Pulpal response to mineral trioxide aggregate containing phosphorylated pullulan-based capping material

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INTRODUCTION

The preservation of vital pulp tissue is the goal of vital pulp therapy, which is an important minimal intervention protocol. Direct pulp capping (DPC), pulpotomy or pulpectomy are used to treat pulp exposure caused by trauma, caries or iatrogenic procedures, depending on the size, duration of time the pulp was exposed and the surrounding environment of the exposure site. DPC is a treatment procedure for exposed pulp when the pulp is visibly exposed due to trauma or caries, or due to a misadventure during tooth preparation or caries removal, and it is directly applied bioactive material over the exposed pulp tissue to facilitate pulp healing and repair dentin generation.

Formation of a complete mineralized tissue has been interpreted as a sign of healing and considered as a positive reaction in terms of prognosis of the exposed pulp treated with bioactive materials.

Calcium hydroxide (CH) has been considered the gold standard of DPC, owing to its biocompatibility, highly antimicrobial properties, and ability to form a mineralized tissue barrier. However, it still has several drawbacks: insufficient adherence to dentin wall, multiple tunnel defects in the mineralized tissue barrier, poor sealing ability and dissolution over time. Several bioactive materials have been introduced to overcome these drawbacks and it was reported that the success rates of bioactive materials are higher and results in less pulpal inflammation and superior quality of mineralized tissue formation (MTF) when compared to CH. One such material is mineral trioxide aggregate (MTA), which has demonstrated an excellent biocompatibility, induce homogenous MTF. Nevertheless, poor adhesion to tooth structure, difficulty in handling, long setting time, discoloration of crown and high costs were reported as the major drawbacks for MTA.

Therefore, the search for ideal biocompatible materials that can overcome these drawbacks for CH or MTA is of supreme importance. Pullulan (PL) is a polymer which is obtained from the fermentation of black yeast. As a biomaterial it is advantageous because, it is non-toxic and the hydroxyl groups within the pyranose rings of PL are available for substitution with...
phosphate groups\textsuperscript{12}. Therefore, phosphorylated pullulan (PPL) has been developed as a carrier for growth factors for bone tissue engineering, where its strong chemical bond that forms between phosphate group and hydroxyl group contributes to adhesion with hard tissue\textsuperscript{13}. Based on the above-mentioned properties of PL, a novel mineral trioxide aggregate containing phosphorylated pullulan (MTAPPL) pulp capping material was recently introduced to overcome the disadvantages of MTA specially the setting time, sealing ability and handling difficulty. The present study aimed to evaluate the pulpal responses of monkey’s pulp after DPC with the novel MTAPPL based material. The null hypothesis was that there is no difference in pulpal inflammation and MTF between the tested materials.

**MATERIALS AND METHODS**

This study was approved by the Institutional Ethical Committee of Hokkaido University (# 150-76) as well as by the Committee for Laboratory Animals and Breeding Facility in HAMRI CO. LTD (# IB14015).

The present investigation was designed with four experimental groups which are tabulated in Table 1. An experimental MTAPPL material was used in this study. NEX-MTA (NX) which is an MTA-based material, has been popularly employed as a suitable material in pulp capping agent was chosen as another experimental material. TheraCal LC (TH) is a calcium silicate-filled light-cured resin-modified material that has a higher calcium-releasing ability and lower solubility that was used in this study. Dycal (DY), whose main composition is CH, was chosen because it is the most used material due to its low price and wide availability.

**Study samples**

One hundred twenty teeth of 5 healthy 4 to 5 years’ old cynomolgus female monkeys were used in this study. Each monkey’s (non-carious) maxillary and mandibular incisors, premolars, and molars (first and second) teeth were treated. After the DPC procedure, 72 teeth were finally selected for histological evaluation, as described in the following section.

| Direct pulp capping materials | Manufacturers | Components | Preparation of materials |
|-------------------------------|--------------|------------|--------------------------|
| Newly developed mineral trioxide aggregate containing phosphorylated pullulan (MTAPPL) | GC | Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide, Phosphorylated pullulan | The powder was mixed according to the manufacturer instructions at water to powder ratio of 1:3 on a sterile glass slab for 1 min. |
| NEX-MTA (NX) | GC | Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide | The powder was mixed according to the manufacturer instructions at water to powder ratio of 1:3 on a sterile glass slab for 1 min. |
| TheraCal LC (TH) | Bisco | Portland cement, Bis-GMA, Strontium glass, Camphor Quinone | TH paste was directly applied on the pulp exposure site and then photo polymerized with a light-curing unit for 10 s. |
| Dycal (DY) | Dentsply Caulk | Base paste: 1,3-Butylene glycol disalicylate, Zinc oxide, Calcium phosphate, Calcium tungstate, Iron oxide pigments Catalyst paste: Calcium hydroxide, N-ethyl-o/p-toluene sulfonamide, Zinc oxide, Titanium dioxide, Zinc stearate, Iron oxide pigments (dentin shade only) Calcium hydroxide, N-ethyl-o/p-toluene sulfonamide, Zinc oxide, Titanium dioxide, Zinc stearate, Iron oxide pigments (dentin shade only) | Dycal was mixed according to the manufacturer instruction and applied directly on the pulp exposure site and was left undisturbed for 3 min. |

Bis-GMA: bisphenol A-glycidyl methacrylate
**DPC procedure**  
The monkeys were anesthetized by intramuscular injection of 2 mg/kg ketamine (Daiichi Sankyo Propharma, Kanagawa, Japan) and xylazine (Bayer Yakuhin, Osaka, Japan). After being anesthetized, the monkey's teeth were cleaned with physiological saline (Otsuka Pharmaceutical, Tokyo, Japan). Bowl-shaped cervical Class V cavities were prepared using a sterile FG #001 regular diamond bur (Horico Dental, Berlin, Germany) with a high-speed handpiece under copious amounts of water spray. Each cavity was then rinsed with copious amount of physiological saline to remove the cutting debris. Pulp was directly exposed with FG #001 regular diamond bur. Bleeding was controlled by pressing sterile cotton pellets for 2–3 min. After controlled the bleeding, the cavity was dried by gentle air blow and directly capped with each test material and was left undisturbed for 5 min. After DPC, G-BOND PLUS (GC, Tokyo, Japan) was applied to the cavity walls and left undisturbed for 10 s, followed by strong air blowing for 5 s and light cured for 10 s. All the cavities were then restored with MI FLOW II (GC) and light cured for 20 s followed by its manufacturer instruction. After the DPC, antibiotics (Enrofloxacin, Bayer Medical, Tokyo, Japan) were intramuscularly administered at 10 mg/0.4 mL/kg for 3 days to prevent suppuration. To reduce the pain, buprenorphine hydrochloride injection 0.2 mg, (Otsuka Pharmaceutical) was intramuscularly administered at 20 μg/0.1 mL/kg once a day for 3 days. The monkeys were observed postoperatively after DPC at three observational time intervals of 3, 7, and 70 days. Six teeth were allocated for each experimental period. Out of 120 teeth, 48 teeth were excluded due to excessively large cavities, large pulp exposure, did not have hemorrhage at the time of pulp exposure, and having dislodge restoration.

**Fixation**  
After completion of each observation period, the monkeys were sacrificed using an intraperitoneal injection with an overdose of the anesthetic solution. Each jawbone was dissected along with all teeth and then the teeth were removed carefully. After that, the teeth were fixed with 10 % neutral buffered formalin solution (Wako Pure Chemical, Tokyo, Japan) at 4°C for 7 days.

**Tissue preparation**  
The teeth were decalcified with Plank Rychlo's decalcifying solution (combination of aluminium chloride-70 g, 95% formic acid-50 mL, 36% hydrochloric acid-85 mL, distilled water-1,000 mL) at room temperature for 3 days. After decalcification, the resin composite was carefully removed from the cavity and then rinsed with running water for 4 days. They were then dehydrated in ascending grades of ethanol, dealcoholized by xylene, and embedded in paraffin. Serial sections of 4 μm thicknesses were cut using a sliding microtome (Retoratome REM-710, Yamato Kohki Industrial, Saitama, Japan) and subsequently stained with Mayer’s hematoxylin-eosin.

**Evaluation criteria for observations**  
The stained sections were observed under a light microscope (Nikon, Tokyo, Japan) for inflammatory cell infiltration (ICI) and MTF for all time point, and odontoblast-like cell layer formation (OCL) were used for only 70 days. The criteria set for the observations were as shown in Table 2 which was followed by Lee et al.

**Statistical analysis**  
The results of the histopathological evaluation were analyzed using the Kruskal-Wallis test and Mann-Whitney U test followed by Bonferroni's post-hoc, for differences between the groups during each observation period.
period. The significance level was set at 0.05.

**Observation of DPC material and dentin interface**

Four extracted non-carious human molars were collected for this study under a protocol reviewed and approved by the Institutional Ethical Committee (# 2014-1) in Hokkaido University. The teeth were thoroughly cleaned and kept in a 0.5% Chloramine-T solution, under refrigeration at 4°C and used within 6 months of extraction.

Flat dentin surfaces were then ground with #600 SiC paper (Sankyo Rikagaku, Saitama, Japan) for 60 s under continuous water-cooling to produce a standardized smear layer prior to the application of DPC materials. After DPC, bonding and cavity filling with resin composite were done in the same method as mentioned before. Then the specimens were stored in water at 37°C for 24 h. They were then sectioned perpendicular to the resin-dentin interface to obtain two parallel 2 mm-thick slabs from each tooth. The exposed interfaces were subsequently polished with #600, 800 and 1000 SiC papers under running water, followed by polishing with 6, 3 and 1 μm diamond pastes (DP-Paste, Struers, Tokyo, Japan), and cleaned with an ultrasonic device between each step of polishing. The specimens were then immersed in 1 M hydrochloric acid for 30 s and 5 percent sodium hypochlorite for 5 min, followed by rinsing with water. After drying in a desiccator overnight, the specimens were sputter coated with Pt-Pd and observed under SEM (S4000, Hitachi, Tokyo, Japan) with an accelerating voltage of 10 kV.

**RESULTS**

**Inflammatory cell response**

The results of the post-surgical histopathological evaluation after 3, 7 and 70 days were summarized in Fig. 1. Representative images obtained from HE stained sections are shown in Figs. 2–4.

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**Fig. 1** Box plot of data.

(A) Inflammatory responses at day 3. (B) Inflammatory responses at day 7. (C) Inflammatory responses at day 70. (D) MTF at day 3. (E) MTF at day 7. (F) MTF at day 70. (G) Odontoblast-like cell formation at day 70. Different lowercase letter (a–d) indicates statistically significant difference between the groups (p<0.05). MTAPPL, mineral trioxide aggregate containing phosphorylated pullulan; NX, NEX MTA; TH, Theracal; DY, Dycal.
On day 3, MTAPPL showed no to mild inflammatory cell responses compared to other experimental groups. Two specimens out of six showed mild inflammatory cell responses and four specimens showed no inflammation. NX group showed no to moderate inflammatory responses. One specimen out of six showed no inflammatory cell responses and one specimen showed mild inflammatory responses, whereas four specimens showed moderate inflammatory cell responses. TH groups showed no to moderate inflammatory responses. Two specimens out of six showed no inflammatory cell responses, two specimens showed mild inflammatory responses, and two specimens showed moderate inflammatory cell responses. DY groups showed mild to moderate inflammatory cell responses. Two specimens out of six showed mild inflammatory cell responses and four specimens showed moderate inflammatory responses. There is a significant difference between MTAPPL with NX and DY (p<0.05). In addition, the NX and DY groups showed partial necrosis at the exposure site. At the exposure site, there were scattered inflammatory cells, mostly polymorphonuclear leucocytes, indicating an acute inflammatory response.

On day 7, MTAPPL showed no inflammatory cell responses compared to other experimental groups. All specimens showed no inflammatory cell responses. NX showed no to mild inflammatory cell responses. Five specimens out of six showed no inflammatory cell responses and one specimen showed mild inflammation. TH showed no to mild inflammatory cell responses. Five specimens out of six showed no inflammatory cell responses and one specimen showed mild inflammation.
DY showed no to mild inflammatory cell responses. Three specimens out of six showed no inflammatory cell responses and three specimens showed mild inflammation. No significant difference among the groups was observed ($p>0.05$).

No ICI was observed in all the groups at day 70. No significant difference was observed between the groups.

**MTF**
On day 3, no MTF was observed in all the groups.

On day 7, MTAPPL group showed no to partial mineralized tissue deposition. One specimen out of six showed no mineralized tissue deposition and four specimens showed initial mineralized tissue deposition and one specimen showed partial mineralized tissue deposition. NX group showed no to initial mineralized tissue deposition. Three specimens out of six showed no mineralized tissue deposition and three specimens showed initial mineralized tissue deposition. TH group of all the specimens showed initial mineralized tissue deposition. DY group of all specimens showed no mineralized tissue deposition. Significant difference was observed between MTAPPL and DY, TH and DY ($p<0.05$).

On day 70, MTAPPL, NX and TH group showed complete MTF at the exposure site. DY group showed initial to complete MTF. Two specimens out of six showed initial mineralized tissue deposition, one specimen showed partial mineralized tissue deposition and three specimens showed complete MTF. Significant difference was observed between MTAPPL and DY, NX and DY, TH and DY ($p<0.05$).

**OCL**
On day 70, complete OCL was observed in the group MTAPPL, NX and TH. DY group showed no to initial OCL beneath the mineralized tissue. Four specimens out of six showed no OCL and two specimens showed initial OCL, significantly different from MTAPPL, NX and TH ($p<0.05$).

**Observation of the DPC material and dentin interface**
SEM images obtained from the interface between the pulp capping material and dentin are shown in Fig. 5. MTAPPL adhered well to the dentin surface indicating superior sealing ability (Fig. 5A). Other capping materials did not adhere to the dentin surface and showed separation at the interface under the high vacuum condition of SEM sample chamber (Figs. 5B, C and D).

**DISCUSSION**
Even though many researchers have been looking for an ideal pulp capping material for a long time, the search continues with the development of newer materials. MTA, which was introduced over 20 years ago, has been widely accepted among dental clinicians in spite of its high cost\(^1\). PPL is a biomaterial that has recently gained attention because of its bioadhesive behavior, high biocompatibility, and tissue regeneration ability. PL has high biocompatibility and biosafety\(^2\). It has been used as a coating of food and packaging material in food industry and pharmaceuticals, such as the cover of capsules\(^3\). Moreover, it can be molded into nanoparticles or nanogels which have been utilized for efficient drug delivery\(^4,5\). On the other hand, MTA is proven as a DPC material with excellent biocompatibility and higher success rate\(^6,7\). However, it has some drawbacks such as long setting time, high cost, poor sealing ability and difficult handling characteristics\(^8,9\). Since PL can form steady and monodisperse nanogels\(^10\), therefore, PPL self-aggregate might have the potential for making a stable complex with MTA thus improving the handling properties. Furthermore, PPL can also adhere to the hydroxyapatites of mineralized tissues, such as a bone\(^11\). Therefore, PPL mixed with MTA might have the potential as a pulp capping material with a better adhesive performance thus improving the sealing ability. According to the manufacturer, PPL can set within 5 min. Adding PPL to the MTA combination might contribute to reduction of setting time and treatment time.

In this present study, pulpal response to DPC with newly developed MTAPPL was evaluated using monkey teeth. Based on the results of the present study, statistically significant differences were observed between the materials ($p<0.05$), therefore, rejecting null hypothesis. After 3 days of post-surgical evaluation, ICI was observed in all the groups. MTAPPL group showed less ICI whereas an extensive inflammatory cell with local infiltration of polymorphonuclear leucocytes was observed at the exposed pulp in the NX and DY groups.
(p<0.05). It was reported that any material intended to be directly exposed to pulp tissue should be able to maintain the inflammatory response to a minimum level, not passing beyond the stage that will lead to pulp necrosis\textsuperscript{29}. Based on the results of the present study, MTAPPL showed less inflammatory responses compared to other experimental groups in all time point. Therefore, our results confirm that the MTAPPL biomaterial is a safe option for DPC material.

This study also showed that the initial deposition of the mineralized tissue in the MTAPPL, NX, TH group except for DY group. This observation can increase the sealing ability, prevent bacterial leakage, and stimulate the reparative capacity of pulp cells through the deposition of calcium phosphate minerals along with the dentin-material interface\textsuperscript{30}. Similar studies were shown that pulp capped with MTA after DPC can induce initial deposition of mineralized tissue at day 7\textsuperscript{31-33}. In comparison to the DY, MTAPPL, NX and TH showed significantly better MTF after 70 days. Layers of well-arranged odontoblast and odontoblast-like cells were found, and tubular dentin formed under the osteodentin. No tunnel defects were observed in all the groups, but some pulp tissue was observed in the vicinity of the mineralized tissue barrier in MTAPPL group. The observation could be attributing the faster formation of the mineralized tissue. These areas of pulp tissues tended to decrease over time\textsuperscript{34}. Reported studies showed that these pulp tissues could be more mineralized and regular as barrier matures and begins tubular dentin formation with time\textsuperscript{34,35}. With continuing mineralization and maturation, the irregular pattern of the mineralized tissue could be replaced by a regular tubular pattern and the entrapped pulp tissue’s disappearance\textsuperscript{36}. Observation of a complete MTF agrees with previous studies that observed similar results with other MTA-based pulp capping materials\textsuperscript{37,38}.

The sealing ability of MTAPPL to the human dentin was also compared with NX, TH and DY (Fig. 5). In the present study, the SEM observation showed no separation between MTAPPL and dentin even under the high vacuum conditions of the SEM sample chamber, suggesting its excellent sealing performance as a pulp capping material. On the other hand, NX, TH and DY did not adhere well to dentin and showed separation in the dentin-interface. These gaps may subsequently lead to leakage and bacterial infection. Therefore, MTAPPL biomaterial exhibit excellent sealing ability to dentin and excellent pulpal response in this study.

**CONCLUSION**

Based on this study, we can conclude that MTAPPL showed excellent healing reaction to the pulp and can induce complete MTF. The MTAPPL showed excellent handling properties, better sealing ability and reduction of setting time. Therefore, MTAPPL could be a promising pulp capping material.

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