博 士 論 文

Pulpal response to capping with MTA containing phosphorylated pullulan
（リン酸化プルラン含有 MTA セメントの歯髄に対する反応）

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Abstract

Direct pulp capping materials ideally promote the formation of dentin bridge. Mineral trioxide aggregate is mostly used for direct pulp capping. However, this material showed difficult sealing ability and poor handling. Phosphorylated pullulan has recently gained attention because of its high biocompatibility, bioadhesive behavior and dentin regeneration ability. It shows high biocompatibility and is capable to act as a carrier and can adhere to hard tissue. The purpose of this study was to histologically evaluate monkeys' pulpal responses to a newly developed material phosphorylated pullulan containing mineral trioxide aggregates as a direct pulp capping and to evaluate its sealing ability using scanning electron microscopy (SEM). Cavities were prepared in monkey's teeth. The pulps were intentionally exposed and randomly divided into four groups according to the application of pulp capping materials – a newly developed direct pulp capping material containing phosphorylated pullulan (PL), conventional direct pulp capping materials such as NEX-MTA cement (NX), Theracal LC (TH) and Dycal (DY). The teeth were then extracted after 3, 7 and 70 days, fixed and prepared according to routine histological

techniques to observe pulpal reactions. Tissues were demineralized and subsequently sectioned. Sections were stained with hematoxylin / eosin for micromorphological observation. SEM observation was performed to study the pulp capping material / dentin interface.

No serious inflammatory reactions of the pulp, such as necrosis or abscess formation were observed in PL group. For PL, dentin bridge was very thick and dentinal tubule and odontoblast cells were observed at 70 days. SEM observation revealed good sealing ability of PL.

Key Words: Restorative dentistry, Adhesives, Bioengineering, Biocompatibility, Biomaterials

INTRODUCTION

Dental pulp is the neurovascular bundle providing nutritive function and protective system including immune defense reactions and subsequent tissue repair [1]. When dental prevention and conservative management fails to contain caries progression, it requires operative intervention to prevent lesion advance. When caries penetrates into dentin, the carious dentin with bacterial infection zone needs to be excavated. Excavation of deeper layers of carious lesion sometimes results in pulp exposure. Direct pulp capping is indicated for such mechanical exposures [2, 3]. In such conditions, dental materials as therapeutic base or liner are placed over the pulpal wound to facilitate the formation of dentin bridge [4].

Direct pulp capping material containing calcium hydroxide ($Ca(OH)_2$) and mineral trioxide aggregate (MTA) are mostly used for direct pulp capping [5]. Formation of a dentinal bridge has been interpreted as a sign of healing and considered a positive in terms of prognosis of the exposed pulp. The success rate of direct pulp capping procedure ranges from 30 to 85 percent in two- to 10-year retrospective studies respectively [6, 7].

Till date, $Ca(OH)_2$ is considered the gold standard because of its

antibacterial properties due to its high pH value (approximately 10) and its ability to stimulate the pulp to form dentin bridge through cellular differentiation, extracellular matrix secretion and subsequent mineralization [8, 9]. However, reports suggest faster dissolution of $Ca(OH)_2$ when compared to MTA. Moreover, $Ca(OH)_2$ shows thin dentin bridge formation and tunnel defect within dentin bridge [10].

MTA has been recognized as a bioactive material and has been used for pulp capping with high success rate [11-13]. It contains calcium oxide, silicon dioxide, and bismuth oxide [14]. When MTA powder is mixed with water, calcium hydroxide and calcium silicate hydrate are initially formed sustaining high alkaline pH [15]. MTA showed better biocompatibility over $Ca(OH)_2$ by promoting complete dentin bridge formation with less pulp inflammation [16, 17]. Nevertheless, long setting time, poor handling characteristics and discoloration of crown and gingiva were reported as the major drawbacks for MTA [18, 19].

The search for biocompatible materials and scaffolds that can stimulate the cells for dentin bridge engineering is a matter of concern. A recently developed material named phosphorylated pullulan (PL) has several

characteristic to improve these defects. Pullulan is the polymer obtained from the fermentation medium of black yeast [20]. Its advantage as a biomaterial is it is non-toxic [21]. Moreover, hydroxyl groups within the pyranose rings of pullulan are available for substitution [22]. PL from strong chemical adhesion with hard tissue by replacing the hydroxide group of hard tissue with its phosphate groups and acts as a carrier for growth factors for bone tissue engineering [23].

Considering the potential of phosphorylated pullulan as a direct pulp capping material, attempt was made to develop a novel pulp capping material containing phosphorylated pullulan and MTA. The purpose of the present study was (i) to evaluate the morphologic response of newly developed PL as a direct pulp capping material comparing with other conventional materials, and (ii) to evaluate their sealing ability. It was hypothesized that this novel material would show high biocompatibility, adhesive ability to hard tissue, better dentin bridge formation and less inflammation as a direct pulp capping material.

MATERIALS AND METHODS

1. Experimental Animals

Ninety two teeth of five healthy 3 or 4-year-old cynomolgus male monkeys were used in this experiment which was approved by the local laboratory animal committee of breeding facility (Breeding facility exam number is IB14015, Ethical committee number is 150-76). The teeth were divided into 4 groups and each group was allocated randomly to the experimental materials. The monkeys were fed one time in a day between 9 and 11 in the morning and allowed to drink water freely in the cages of the animal research center.

2. Experimental Materials

The materials used in this study are shown in Table 1. As the direct pulp capping material, a newly developed MTA direct pulp capping material containing PL (GC), conventional direct pulp capping materials such as NEX-MTA cement (NX, GC), Theracal LC (TH, Bisco) and Dycal (DY, DENTSPLY) were used.

As bonding material, one-step self-etch adhesive (G bond plus; GC) was used. After dental surface treatment was completed in all the experimental

groups, flowable composite (MI flow 2; GC) was applied. After direct pulp capping, the monkeys were observed postoperatively for three observation periods of 3, 7 and 70 days. They were sacrificed on these days to provide specimens for histopathological examination.

3. Cavity preparation

The monkeys were anesthetized by intramuscular injection of 2 mg/kg ketamine (KETALAR, Daiichi Sankyo Propharma) and xylazine (Selactar, Bayer Yakuhin). The teeth were rinsed with physiological saline for the removal of remaining debris on the tooth surface (Otsuka Pharmaceutical). Bowl-shaped cervical class V cavities, with diameters of 0.5mm approximately, were prepared on the buccal marginal ridge of all teeth using a FG #1 regular cut diamond point with a high-speed electric handpiece under copious amounts of water spray. Each cavity was then rinsed with copious amounts of physiological saline (Otsuka Pharmaceutical) and gently air dried. Then direct pulp capping was done in the following procedure: PL or NX was applied directly to the pulp, and the surface was left undisturbed for 180 s. TC was applied to the pulp, and then photo polymerized with a light-curing unit (PENCURE, Morita) for 10 s. DY

was applied drectly to the pulp, and the surface was left undisturbed for 180 s. After direct capping, adhesive material (G-BONDPLUS, GC) was applied to the cavity and was left undistrubed for 10 s followed by strong air blowing. After that, adhesive was light cured for 10 s. All the cavities were restored with a hybrid restorative resin composite (MI FLOW, GC) and light cured for 20 s. The cavities for each experimental material were subdivided into 4 subgroups for the experimental periods set at 3, 7, and 70 days. The teeth which had excessively large cavities and pulp exposure, did not have hemorrhage at the time of pulp exposure, and those suspected of having fractures were excluded from this study.

4. Perfusion fixation

After completion of each observation period, the monkeys were sacrificed using intraperitoneal injections and with an overdose of anesthetic solution mentioned above. Each jaw bone was removed. And teeth were removed carefully from each haw bone. After that, teeth were fixed by transcranial vital perfusion with 10% buffered formalin solution (10% Formalin Solution, Wako Pure Chemical Industries) at 4℃ for further fixation.

5. Tissue preparation and serial sectioning

Tissues were decalcified with Plank Rychlo's decalcifying solution ($AlCl_3.H_2O$: 70g; 95% formic acid: 37% hydrochloric acid: 85mL; distilled water: 865mL) at room temperature for 1 day. After decalcification, the resin composite was carefully removed from the cavity and rinsed with PBS solution for 4 days. They were then dehydrated in ascending grades of ethanol, dealcoholized by xylene, and embedded in paraffin. Serial sections of 4μm thickness were cut using a sliding microtome (Retoratome REM-710, YAMATO KOHKI INDUSTRIAL) and alternatingly stained with Mayer's hematoxylin-eosin, reticulin silver impregnation stain.

6. Observation items and evaluation criteria

The stained sections were observed under a light microscope (Eclipse E800, NIKON INSTRUMENTS), and the following parameters were evaluated: inflammatory cell infiltration (ICI), odontoblast-like cell layer (OCL), dentin bridge formation (DBF).

6.1 Inflammatory cell infiltration (ICI) at 3, 7 and 70 days

0. No reaction was characterized by an absence of inflammatory cells (none);

1. Mild reaction was characterized by the scattering of a small number of inflammatory cells (mild);

2. Moderate reaction was characterized by a distinct increase in inflammatory cells (moderate);

3. Severe reaction was characterized by abscess formation in the pulp or pulpal necrosis (severe);

6.2 Odontoblast-like cell layer (OCL) at 3, 7 and 70 days

0. No odontoblast-like cell layer formation (none).

1. Initial odontoblast-like cell layer extending to not more than one-half of the exposure site (initial).

2. Partial/incomplete odontoblast-like cell layer extending to more than one-half of the exposure site but not completely closing the exposure site (partial)

3. Complete odontoblast-like cell layer formation (complete)

6.3 Dentin bridge formation (DBF) at 3, 7 and 70 days

0. No dentin bridge formation (none)

1. Initial dentin bridge formation extending to not more than one-half of the exposure site (initial).

2. Partial/incomplete dentin bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site (partial).

3. Complete dentin bridge formation (complete).

7. Observation of direct pulp capping material and dentin interface

Four extracted non-carious human molars were collected for this study under a protocol reviewed and approved by the local Ethical Committee (2014-1).

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 surface was obtained by removing the coronal enamel of each tooth with a gypsum model trimmer under water coolant, leaving the surrounding enamel. The dentin surfaces were then ground with #600 SiC paper for 60 s under continuous water-cooling to produce a standardized smear layer prior to the application of direct pulp capping materials.

After direct pulp capping, bonding and composite resin build up were done following the manufacturer's instruction. After storage in 37°C water for 24 h, they were then sectioned perpendicular to the resin-dentin interface

to obtain two parallel 2 mm-thick slabs. 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), and cleaning with an ultrasonic device between each step of polishing. After polishing, specimens were immersed in 1 M hydrochloric acid for 30 s and 5% 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 scanning electron microscope (SEM; S4000, Hitachi) with an accelerating voltage of 10 kV.

8. Statistical analysis

The results of the histopathological evaluation were statistically analyzed using the Kruskal-Wallis test and the Bonferroni-corrected Mann-Whitney U test post-hoc, testing for differences between the groups during each observation period. Statistical procedures were performed at significance level of 0.05 using the statistical software (SPSS 17.0J Base System SX, IBM SPSS Japan).

RESULTS

1. Inflammatory cell infiltration findings

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

After 3 days, the total examined of the pulps for all the experimental materials showed slight to moderate inflammatory cell infiltrations at the exposure site. The obtained score of reactions were ranged from 0.56 to 1.5. The number of pulps with the reactions was dependent on the materials and significant difference was observed. PL showed significantly less inflammatory reactions ($p < 0.05$). When the pulp exposure was covered by PL, 5 pulps out of 9 teeth showed slight inflammatory cell infiltrations and 4 pulps showed no reactions. After 7 days, the reactions for all the materials were gradually reduced and no significant difference among the groups was observed. The average score of reactions at 7 days were ranged from 0.14 to 0.67. At 70 days, all the groups showed no inflammatory reaction.

2. Formation of odontoblast-like cell layer

After 3 days, no odontoblast-like cell layer was formed at the wound area for any capping material. After 7 days, PL induced formation of odontoblast-like cell layer in the pulps of 5 out of 6 teeth. The average score

of reactions at 7days were ranged from 0 to 2.83. PL caused significantly higher amount of odontoblast-like cell layer formation after 7 days in comparison with other materials ($p < 0.05$). At 70 days, direct pulp capping materials containing MTA exhibited significantly higher scores ($p < 0.05$). The average score of reactions at 70 days were ranged from 0.5 to 3.

3. Dentin Bridge Formation findings

The dentin bridge formations observed in this experiment were shown in Fig. 1. No dentin bridge formation was formed after 3 days. However all the exposure site of the pulp was occluded by the dentin bridge after 70 days. The average score of reactions at 7 days were ranged from 0 to 1. PL showed significantly prompt dentin bridge formation over the other capping materials. After 70 days, PL, NX and TH showed thick dentin bridge formation with dentinal tubule like structure in many cases. Although not significantly different from other materials, average dentin bridge formation score of DY was lowest (2.17; Table.2).

4. Observation of the direct pulp capping material and dentin interface

SEM images obtained from the interface between the pulp capping material and dentin are shown in Fig. 2. PL adhered to the dentin surface and

showed superior sealing ability. Other capping materials containing MTA, *e.g.* NX and TH, did not adhere to the dentin surface and showed the separation at the interface under the highly vacuum condition within the SEM sample chamber. DY showed gap formation within the material near the interface to left the remnant of material on the dentin surface.

DISCUSSION

In this experiment, pulpal response to direct pulp capping with newly developed material containing MTA and phosphorylated pullulan was evaluated using monkey teeth. The sealing ability of the material to the human dentin was also compared with the commercially available dental component containing MTA or $Ca(OH)_2$ (Fig. 2). The material containing MTA and phosphorylated pullulan showed excellent healing reaction to the exposed monkey pulp. When the pulp was sealed with phosphorylated pullulan, the lightest inflammatory reaction was observed after 3 days and showed significant amount of dentin bridge formation over DY after 70 days.

Pullulan has several advantages as a pulp capping material. It has high biocompatibility and biosafety [24]. It can be used as a coating of food and

packaging material in food industry [25, 26]. Moreover, it can be derived via various chemical reactions to increase its utility in the field of pharmaceuticals [27, 28]. It can be molded in nanoparticles or nanogels which have been reported for efficient drug delivery [29, 30].

MTA is considered as a direct pulp capping material with excellent biocompatibility and higher success rate [16, 31]. However, it has some known drawbacks such as long setting time, high cost, and difficult handling characteristics [32-34]. The handling of MTA requires practice [35]. On the other hand, pullulan forms steady and monodisperse nanogels [36]. The phosphorylated pullulan self-aggregate is potent for making a stable complex with MTA. This characteristic provides the opportunity of one lump application of MTA. It might contribute to reduction of treatment time, covering the whole pulpal surface area and sealing all the cavity walls. Furthermore phosphorylated pullulan adheres to the hydroxyapatite [37]. The phosphorylated pullulan mixed with MTA appears to have the potential as a pulp capping material with an adhesive performance please define acceptable. Indeed our SEM observation showed no separation between PL and dentin even under the highly vacuum condition, suggesting its excellent

sealing performance as a pulp capping material (Fig. 2).

In most of the cases, post-surgical evaluation after 3 days showed distinct amount of hemorrhage within the pulp due to the pulpal exposure. Inflammatory cell infiltrations were observed in many cases, though its level was significantly different among the capping materials. PL showed the lowest level of inflammatory cell infiltration, whereas DY showed the highest. After 7 days, the exposed pulp sealed with PL showed the migration of odontoblast-like cells underneath the material, suggesting the healing process has taken place. It is noteworthy that 5 pulps out of 6 showed dentin bridge formation with partially covering the wound surface despite the thickness was thin. These finding are in accordance with previous studies that showed excellent biocompatibility of this material. Other materials (NX, TH, DY) could not induce odontoblast-like cell formation at the exposure site and did not show dentin bridge formation at the 7 days postoperative interval.

MTA materials have been shown to have a biocompatible nature and have excellent potential for direct pulp capping [38]. Comparing to the calcium hydroxide, the materials containing MTA cements showed significantly

better formation of dentin bridge after 70 days. Layers of well-arranged odontoblast and odontoblast-like cells were found to form tubular dentin under the osteodentin. It was reported that MTA significantly upregulated the level of bone morphogenetic protein (BMP)-2 [39]. Moreover, it was indicated that MTA is suitable to stimulate Nestin and Osteopontin [40] .

Nonetheless, $Ca(OH)_2$ and MTA do not adhere to the hard tissue such as bone and dentin and degrades gradually. Because of the marginal leakage, bacterial infection and inflammation also occur. Bacterial contamination is a significant issue. To increase the success rate of direct pulp capping, it is necessary to prevent bacterial invasion. Since PL showed excellent sealing ability to dentin and excellent pulp response in the monkey study, it is reasonable to conclude that PL is a suitable material for the direct pulp capping.

This in vivo study, only evaluated the pulpal responses and sealing ability after 3, 7 and 70 days. In future, experiment should be aimed to observe the much earlier and medium days groups.

Further experiments are also necessary to clarify the healing mechanism. Immunostaining method shows specific cells such as dentin morphogenetic

cells. By staining signal producing cytokines, the differences of dentin bridge developing ability between PL and other groups could be detected.

CONCLUSION

PL provided acceptable pulpal responses and biological compatibility to the monkey pulp, comparable with that of calcium hydroxide (DY). Furthermore it showed good sealing ability.

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Table. 1. Direct pulp capping materials used in the present study

Direct pulp capping materials	Manufacturers	Components	
Newly developed direct pulp capping material containing Phosphorylated pullulan (PL)	GC	Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide, Phosphorylated pullulan	
NEX-MTA (NX)	GC	Calcium oxide, Bismuth oxide, Silicon dioxide, Aluminum oxide	
TheraCal LC (TH)	Bisco	Portland cement, Bis-GMA, Strontium glass, Camphor Quinone	
Dycal (DY)	DENTSPLY	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)

Table. 2. ICI, OCL and DBF

		n	Inflammatory cell infiltration					Odontoblast-like cell layer or odontoblast cell layer					Dentin bridge formation				
			0	1	2	3	Av	0	1	2	3	Av	0	1	2	3	Av
PL	3 days	9	4	5	0	0	0.56	6	0	0	0	0	9	0	0	0	0
	7 days	6	6	0	0	0	0	0	0	1	5	2.83	1	4	1	0	1
	70 days	9	9	0	0	0	0	0	0	0	9	3	0	0	2	7	2.78
NX	3days	6	1	1	4	0	1.5	6	0	0	0	0	6	0	0	0	0
	7days	10	9	1	0	0	0.1	9	0	0	0	0	9	0	0	0	0
	70 days	8	8	0	0	0	0	0	0	1	7	2.88	0	0	1	7	2.88
TH	3 days	9	5	2	2	0	0.67	9	0	0	0	0	9	0	0	0	0
	7 days	7	6	1	0	0	0.14	7	0	0	0	0	7	0	0	0	0
	70 days	8	8	0	0	0	0	0	0	0	8	3	0	0	0	8	3
DY	3days	7	1	2	4	0	1.43	7	0	0	0	0	7	0	0	0	0
	7days	7	4	3	0	0	0.43	7	0	0	0	0	7	0	0	0	0
	70 days	6	6	0	0	0	0	3	3	0	0	0.5	0	2	1	3	2.17

FIGURES

Fig. 1

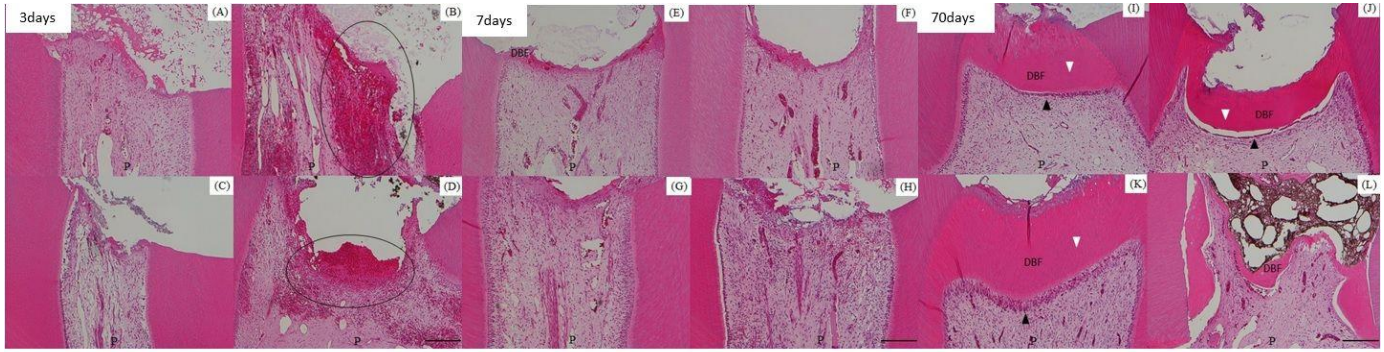
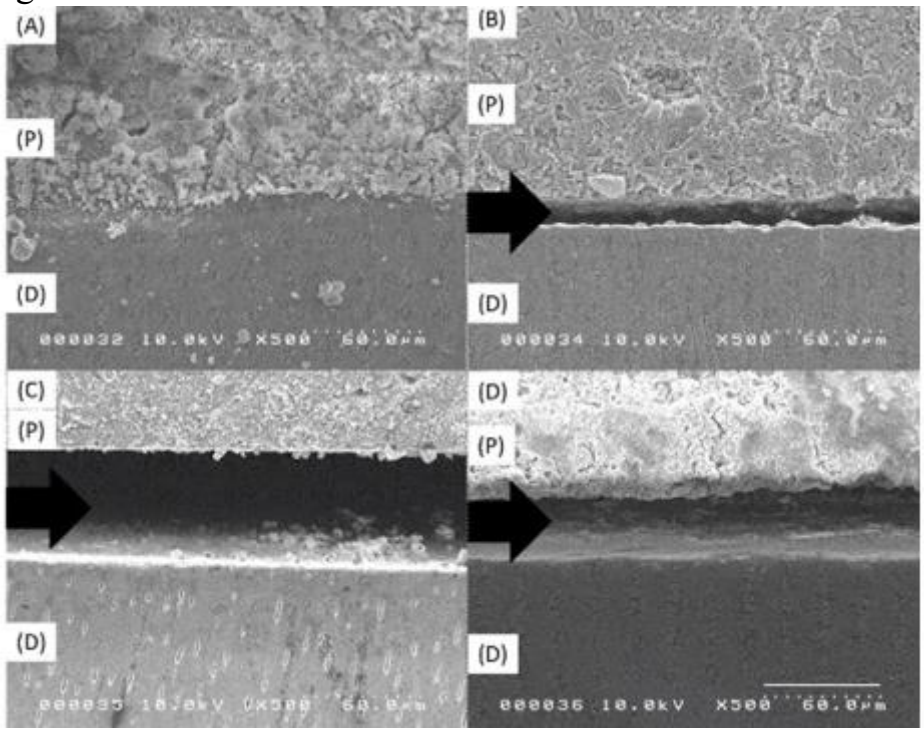


Fig. 2


(A)
(P)
(D)
000032 10.0kV X500 60.0µm
(B)
(P)
(D)
000034 10.0kV X500 60.0µm
(C)
(P)
(D)
000035 10.0kV X500 60.0µm
(D)
(P)
(D)
000036 10.0kV X500 60.0µm

FIGURES AND LEGENDS

Fig. 1 – ICI, OL, DBF findings were shown. At 3 days, the least inflammatory cell reactions were observed. ; (original magnification, ×200) **(A)**. NX; more inflammatory reactions were observed. (original magnification, ×200) **(B)**. TH; less inflammatory reactions were observed. ; (original magnification, ×200) **(C).** DY; more inflammatory reactions were observed. ; (original magnification, ×200) **(D)**. At 7 days, PL, NX and DY; less inflammatory reactions were observed. ; PL showed dentin bridge formation. ; (original magnification, ×200) (**E**) (**F**) (**G**) (**H**). At 70 days, PL, NX and TH; complete thick dentin formation, dentinal tubule like structure and regularly arrangement of the odontoblast cells were observed. ; (original magnification, ×200) **(I) (J) (K)**. DY; complete dentin formation was observed. However, dentin formation was thin and regularly arrangement of the odontoblast cells was not observed. ; (original magnification, ×200) **(L)**. P, pulp; DBF, dentin bridge formation, black circle; inflammatory reaction, white triangles; dentinal tubule like structure, black triangles; regularly arrangement of the odontoblast cells, arrows; gap formation. The bars represent 100 μm.

Fig. 2 - SEM images of dentin-pulp capping material interface. PL; adhered to the dentin surface and showed no gap formation, **(A)**; (original magnification, ×500), NX, TH and DY showed gap formation to the dentin surface. **(B)**, **(C)**, **(D)**; (original magnification, ×500), D, Dentin; P, Pulp capping material. The bar represents 60 μm.