in human dental pulp cells. Therefore, the aim of this study was to evaluate whether OST has a synergistic effect with MTA on hard tissue formation in vivo.

Materials and Methods: Thirty-two maxillary molars of Spraque-Dawley rats were used in this study. An occlusal cavity was prepared and the exposed pulps were randomly divided into 3 groups: group 1 (control; ProRoot MTA), group 2 (OST 100 μM + ProRoot MTA), group 3 (OST 1 mM + ProRoot MTA). Exposed pulps were capped with each material and cavities were restored with resin modified glass ionomer. The animals were sacrificed after 4 weeks. All harvested teeth were scanned with micro-computed tomography (CT). The samples were prepared and hard tissue formation was evaluated histologically. For immunohistochemical analysis, the specimens were sectioned and incubated with primary antibodies against dentin sialoprotein (DSP).

Results: In the micro-CT analysis, it is revealed that OST with ProRoot MTA groups showed more mineralized bridge than the control (p < 0.05). In the H&E staining, it is showed that more quantity of the mineralized dentin bridge was formed in the OST with ProRoot MTA group compared to the control (p < 0.05). In all groups, DSP was expressed in newly formed reparative dentin area.

Conclusions: OST can be a supplementary pulp capping material when used with MTA to make synergistic effect in hard tissue formation.

Keywords: Direct pulp capping; Mineralization, MTA; Osteostatin

INTRODUCTION

Dental pulp exposure can be occurred by accidental trauma, iatrogenic tooth preparation or caries excavation process. In certain cases, direct pulp capping has been used to maintain pulp vitality and function. When direct pulp capping is performed, the selection of the capping material is a key to produce good treatment outcomes [1,2]. The ideal direct pulp capping material should have an ability regarding disinfection, adhesiveness to dentin to prevent microleakage, easy handling and dentin bridge formation [3,4].
Author Contributions
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Mineral trioxide aggregate (MTA) is a biocompatible material which has been widely used in clinical endodontic practice due to its low cytotoxicity, good biocompatibility and the capacity to induce new dentin formation [5-7]. Various clinical trials and histological data have verified the superior effects of MTA in many endodontic treatments including pulp capping, pulpotomy, root-end filling, perforation repair, apexogenesis and apexification [8-10]. Furthermore, several studies have demonstrated that a combination of MTA and a potent odontogenic protein can promote rapid odontogenic/osteogenic differentiation of human dental pulp cells (hDPCs) than MTA alone [11-14].

Parathyroid hormone-related protein (PTHrP) is a secreted factor expressed in almost all normal fetal and adult tissues [15]. It has been reported that the N-terminal fragment of PTHrP plays an important role in bone development and bone remodeling resulting in bone anabolic actions [16,17]. Recently, the C-terminal 107-111 domain, also known as osteostatin (OST), of PTHrP exhibits osteogenic effects in the in vitro study and stimulates bone formation in the in vivo study [18,19]. Furthermore, several studies reported that the combination of OST with bioceramic materials such as silicone-doped hydroxyapatite and gelatin-glutaraldehyde biopolymer-coated hydroxyapatite showed increased osteogenic potential in osteoblastic cells [16,20,21].

However, there were few studies regarding the odontogenic potential of OST and the combination effect with bioceramic materials on hDPCs. In the previous study, it was reported that the combination of MTA and OST showed a synergistic effect on odontogenic differentiation of hDPCs compared with MTA alone [22].

Based on the results of Han et al. [22], this study was planned to evaluate hard tissue formation of the dental pulps in rat maxillary molars after immediate direct pulp capping and to investigate whether OST has a synergistic effect with MTA on hard tissue formation in vivo.

MATERIALS AND METHODS

Surgical procedures
All procedures were approved by the Ethics Committee for Animal Use, College of Veterinary Medicine, Chonnam National University (CNU IACUC-YB-2017-30).

Intact maxillary first molars without caries of 32 Spraque-Dawley rats (male, 6–8 weeks old) were used in this study. The rats were divided into 3 groups based on 3 different pulp capping materials: group 1 (control; ProRoot MTA [Dentsply, Tulsa, OK, USA], n = 8), group 2 (OST [Bachem, Bubendorf, Switzerland] 100 μM + ProRoot MTA, n = 12), group 3 (OST 10 mM + ProRoot MTA, n = 12). The rats were anesthetized with a combination of 50 mg/kg of zolazepam/tiletamine (Zoletil50; Virbac, Carros, France), 4 mg/kg Alfaxan (Jurox, Rutherford, Australia) and 5 mg/kg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany) by intraperitoneal injection. Class I cavities were prepared to induce pinpoint pulp exposure on the occlusal surfaces of both maxillary first molars using 1/2 round carbide burs (diameter = 0.6 mm). The depth of the cavity was approximately the size of the bur head and the final exposure was performed using the tip of the endodontic explorer (Hu-Friedy Mfg Co, Chicago, IL, USA). Bleeding control was done by using light pressure with wet cotton pellets and irrigation with sterile normal saline solution.
The prepared cavities were covered with 3 pulp capping materials: ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA), OST 100 μM + ProRoot MTA, OST 10 mM + ProRoot MTA. MTA was mixed according to the manufacturers’ instructions under aseptic condition. The mixing ratio of MTA powder-to-OST liquid was 3:1. All cavities were restored with resin-modified glass ionomer (Fuji II LC; GC America Inc., Alsip, IL, USA).

After 4 weeks, the animals were sacrificed using an overdose of CO₂ gas.

**Micro-computed tomography (CT) imaging**

All maxillae were dissected into 2 block sections including each molar and the specimens were placed in 10% formalin for storage before micro-CT scanning. Fixed block sections were scanned using a high-resolution micro-CT system (SkyScan 1172, SkyScan, Aartselaar, Belgium). Each section was mounted on a plastic container with the root oriented vertically. The X-ray transmission was set at 180 degrees of rotation, with the X-ray source set at 70 kV/141 μm. A 0.5 mm aluminum filter was used to cut off the softest X-ray. The raw data were reconstructed into images using SkyScan’s cluster reconstruction software (NRecon/NRecon Server).

Quantitative analysis for hard tissue formation was conducted by using image J program (version 1.47, National Institutes of Health, Bethesda, MD, USA). Areas of newly formed reparative dentin and pulp cavity were measured in transverse section view near cementoenamel junction. The relative ratio of reparative dentin to pulp cavity was measured.

**Histological examination**

All specimens were fixed in 4% paraformaldehyde for 24 hours and demineralized using a decalcifying agent (Calci-Clear Rapid, National Diagnostics, Atlanta, GA, USA) at 4°C for 6 weeks. The samples were treated with ethanol dehydration and embedded in paraffin. Sagittal sections (5-μm thickness) were obtained and stained with hematoxylin and eosin for evaluation of hard tissue formation. Quantitative analysis for hard tissue formation was conducted by using image J program (National Institutes of Health). The relative ratio of reparative dentin to pulp cavity was measured.

**Immunohistochemical examination**

Immunohistochemical examinations were performed with a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. The specimens were de-waxed in xylene, rehydrated in a graded alcohol series, placed in 3% hydrogen peroxide in methanol for 50 minutes, and washed with phosphate-buffered saline (PBS). After treatment of horse normal serum for 2 hours, the primary anti-dentin sialoprotein (DSP) antibody (dilution 1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was incubated at 4°C for 24 hours, followed by the biotin-streptavidin peroxidase complex as a secondary antibody. The tissues were counterstained with hematoxylin. Negative controls were incubated with normal rabbit IgG (Cell Signaling Technology) instead of primary antibody. The immunostained samples were scanned and reviewed using Upright microscope ECLIPSE NI-U (Nikon corporation, Tokyo, Japan).

**Statistical analysis**

Kruskal-Wallis and Mann-Whitney U test were used for all data by using SPSS 23.0 (IBM Corp, Armonk, NY, USA). A significant difference was determined at values of p < 0.05.
RESULTS

Micro-CT analysis
All groups in micro-CT analysis revealed the formation of a reparative mineralized bridge in the residual pulp (Figure 1). The relative ratios of newly formed reparative dentin to pulp cavity were 0.23 ± 0.03, 0.42 ± 0.12, 0.52 ± 0.14 for group 1, group 2 and group 3, respectively. Group 2 (OST 100 µM + MTA) and group 3 (OST 10 mM + MTA) showed significantly higher reparative dentin formation compared to the control group (MTA) (p < 0.05) (Figure 2).

Figure 1. Micro-computed tomography images of pulp capped rat molar teeth using mineral trioxide aggregate (MTA) with or without Osteostatin (OST). OST groups (100 µM, 10 mM) showed more hard tissue formation than MTA group. (A) ProRoot MTA, sagittal view (B) OST 100 µM + ProRoot MTA, sagittal view (C) OST 10 mM + ProRoot MTA, sagittal view (D) ProRoot MTA, axial view (E) OST 100 µM + ProRoot MTA, axial view (F) OST 10 mM + ProRoot MTA, axial view. White arrow indicates reparative dentin.

Figure 2. The relative ratio of newly formed reparative dentin to pulp cavity in micro-computed tomography results. Area of mineralized tissue and pulp cavity was measured by Image J. Mineral trioxide aggregate (MTA) with osteostatin (OST) group significantly showed more reparative dentin formation than MTA alone group. *p < 0.05.
Histological findings
In histological analysis, the dentin bridge was created but few inflammatory cells were observed in the pulp cavity in all 3 groups. Specimens in the group 1 (MTA) showed complete dentin bridge formation (Figure 3). Newly formed hard tissues had an irregular pattern near the exposure site and necrotic changes were not observed. The group 2 (OST 100 μM + MTA) and group 3 (OST 10 mM + MTA) showed similar hard tissue formation pattern with the control MTA group but showed significantly more hard tissue formation ($p < 0.05$) (Figure 4). Specimens showed dense calcified tissue formation at the pulp exposure area and canal orifice without any necrotic change.

Immunohistochemical findings
Newly formed hard tissues from all three groups showed positive immunoreactivity for DSP. In the MTA group, strong immunopositive layer was observed along with the odontoblastic layer of the new dentin. In the group 2 (OST 100 μM + MTA), immunopositive area was observed in the new dentin bridge. In the group 3 (OST 10 mM + MTA), positive DSP expression was observed in both odontoblastic layer of the new dentin and dentin bridge area around the pulp exposure site (Figure 5).

Figure 3. Histological appearance of pulp capped rat molar teeth using MTA with or without OST (×40 magnification). Hematoxylin and eosin-stained sections showed reparative dentinal bridge formation in all groups. Reactionary dentin could be seen around canal orifices in all groups. (A) ProRoot mineral trioxide aggregate (MTA) (B) osteostatin (OST) 100 μM + ProRoot MTA (C) OST 10 mM + ProRoot MTA. P, pulp; RD, reparative dentin.
* Capping material.
DISCUSSION

The current study showed that both MTA and the combination of MTA and OST as a pulp capping material lead to favorable outcomes in rat model.

We used micro-CT to evaluate newly formed reparative dentin. Micro-CT is a noninvasive technique that shows detailed morphological features of the hard tissues in a nondestructive manner [23]. In micro-CT analysis, the newly formed hard tissue areas of group 2 (OST 100 μM + MTA) and group 3 (OST 10 mM + MTA) were significantly higher than those of the control group (MTA). This finding suggests that MTA and the combination of MTA and OST have a dentinogenic capacity, but the combination of MTA and OST has superior synergistic effects, when compared with MTA alone. These results are thought to be due to the fact that OST has odontogenic potential and has a synergistic effect with MTA [22].

In histological findings, the newly formed dentin in all three groups had the characteristics of homogeneous reparative dentin but few engulfed cellular inclusions within newly formed hard tissues in the MTA group. This homogeneous reparative dentin formation could have been the result of the rapid initial formation of the reparative dentin. The nature of new hard tissue produced under pulp-capping materials is not completely known. Newly formed dentin bridges often contain multiple perforations and imperfections as seen in our histologic results (engulfed cellular inclusions), so the term “dentin bridge” may be inappropriate [24]. Recently, the newly formed hard tissue has been described as dentin-like [25], bone-like [26], and as a reparative dentin bridge [27].

During dentin formation, odontoblasts synthesize and secrete several non-collagenous proteins into dentin extracellular matrix [28]. DSP is a special sialic acid-rich glycoprotein that is synthesized by these terminally differentiated odontoblasts [23]. Several immunohistochemical and molecular studies demonstrated that DSP is expressed exclusively

Figure 4. The relative ratio of newly formed reparative dentin to pulp cavity in hematoxylin and eosin histologic results. Area of mineralized tissue and pulp cavity was measured by Image J. Mineral trioxide aggregate (MTA) with osteostatin (OST) group significantly showed more reparative dentin formation than MTA alone group. *p < 0.05.
by odontoblasts and can serve as a specific marker for the odontoblast phenotype [28]. In the present study, positive DSP expressions were observed in all three groups.

For the success of vital pulp therapy, compact hard tissue formation without bacterial invasion is important. To achieve this, the sealing ability of the capping material is a crucial factor. Many studies demonstrated good sealing ability of MTA [29,30], but it was unknown that the combination of OST with MTA degrade the sealing ability of MTA. In the current study, all 3 groups formed a tight dentin bridge, and this suggests that the combination of OST with MTA could not affect the sealing ability of MTA.

Further studies will be needed to demonstrate the exact mechanism regarding the combined effect of MTA and OST and the odontogenic potential of OST alone on hDPCs in vivo.
CONCLUSIONS

The result of the current study suggests that OST can be a supplementary pulp capping material when MTA to make synergistic effect in hard tissue formation.

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