Histopathological and immunohistochemical study on the effects of a direct pulp capping experimentally developed adhesive resin system containing reparative dentin-promoting agents

Chikage KATO¹, Masaya SUZUKI², Koichi SHINKAI² and Yoshiroh KATO²

¹Advanced Operative Dentistry-Endodontics, Graduate School of Life Dentistry at Niigata, The Nippon Dental University, 1-8 Hamaura-cho, Chuo-ku, Niigata 951-8580, Japan
²Department of Operative Dentistry, School of Life Dentistry at Niigata, The Nippon Dental University, 1-8 Hamaura-cho, Chuo-ku, Niigata 951-8580, Japan

Corresponding author, Chikage KATO; E-mail: chigeru1514@yahoo.co.jp

INTRODUCTION

In the clinical setting, we often encounter cases of exposed dental pulp and, at times, those of not overtly exposed pulp as well. For the treatment of such cases, it is common practice to perform either direct or indirect pulp capping using calcium hydroxide or its preparations. This procedure has long been supported by the majority in dental practice. Having said that, it should be noted that this chemical irritates vital tissue because of its high alkalinity, although irritation lasts only 24 hours or so¹,²). This may result in the formation of a necrotic pulp layer near the exposed pulp area where calcium hydroxide or its preparation has been applied and materially reduce the volume of the remaining vital pulp — and this is one drawback of this procedure³-⁵). Moreover, it has been reported that the exacerbation of pulpal tissue inflammation⁶-⁸), an extensive formation of an abscess and tissue loss could ensue. Thus, today, many researchers have come to perceive that this pulp capping technique is not always a reliable therapeutic means⁹). Besides, the mechanical strength of any calcium hydroxide agent is low, and its adhesiveness to dentin can hardly be expected. Such being the properties, it is almost impossible to apply the calcium hydroxide agent to pulp that has accidentally been exposed during preparation of the abutment teeth and external cavities.

On the other hand, resin composites used for restorative purposes have now become indispensable in dental offices because they excel in mechanical strength as well as adhesiveness. Formerly it had been thought that resin composite had a tendency to irritate pulp tissue by polymerization catalysts such as tert-amine, high coefficient of thermal expansion and polymerization shrinkage. As a result, direct pulp capping of resin composite has not been recommended. But recently basic concepts have been changing due to accumulation of advances in research. It is now understood that the main cause of pulp irritation of resin composite restoration is bacterial infection or chemical irritation induced by microleakage and gap formation between the cavity wall and the restoration⁹-¹¹). Some reports say that the use of resin composites as either pulp capping or restorative material is valid, depending on the type of resin. Many researchers are interesting in using resin composites in place of calcium hydroxide preparations for capping exposed pulp¹²-¹⁷).

The studies so far conducted by our colleagues in relation to dental pulp capping using adhesive resins revealed that the adhesive resins are useful for capping exposed pulps but are a little slower to take effect on the injured pulp tissue during the initial stage: up to 90-days after pulp exposure, compared with calcium hydroxide and its preparations. In the present study, an experimentally developed adhesive resin system was applied in direct pulp capping and restoration and the healing process was examined histopathologically and immunohistochemically. The resin system was manufactured with calcium phosphate added into the bonding material for the purpose of accelerating the healing process. The largest amount of reparative dentin was formed by SE5 (whitlockite 5 wt%), followed by SE9 (hydroxyapatite 5 wt%, whitlockite 5wt%), SE1 (hydroxyapatite 5 wt%), and SE2 (hydroxyapatite 10 wt%). Generally, it could be said that the experimental groups using whitlockite and hydroxyapatite had the tendency to produce a larger amount of reparative dentin.

Keywords: Direct pulp capping, Bonding agent, Adhesive resin monomer, Reparative dentin-promoting agents, Calcium-phosphate powder
MATERIALS AND METHODS

Experimental Animals
Six week old Sprague-Dawley male rats and about 180g in weight were raised for about two weeks. They were allowed to eat solid food (MF; Oriental Yeast Co, Tokyo, Japan) and drink water freely in the cages of the animal research center affiliated with our university. Those maxillary first and second molars which looked free from dental caries to the naked eye were selected. Excluded were those upper first and second molars with excessively large cavities and pulp exposure, those without hemorrhaging at the time of pulp exposure, and those suspected of having fractures. The experimental groups ($n=5$) were arranged carefully considering animals’ individual variations and the type of teeth. This study was approved by the Laboratory Animal Committee of The Nippon Dental University School of Life Dentistry at Niigata, Japan (Receipt number: 191, December 28th, 2004).

Experimental Materials
All the materials used in this experiment are shown in Table 1. AS the primer, Clearfil SE Bond Primer (Kuraray Medical Inc, Tokyo, Japan; SEP) was used. The bonding materials were prepared with a experimentally manufactured monomer liquid of SE thanks to the cooperation of Kuraray Medical Inc. As agents that promote the production of reparative dentin, at least one of the following four types of calcium phosphates: hydroxyl-calcium phosphate (hydroxyapatite; OHAp), dicalcium phosphate dihydrate (brushite; DCPD), beta-tricalcium phosphate (whitlockite; $\beta$-TCP) and octacalcium phosphate (OCP) were added to the bonding materials in the ratios as shown in Tables 2 and 3.

SE4, with SEP as its base was exclusively used as the experimentally manufactured primer containing 2 wt% salicylic acid derivative monomer 5-NMSA (MP3), which is known to be effective in augmenting adhesiveness to dentin and in allaying dentin hypersensitivity.

The control group was directly capped with Dycal (Dentsply Sankin, Tokyo, Japan; DY), a calcium hydroxide preparation, and then treated with SEP, the Clearfil SE Bond bonding agent (Kuraray Medical Inc, Tokyo, Japan).

Table 1. As the primer, Clearfil SE Bond Primer (Kuraray Medical Inc, Tokyo, Japan; SEP) was used. The bonding materials were prepared with a experimentally manufactured monomer liquid of SE thanks to the cooperation of Kuraray Medical Inc. As agents that promote the production of reparative dentin, at least one of the following four types of calcium phosphates: hydroxyl-calcium phosphate (hydroxyapatite; OHAp), dicalcium phosphate dihydrate (brushite; DCPD), beta-tricalcium phosphate (whitlockite; $\beta$-TCP) and octacalcium phosphate (OCP) were added to the bonding materials in the ratios as shown in Tables 2 and 3.

SE4, with SEP as its base was exclusively used as the experimentally manufactured primer containing 2 wt% salicylic acid derivative monomer 5-NMSA (MP3), which is known to be effective in augmenting adhesiveness to dentin and in allaying dentin hypersensitivity.

The control group was directly capped with Dycal (Dentsply Sankin, Tokyo, Japan; DY), a calcium hydroxide preparation, and then treated with SEP, the Clearfil SE Bond bonding agent (Kuraray Medical Inc, Tokyo, Japan).

Table 1 Experimental materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Abbr.</th>
<th>Lot #</th>
<th>Classifications</th>
<th>Compositions</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearfil SE Bond Primer</td>
<td>SEP</td>
<td>00518A</td>
<td>Two-step self-etching primer</td>
<td>10-MDP, HEMA, hydrophilic dimethacrylate, CQ, tert-amine, water, dyes</td>
<td>Kuraray Medical Inc (Tokyo, Japan)</td>
</tr>
<tr>
<td>Experimental Primer</td>
<td>MP3</td>
<td>021009</td>
<td></td>
<td>SEP containing 2wt% 5-NMSA</td>
<td></td>
</tr>
<tr>
<td>Clearfil SE Bond Bond</td>
<td>SEB</td>
<td>0373AA</td>
<td></td>
<td>10-MDP, HEMA, Bis-GMA, hydrophobic aliphatic dimethacrylate, tert-amine, CQ</td>
<td></td>
</tr>
<tr>
<td>Experimental Monomer</td>
<td>EML</td>
<td>050705</td>
<td>Experimental manufactured</td>
<td>Experimentally manufactured monomer liquid of SE. Basic composition is the same as the SEB.</td>
<td>Dentsply Sankin (Tokyo, Japan)</td>
</tr>
<tr>
<td>Monomer Liquid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearfil AP-X (A3)</td>
<td>APX</td>
<td>01065A</td>
<td></td>
<td>Bis-GMA TEG-DMA barium glass silanated colloidal silica CQ</td>
<td></td>
</tr>
<tr>
<td>Dycal</td>
<td>DY</td>
<td>020121</td>
<td>Catalyst paste:</td>
<td>ethylene toluene sulfon amide Ca(OH)$_2$ ZnO</td>
<td>Dentsply Sankin (Tokyo, Japan)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ester glycol salicylate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>calcium phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca tungstate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ZnO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tl$_2$O</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zn stearate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5-NMSA: N-methacryloyl 5-aminosalicylic acid (adhesion promoter)
Tokyo, Japan; SEB). After dental surface treatment was completed in all the experimental groups, resin composite restoration was performed with Clearfil AP-X A3 (Kuraray Medical Inc, Tokyo, Japan; APX).

The data for SE4 groups of 3 days and 7 days and control groups of 3 days, 7 days and 14 days were those obtained by previous studies carried out by colleagues of our laboratory 31,32). The observations of the SE5 to SE12 groups of 3 days and 7 days and the SE1 to SE12 groups of 14 days were data obtained by the present study. Therefore the observation of the SE1 to SE3 groups in the present study covered a period of only 14 days.

### Experimental Methods

1) Observation Periods

Three postoperative observation terms were set: 3 days, 7 days and 14 days. (only 14 days for SE1-SE3).

2) Treatment methods

Each of the SD rats was etherized (Diethyl Ether, Wako Pure Chemical Industries Ltd, Osaka, Japan) and later anesthesized by an intraperitoneal injection of 5% pentobarbital sodium (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). Thereafter, the rodents were fixed to the operating table, and their mouths were kept open with a mouth gag. The oral cavity of each rat was cleansed with a 3% H2O2 (Oxydol, Yoshida Pharmaceutical Co, Tokyo, Japan) solution and a sterile physiological saline solution (Physisolz PL-D, Fuso.  

<table>
<thead>
<tr>
<th>Materials</th>
<th>Abbr.</th>
<th>Chemical Structures</th>
<th>Molar Ratio Ca / P</th>
<th>Lot #</th>
<th>Manufactures</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite</td>
<td>OHAp</td>
<td>Ca₁₀(PO₄)₆(OH)₂</td>
<td>1.67</td>
<td>030605</td>
<td>Ube Materials Industries Ltd (Ube-City, Japan)</td>
<td>Experimental Product (Sintering at 1200°C)</td>
</tr>
<tr>
<td>Dicalcium phosphate dihydrate (Brushite)</td>
<td>DCPD</td>
<td>Ca₅(PO₄)₂•2H₂O</td>
<td>1.00</td>
<td>M7H6573</td>
<td>Nacalai Tesque Inc (Kyoto, Japan)</td>
<td>The First Class Reagent</td>
</tr>
<tr>
<td>Beta-tricalcium phosphate (Whitlockite)</td>
<td>β-TCP</td>
<td>Ca₅(PO₄)₂</td>
<td>1.50</td>
<td>04080401</td>
<td>Taihei Chemical Industrial Co Ltd (Osaka, Japan)</td>
<td>Experimental Product</td>
</tr>
<tr>
<td>Octacalcium phosphate</td>
<td>OCP</td>
<td>Ca₈H₂(PO₄)₆•5H₂O</td>
<td>1.33</td>
<td>SA3131</td>
<td>Taihei Chemical Industrial Co Ltd (Osaka, Japan)</td>
<td>Experimental Product</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Compositions (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SE1</td>
</tr>
<tr>
<td>EML Experimental monomer</td>
<td>100</td>
</tr>
<tr>
<td>OHAp Hydroxyapatite</td>
<td>5</td>
</tr>
<tr>
<td>DCPD Brushite</td>
<td>5</td>
</tr>
<tr>
<td>β-TCP Whitlockite</td>
<td>5</td>
</tr>
<tr>
<td>OCP Octacalcium</td>
<td>5</td>
</tr>
</tbody>
</table>

Control group: DY + SEP + SEB. SE4: Only MP3 was used as a primer. 

\( n=5 \)
and a 3% H2O2 solution, alternately. After this procedure, the prepared cavities were treated with a 10%NaClO solution (AD-Gel, Kuraray Medical Inc, Tokyo, Japan) for 2 to 6 minutes and irrigated with a distilled water for injection use (Wasser Free, Fuso, Tokyo, Japan). The extracted specimens were immersed in a 4% PFA phosphate buffer solution (PFA: pH 7.4) buffered fixation solution for three days for further fixation, washed under running water, and then decalcified with a 10% EDTA-2Na solution (pH 7.4) at room temperature for three to four weeks. After decalcification, APX was removed. Then, the specimens were washed under running water for 24 hours and dehydrated in ascending grades of ethanol. The alcohol content was removed with xylol. The specimens were embedded in paraffin in the usual manner and sliced in 5 µm-thick sections serially with a sliding microtome (Jung Histoslide 2000R, Leica Microsystems Vertrieb GmbH, Wetzlar, Germany).

5) Staining methods

Mayer’s hematoxylin-eosin staining (H-E) for histopathological observation, tissue and bacterium staining by the Hucker-Conn method for bacteriological observation, and reticulin silver impregnation staining by a modified NF-Watanabe method (NF) for reticular collagen fiber observation were carried out. Stained sections were examined under an optical microscope. For immunohistochemical staining, anti-DMP1 antibody (Anti-Dentin Matrix Protein, Polyclonal, Lot # 002FD, Takara Bio Inc, Shiga, Japan) and anti-TGF-β1 antibody (Anti-TGFb1(V), Lot #3 F2306, Cosmo Bio, Tokyo, Japan) as the primary antibodies were used. For this staining, we adopted the sABC (avidin-biotin horseradish peroxidase complex) method following the instructions of the manufacturer of the immunochemical reagent (Histofine SAB-POR kit, Lot #0609, Nichirei Bioscience Inc, Tokyo, Japan). Coloring matter was formed by an adjuvant reagent tradenamed DAB (Simple Stain DAB Solution, Lot # H0610, Nichirei Bioscience, Tokyo, Japan). Hematoxylin was used as the dye for cell nuclei. In the negative control, PBS was used instead of the antibody. As an assessment criterion, the presence or absence of coloring matter was ascertained under a light microscope (Eclipse E1000, Nikon Co, Tokyo, Japan).

6) Observation items and evaluation criteria

The stained sections were examined under an optical microscope to evaluate (1) pulp tissue disorganization (PTD), (2) inflammatory cell infiltration (ICI), (3) reparative dentin formation (RDF) and (4) bacterial penetration (BP). The findings were graded according to following evaluation criteria made by Medina III-Katoh (2002)29.

1) Pulp tissue disorganization (PTD)

- Normal or almost normal tissue morphology (none).
- Odontoblast layer disorganization but the deep part of the pulp was normal (mild).
- Loss of general tissue morphology (moderate).
- Necrosis in the coronal third or more of the pulp (severe).

2) Inflammatory cell infiltration (ICI)

- Absence or presence of a few scattered inflammatory cells in the pulp (none).
- Mild acute/chronic cell lesions (mild).
- Moderate inflammatory cell lesions seen as an abscess or densely stained infiltrates of polymorphonuclear leucocytes, histiocytes and lymphocytes in one-third or more of the coronal pulp and/or the mid-pulp (moderate).
- Pulp necrosis due to a severe degree of infection or lack of tissue in half or more of the pulp (severe).

3) Reparative dentin formation (RDF)

- No dentin bridge formation (none).
- Initial dentin bridge formation extending to not more
than one-half of the exposure site (initial).
3- Partial/incomplete dentin bridge formation extending
to more than one-half of the exposure site but not
completely closing the exposure site (partial).
4- Complete dentin bridge formation (complete).

(4) Bacterial penetration (BP)
1- Absence of stained bacterial profiles in any of the
sections (none).
2- Presence of stained bacterial profiles along the coronal
or apical walls of the cavity (mild).
3- Presence of stained bacterial profiles within the cut
dentinal tubules or axial wall of the cavity (moderate).
4- Presence of stained bacterial profiles within the dental
pulp (severe).

7) Measurement of pulp exposure area
The largest diameter of every specimen’s pulp exposure
area was measured with the aid of a microscope
(Measuring Microscope MM-40, Lot #2104048, Nikon
Co, Tokyo, Japan).  The mean values and standard
deviations (mm) were worked out in each of the
experimental groups according to the period of
observation.

8) Statistical analyses
(1) On diameters of pulp exposure area
Using statistic software (Microsoft Excel 2010 for
Windows, SSRI Co Ltd, Tokyo, Japan), two-way ANOVA
was performed on the mean exposure size in each group
with different observation periods and SE1 to control
groups as the main effects at a significance level of 0.05.

(2) On pathological results
The Kruskal-Wallis H-test was conducted on the results
of each test item at a significance level of 0.05. Significant
differences were noted between the control group and
experimental groups. After that the Steel test was
carried out as a post-hoc test for differences between
each experimental group and the control during each
observation period at a significance level of 0.05.

RESULTS

Measurement of pulp exposure size
The mean values and standard deviations of the pulp
exposure diameter (mm) for each experimental group are
shown in Table 4 according to the length of the
observation period.  The overall mean diameter and
standard deviation were 0.240 mm and 0.096 mm. The
maximum and the minimum diameters were, 0.701 mm
and 0.044 mm. Two-way ANOVA was performed to
assess the mean values of the diameters of the pulp
exposure areas with the different observation periods (3,
7 and 14 days) and the control and all the experimental
groups from SE1 to SE12 as the main effects at a
significance level of 0.05. The results did not show any
statistically significant difference either in the main
effects or mutual action effects (p>0.05).

Histopathological and immunohistochemical findings
The summary of the results of histopathological and
immunohistochemical evaluation is shown in Figs. 1, 2
and 3. Results of statistical analysis on histopathological
evaluation were as follows: The Kruskal-Wallis H-test
revealed significant differences in the incidence of ICI
among the experimental groups observed for 3 days and
7 days, and in the incidence of PTD among the groups
after 7 days of observation, at a significance level of 0.05.
The Steel test did not show any significant difference
between the control and the groups in which the Kurskal-
Wallis H-test found significant differences (p>0.05). This
was presumably due to statistically significant
differences between the experimental groups.

Table 4  Diameters of exposed pulp area

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>3 days</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE1</td>
<td>–</td>
<td>–</td>
<td>0.180 (0.093)</td>
</tr>
<tr>
<td>SE2</td>
<td>–</td>
<td>–</td>
<td>0.171 (0.065)</td>
</tr>
<tr>
<td>SE3</td>
<td>–</td>
<td>–</td>
<td>0.191 (0.058)</td>
</tr>
<tr>
<td>SE4</td>
<td>0.312 (0.059)</td>
<td>0.271 (0.036)</td>
<td>0.322 (0.064)</td>
</tr>
<tr>
<td>SE5</td>
<td>0.230 (0.105)</td>
<td>0.310 (0.208)</td>
<td>0.252 (0.065)</td>
</tr>
<tr>
<td>SE6</td>
<td>0.199 (0.044)</td>
<td>0.255 (0.115)</td>
<td>0.171 (0.038)</td>
</tr>
<tr>
<td>SE7</td>
<td>0.194 (0.035)</td>
<td>0.315 (0.078)</td>
<td>0.253 (0.111)</td>
</tr>
<tr>
<td>SE8</td>
<td>0.172 (0.027)</td>
<td>0.197 (0.033)</td>
<td>0.354 (0.143)</td>
</tr>
<tr>
<td>SE9</td>
<td>0.223 (0.068)</td>
<td>0.254 (0.057)</td>
<td>0.263 (0.146)</td>
</tr>
<tr>
<td>SE10</td>
<td>0.173 (0.020)</td>
<td>0.219 (0.029)</td>
<td>0.252 (0.111)</td>
</tr>
<tr>
<td>SE11</td>
<td>0.186 (0.033)</td>
<td>0.294 (0.084)</td>
<td>0.251 (0.086)</td>
</tr>
<tr>
<td>SE12</td>
<td>0.169 (0.025)</td>
<td>0.172 (0.020)</td>
<td>0.210 (0.032)</td>
</tr>
<tr>
<td>Control group</td>
<td>0.246 (0.014)</td>
<td>0.295 (0.058)</td>
<td>0.307 (0.068)</td>
</tr>
</tbody>
</table>

m (SD): mm
Fig. 1  Results of histopathological evaluation (3 days).

Fig. 2  Results of histopathological evaluation (7 days).
In other words, it was ascertained that there was no significant difference between the control and experimental groups in the results of the tests regardless of the period of observation.

To look at the correlation between ICI and BP, Kendall's rank correlation test was carried out. The results did not show any correlation between the two, irrespective of the observation period.

Representative histopathological and immunohistochemical micrographs of the groups are shown in Figs. 4–9.

1) Results by group
SE1: Pulp tissue was mostly normal in the specimens which had been under observation for 14 days, and reparative dentin formations in Initial to Partial were evident in all specimens.

SE2: The pulp was restored to normal in every specimen which had been under observation for 14 days. The recovery rate of pulp tissue was the highest in the experiments. Reparative dentin formations in Initial to Partial were also recognized in all specimens.

SE3: When the 14-day observation period was over, pulp tissue was found to be normal in most of the specimens. Reparative dentin formation was seen in four specimens, of which one showed a perfect dentin bridge formation.

SE4: Reparative dentin was formed in two specimens in the group of 7-day observation. In the group of 14-day observation, reparative dentin formation was seen in all specimens. Inflammatory cells had disappeared in every specimen during the 7-day period.

SE5: This group was notably high in the incidence of PTD and ICI compared to the other experimental groups, indicating a very slow recovery of pulpal tissue. Nonetheless, reparative dentin formation was seen in some specimens of 7-day observation and in all the specimens of 14-day observation. Two specimens showed a perfect dentin bridge formation and other three specimens showed Partial.

SE6: High rates of PTD and ICI marked the specimens which were observed after seven days. Some specimens exhibited pulpal necrosis, although there was no sign of bacterial invasion. Reparative dentin formation was seen in all the specimens which were observed after 7 days. One showed a perfect dentin bridge after 14 days.

SE7: During the 7-day observation period, reparative dentin formation was recognized in some specimens, whereas reparative dentin was formed in only two of the specimens observed for 14 days.

SE8: Pulpal tissue was slow in recovering, and reparative dentin formation was seen only in three specimens after 14 days. These were one Initial and two Partial.

SE9: The recovery of pulpal tissue was a little slow, but reparative dentin formation was recognized in all specimens after 14 days. One of them showed a perfect dentin bridge formation.
SE10: PTD and ICI rates were lower in many of the specimens observed for 3 days and 7 days, but higher in the specimens observed for 14 days than in the other groups. Reparative dentin formation was recognized in four specimens.

SE11: The recovery from PTD was a little slow, but reparative dentin formation was recognized in four specimens.

SE12: The recovery of pulpal tissue was quick. Bacterial invasion was seen in three of the specimens under observation for 14 days. Probably because of this, only three specimens exhibited reparative dentin formation. Nevertheless, the volume of dentin formed was large. But for bacterial invasion, reparative dentin formation would have been recognized in more specimens.

The control group: Reparative dentin formation was recognized in two specimens observed for 7 days, but only three out of the five specimens observed for 14 days exhibited reparative dentin formation. A check on the mode of reparative dentin formation revealed that a perfect dentin bridge was formed in one specimen and an irregular osteo-dentin bridge in two specimens. It was confirmed that there was a notable reduction in the volume of vital pulp in the control. This was probably due to the formation of necrotic layers.

2) Results according to observation periods
The 3-day observation period: Tissue disorganization and disappearance of the odontoblastic layers were
observed in all the experimental groups and the control. In the adjacent tissue of the pulp exposure sites, reductions in the number of cells, vacuolar degeneration and formation of irritation dentin were recognized. However, deeper tissue was mostly normal. In deeper tissue of some specimens, chronic inflammatory cell infiltration was noted. The Kruskal-Wallis\textit{H}-test showed a significant difference in ICI at a significance level of 0.05, but the Steel test did not find any significant difference between the control and experimental groups. Kendall’s rank correlation test did not reveal any correlation between ICI and BP, either.

The 7-day observation period: Although dental pulp tissue was in a stable condition since the 3rd day, reductions in the number of cells and vacuolar degeneration were seen. Many specimens showed that predentin had formed in the area around the pulp exposure site. Some showed reparative dentin at an incipient stage of formation, which was also confirmed by reticulum silver impregnation. The Kruskal-Wallis\textit{H}-test demonstrated a significant difference in PTD and ICI at a significance level of 0.05, but the Steel test did not show any significant differences between the control and experimental groups. Kendall’s rank correlation test did not find any correlation between ICI and BP.

The 14-day observation period: Although various findings were found among the specimens, reparative dentin formation was evident and active restorative
changes on the surface of the wounds were seen in every group. Pulpal tissue was almost normal and inflammatory cell infiltration had disappeared in many specimens. The Kruskal-Wallis H-test did not find any significant differences between the control and experimental groups. No correlation between ICI and BP was found by Kendall’s rank correlation test.

Observations on PTD: Pulp tissue was being restored to normalcy over time.

Observations on ICI: Pulp tissue was being restored to normalcy over time. The reaction was stronger in SE5 and SE6 than in other experimental groups during the 7-day observation period.

Observations on RDF: The amount of reparative dentin formation increased as time elapsed. In some specimens, the production of reparative dentin started on the 7th day. On day 14, reparative dentin formation was confirmed in all groups. Nevertheless, the amount of reparative dentin production varied widely from one specimen to another. What was worthy of note was the finding that in the control group, the formation of the reparative dentin was seen in only three out of the five specimens including one specimen which had a complete dentin bridge. There was no sign of reparative dentin in the remaining two specimens.

Fig. 6  a, b, c, d. Representative histological micrographs on the 14th day after direct pulp capping and restoration (SE5).

a: There is a complete but uneven dentin bridge in thickness that is composed of the osteodentin type. A small amount of tubular dentin can be seen at the pulpal side of the dentin bridge. There are recognizable odontoblast-like cells just beneath the dentin bridge. Pulpal morphology is normal (H-E stain, 10×).

b: Positive reaction at the active formation site of reparative dentin can be observed (DMP1 stain, 20×).

c: A thick network formation of reticular fiber at just beneath the area of the dentin bridge can be seen (NF stain, 10×).

d: No positive reaction can be seen (TGF-β1 stain, 10×).
DISCUSSION

Comparison of calcium phosphate concentration
1) Comparison between SE1 and SE2
Inflamed pulpal tissue was restored to normal in many specimens in both groups after the 14-day observation period, and reparative dentin was formed in every specimen. These findings revealed that hydroxyapatite was superb in biocompatibility as well as hard tissue inducibility. A comparison between the two groups showed that the dental pulp was better in SE2, to which 10 wt% hydroxyapatite was added, than in SE1. However, the difference was not significant. Considering that it is impossible to make all experiment conditions exactly the same, it seemed that the concentration made scarcely any difference. These findings suggested that hydroxyapatite was suitable for direct pulp capping and that a concentration of 5 wt% or thereabouts was reasonable.

2) Comparison between SE5 and SE6
PTD and ICI occurred at a higher rate in these groups than in the others. Whitlockite is known as tribasic calcium phosphate and noted for its biocompatibility and ability to induce hard tissue to regenerate, but the results of the present study revealed that this compound was the most stimulating of all the four forms of calcium.

Fig. 7  a, b, c, d. Representative histological micrographs on the 14th day after direct pulp capping and restoration (SE9).

a: A complete dentin bridge is of the very irregular tubular type that is connecting to the irritation dentin. There are reorganized odontoblast-like cells just beneath the area of the dentin bridge. Pulpal morphology is normal (H-E stain, 10×).
b: A positive reaction can be seen at just beneath the area of dentin bridge and at the interface of primary dentin and irritation dentin (DMP1 stain, 10×).
c: A thick network formation of reticular fibers at widely spreaded pulpal tissue can be seen (NF stain, 10×).
d: No positive reaction can be seen. Upper right is a representative micrograph of the negative control (TGF-β1 stain, 10×).
phosphate used in this study. In addition, it was suggested that the single use of whitlockite had caused a delay in allaying inflammatory response in early stages. But, it was ascertained that it was superb in hard tissue inducibility because the formation of reparative dentin in every specimen was excellent. Although the results are apparently contradictory, we rationalized that the continuous application of stimulus promoted the formation of reparative dentin.

Necrosis which was not due to bacterial invasion was noted in SE6, to which 10 wt% whitlockite was added, but not in SE5. The amount of reparative dentin formed was less in SE6 than in SE5. These findings suggested that the amount of 10 wt% whitlockite added might have been excessive. We concluded that whitlockite is a good direct pulp capping agent which can demonstrate excellent hard tissue inducibility if its concentration is closely monitored.

Combination of calcium phosphate agents
1) Comparison between SE1, SE3 and SE8
Compared with SE1 and SE3, SE8 produced unfavorable results. Compared with the other groups containing hydroxyapatite or brushite, SE8 was very slow in the recovery of pulpal tissue. Based on these findings, we assumed that the combined use of hydroxyapatite and

Fig. 8 a, b, c, d. Representative histological micrographs on the 14th day after direct pulp capping and restoration (control group).

a: Complete dentin bridge formation can be observed at the exposed surface. The dentin bridge is composed of 50% osteodentin type and tubular dentin at the pulpal side. In addition, a newly reorganized odontoblastic layer just beneath the area of the dentin bridge can be recognized. Pulp morphology is completely normal (H-E stain, 10×).
b: Positive reaction at the outer layer of dentin bridge can be seen but no reaction was observed at the inner layer of the dentin bridge (DMP1 stain, 10×).
c: A well formed network structure consisting of reticular fibers stained in blue at the outer layer and collagen fiber stained red at the inner layer of dentin bridge can be recognized (NF stain, 40×).
d: No positive reaction can be seen (TGF-β1 stain, 10×).
2) Comparison between SE1, SE5 and SE9
Compared with SE1, which contained only hydroxyapatite, SE9 was slow to restore injured pulp to normal but almost same in hard tissue inducibility. In comparison with SE5, which contained only whitlockite, SE9 was a little quicker to restore pulp, but a little inferior in hard tissue inducibility. In other words, SE9 produced intermediate results between those produced by other groups, which used one of the four forms of calcium phosphate. Although SE9 was surpassed by the other groups concerning some parameters, it was comparatively superior to the others.

3) Comparison between SE3, SE5 and SE10
SE10, which contained whitlockite, was presupposed to promote the formation of reparative dentin briskly. As it turned out, it was on a par with SE3, which contained only brushite. In SE10 as compared to SE5, which contained only whitlockite, the recovery of pulpal tissue was a little faster, but reparative dentin formation was far from satisfactory. From this viewpoint, we considered that the combination of brushite and whitlockite could not be recommended for use in clinic.

4) Comparison of SE8 and SE9
Both groups contained 5 wt% hydroxyapatite. In addition SE8 contained brushite while SE9 whitlockite. So, we examined which would make a better match for hydroxyapatite: brushite or whitlockite. The recovery of pulpal tissue was a little quicker in SE9 than in SE8, and SE9 promoted the formation of reparative dentin much more than SE8 did. These results suggested that the combination of hydroxyapatite and whitlockite was better than the combination of hydroxyapatite and brushite.

5) Comparison of SE8 and SE10
Since both groups contained 5 wt% brushite, we looked into which was more suitable: hydroxyapatite or whitlockite, for use together with brushite by comparison. The recovery of pulpal tissue was slower in SE8 than in SE10, and the formation of reparative dentin was less in the former than in the latter. This clearly indicated that whitlockite was better in combination with brushite.

When those groups using hydroxyapatite or whitlockite were examined for comparison, the recovery of pulpal tissue was quicker in those groups using hydroxyapatite than in those using whitlockite. However, the combined use of hydroxyapatite and brushite caused a delay in the recovery of pulpal tissue. From this, we considered that the combination of hydroxyapatite and brushite was ill-suited for clinical use.

6) Comparison of SE9 and SE10
SE9 contained whitlockite and hydroxyapatite, while SE10 used whitlockite and brushite. Both groups were equal in the recovery speed of pulpal tissue, but SE9 had an edge over SE8, when it came to the formation of reparative dentin. This clearly indicated that the combination of hydroxyapatite and whitlockite was superior.

The ability to induce hard tissue regeneration in the four forms of calcium phosphate
Hydroxyapatite is known for its biocompatibility and stability. Thanks to these properties, this inorganic compound fully has displayed its ability to induce hard
tissue regeneration over a long period of time.

Brushite in the early stages of dental calculus formation gradually changes into whitlockite, octacalcium phosphate, and hydroxyapatite over time. In dental caries, brushite exists in the second layer of enamel decalcification which progresses rapidly at pH 6 or above. It is closely related to the formation of hard tissue at an early stage. SE3, which used brushite, continued to show its ability to induce hard tissue to form over a long period of time. This was presumably not so much due to brushite's own ability as due to its gradual change into hydroxyapatite and others which are more stable chemically.

Whitlockite is said to be present in caries in which decalcification is suspended or at the second layer of decalcification which progresses very slowly at pH6 or above. As the pH level increases, whitlockite supposedly converts itself into hydroxyapatite by degrees, but it has been reported that this conversion rarely occurs because it is chemically stable. In the present study, concerning comparison between PTD and ICI grades, no every experimental group showed a clear indication that whitlockite transformed itself into hydroxyapatite.

Octacalcium phosphate occurs in the body as the precursor of apatite found in bone and teeth and promotes the growth of osteoblasts and the formation of bone. It has been reported that bone formation is faster with octacalcium phosphate than with hydroxyapatite. However, in our study, the addition of octacalcium phosphate did not show any major change.

To summarize the results of this experiment, it was suggested that reparative dentin formation was effectively promoted under the following conditions: a calcification promoting effect by direct contact of calcium phosphate powder or that found in the vicinity of exposed pulp, an ionic effect of Ca and P eluted from calcium phosphate powder in polymerized bonding material used as a direct pulp capping agent and environmental change in the surface area of the exposed pulp through the effect of pH change.

Also it was suggested that reparative dentin formation was particularly promoted which confirmed the effectiveness of wound healing in the early stage after direct capping of exposed pulp.

Impregnation silver staining
Collagen fibers were in evidence in some of the specimens observed after 7 days and 14 days. These specimens were thought to be at the stage where reparative dentin was just about to take form.

Regarding specimen preparation
The presence of bacteria is a stimulation factor affecting the wound healing process after pulp exposure. Bacterial penetration may hamper the assessment of the efficacy of direct pulp capping agents. In our study, Kendall’s rank correlation test did not reveal any correlation between ICI and BP. Nonetheless, we suspected bacterial contamination. As a precaution against this, it is absolutely necessary to take antiseptic measures in the preparatory stages.

As a primer, we used MP3 containing 5-NMSA in SE4. Thus, we could not make a comparison between SE3 and SE4 with respect to the concentration of brushite. It would be possible to assess the efficacy of MP3 if we had used brushite of the same concentration.

CONCLUSIONS
1. In the control group, the formation of necrotic layers was recognized on the 3rd day and continued into the 7th day. Some specimens had formation of reparative dentin from the 7th day on, while some others presented perfect reparative dentin formations on the 14th day. Reparative dentin formations varied widely both in morphology and amount. When it came to the healing process, it was reconfirmed that the volume of vital pulp decreased markedly by the formation of necrotic layers, resulting in pulpal tissue defects.

2. All the four forms of calcium phosphate: hydroxyapatite, brushite, whitlockite and octacalcium phosphate, have the ability to induce hard tissue to regenerate when mixed as promoters of reparative dentin formation into the bonding material.

3. The largest amount of reparative dentin was formed by SE5 (whitlockite 5 wt%), followed by SE9 (hydroxyapatite 5 wt%, whitlockite 5 wt%), SE1 (hydroxyapatite 5 wt%), and SE2 (hydroxyapatite 10 wt%).

4. Generally, it could be said that the experimental groups using whitlockite and hydroxyapatite had a tendency to produce a larger amount of reparative dentin.

ACKNOWLEDGMENT
The authors thank Kuraray Medical Inc, for the experimental adhesive resin systems and other materials they generously provided.

SOURCE OF FUNDING
This study was supported in part by a grant-in-aid for Scientific Research (B)(2): project number: 15390578 from The Japan Society for the Promotion of Science.

REFERENCES
3) Sekine E, Watanabe Y. Clinico-pathological supplement study in relation to vital pulp amputation using calcium


