Cytotoxicity and Genotoxicity of Newly Developed Calcium Phosphate-based Root Canal Sealers

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ABSTRACT

The purpose of this study was to compare the cytotoxicity by MTT test and genotoxicity by Ames test of new calcium phosphate-based root canal sealers (CAPSEAL I, CAPSEAL II) with commercially available resin-based sealers (AH 26, AH Plus), zinc oxide eugenol-based sealers (Tubliseal EWT, Pulp Canal Sealer EWT), calcium hydroxide-based sealer (Sealapex), and tricalcium phosphate based sealers (Sankin Apatite Root Canal Sealer I, II, III).

According to this study, the results were as follows:

1. The extracts of freshly mixed group showed higher toxicity than those of 24 h set group in MTT assay ($p < 0.001$).
2. CAPSEAL I and CAPSEAL II were less cytotoxic than AH 26, AH Plus, Tubliseal EWT, Pulp Canal Sealer EWT, Sealapex and SARCS II in freshly mixed group ($p < 0.01$).
3. AH 26 in freshly mixed group showed mutagenicity to TA98 and TA100 with and without S9 mix and AH Plus extracts also were mutagenic to TA100 with and without S9 mix.
4. Tubliseal EWT, Pulp Canal Sealer EWT and Sealapex in freshly mixed group were mutagenic to TA100 with S9 mix.
5. Among those of 24 h set groups, the extracts of SARCS II were mutagenic to TA98 with and without S9 mix and AH 26 showed mutagenic effects to TA98 with S9 mix.
6. No mutagenic effect of CAPSEAL I and CAPSEAL II was detected.
7. There is no statistically significant difference between CAPSEAL I and CAPSEAL II at MTT assay and Ames test in both freshly mixed group and 24 h set group. (J Kor Acad Cons Dent 31(1):36-49, 2006)

Key words: Root canal sealer, Cytotoxicity, Genotoxicity, Calcium phosphate

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I. Introduction

The biocompatibility of root canal sealers is important for the clinical success of endodontic therapy because they may come into direct contact, especially when extruded, with surrounding soft and hard tissues for a prolonged period of time.

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Various studies have revealed that eluatable substances or degradation or corrosion products from root canal filling materials may gain access to surrounding tissues (periodontal ligament, alveolar bone) through numerous connections, e.g., dentinal tubules, accessory and lateral canals, and apical foramen[1,2]. Araki *et al.*[3] investigated the diffusion of ^{14}C-formaldehyde through radicular dentin 72 h after the application of formocresol into the root canal of cat canines. It was found that formaldehyde was distributed from the pulp space into the body.

There are many brands and types of root canal sealers in use today. They may be divided according to their ingredients: zinc oxide-eugenol (ZOE)-based, resin-based, calcium hydroxide-based, glass ionomer-based, and calcium phosphate-based.

For many decades the root canal sealers most frequently used were those based on ZOE, which, despite their satisfactory physico-chemical properties, do not present a favorable biological behavior. The presence of a chronic inflammatory process is observed in apical and periapical tissues after their use leading to tissue injury attributed to the presence of free eugenol, which may act as a cell depressor[4,5].

Results from these studies showed that endodontic materials possessed both beneficial and undesirable properties. Thus biocompatibility of root canal sealers is as important as physical and chemical features.

The beginnings of the application of calcium phosphate materials as bone substitute or bone graft may be traced to Albee, who reported in 1920 that a triple calcium phosphate compound used in a bony defect promoted osteogenesis or new bone formation[6]. Levin *et al.*[7] reported in 1974 the first dental application of a tricalcium phosphate ceramic in periodontal defects in dogs.

Brown and Chow[8] reported on a self-hardening calcium phosphate cement (CPC) that contained equimolar mixture of finely ground tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) or dicalcium phosphate dehydrate (DCPD) as the solid phase. When mixed with water, the cement forms hydroxyapatite (HA) as the only end product, which is the major mineral component of tooth and bone. Formation of HA in such mixture does not release acidic or basic by-products. The setting reaction of calcium phosphate cement is Eq. 1:

$$Ca_4(PO_4)_2O + CaHPO_4 \xrightarrow{\text{water}} Ca_5(PO_4)_3OH \quad \text{(Eq. 1)}$$

Because the HA is formed in an aqueous environment, it is more similar to biological apatite than is the HA formed in high temperature processes. Since CPC has a neutral pH and contains only calcium phosphates, it was found to be highly biocompatible and osteoconductive[9].

Gruninger *et al.*[10] reported that testing cement (containing TTCP, DCPD, HA, and sodium fluoride) was neither toxic nor mutagenic, and performed implants were well tolerated by the animals and no adverse tissue reaction was reported. Hong *et al.*[11] evaluated the histologic reactions to a calcium phosphate cement composed of TTCP, DCPA or DCPD in the periapical and periodontal tissues. They reported that only a limited inflammatory response to CPC was found after 6 weeks of implantation in the periodontal area, and the bone formation activity and biocompatibility in general were found to be even better in the periapical region in 16 week specimens.

As a result of its ease of use, together with excellent biocompatibility and bone replacing properties, CPC has been investigated for use in a number of medical and dental procedures, including use in reconstruction of frontal sinus and augmentation of craniofacial skeletal defects[12], pulp capping and cavity lining[13,14], repair of periodontal bone defects[15], and endodontics[16].

In vitro studies and animal models have indicated that it is also useful in endodontics as a sealer in root canal treatment[14,16,17]. Krell and Wefel[17] reported that CPC as a root canal sealer appeared similar to Grossman's cement sealer in apical and dentinal tubule occlusions. Sugawara *et al.*[14] showed that CPC had a better sealing ability than Grossman's sealer. In addition to being used as a sealer, *in vitro* studies have shown that CPC can

also seal a furcation perforation and could be used as an apical barrier for apexification[18]. These results suggest that CPC has potential to promote the healing of bone in endodontic treatment.

Recently, we have developed the new calcium phosphate-based root canal sealers (CAPSEAL I, CAPSEAL II) composed of a mixture of TTCP, DCPD and zirconium oxide as solid phase and sodium phosphate buffer as liquid phase, complied with the standard of ISO-6876 (the International Organization for Standardization) applicable to the dental root canal sealing materials. Kim *et al.* showed the new sealers revealed a lower tissue response in the subcutaneous implantation test[19].

Root canal filling materials are usually in close contact with living tissue. Thus, the biological properties of those materials are important as cytotoxic materials can damage periapical tissues, and material with mutagenic potential can induce DNA mutations, possibly causing malignant transformation of the cells. *In vitro* test model to determine the cellular responses is one of the methods for evaluating the biological compatibility of root canal sealers. This has the advantages that many factors and variables can be controlled and the cytotoxicity can be determined with reliability and reproducibility[20].

The short-term Ames test has been recommended as the mutagenesis-screening test for chemicals and environmental samples because of its extensive database and good correlation with carcinogenicity. Also its low cost, simplicity, and speed make the Ames test an important and widespread part of biological examinations of dental materials and of standardization protocols.

The purpose of this study was to compare the cytotoxicity and genotoxicity of newly developed calcium phosphate-based root canal sealers (CAPSEAL I, CAPSEAL II) with another type of commercially available calcium phosphate-based sealers, resin-based sealers, ZOE-based sealers and calcium hydroxide-based sealer using MTT assay and Ames test.

Ⅱ. Materials and Methods

The root canal sealers used in this study were: new calcium phosphate-based sealers (CAPSEAL I, CAPSEAL II), another commercially available calcium phosphate-based sealers (Sankin Apatite Root Canal Sealer (SARCS) I, SARCS II, SARCS III, Sankin kogyo, Tokyo, Japan), resin-based sealers (AH 26, AH Plus, Dentsply DeTrey, Konstanz, Germany), ZOE-based sealers (Pulp Canal Sealer EWT, Tubliseal EWT, Kerr, Detroit, MI, USA) and calcium hydroxide-based sealer (Sealapex, Kerr, Detroit, MI, USA). Components of CAPSEAL I and CAPSEAL II are listed Table 1.

Table 1. Composition of CAPSEAL I and CAPSEAL II

Materials	Components	Ingredients
CAPSEAL I	Powder	Tetracalcium phosphate & Dicalcium phosphate dihydrate
		Portland cement
		Zirconium oxide
		Others
	Liquid	Sodium phosphate solution
CAPSEAL II	Powder	Tetracalcium phosphate & Dicalcium phosphate dihydrate
		White portland cement
		Zirconium oxide
		Others
	Liquid	Sodium phosphate solution

A. Cytotoxicity test

1. Cell Culture

L929 mouse fibroblasts were grown in minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (Gibco, Grand Island, NY, USA). Cells were cultivated in plastic culture flasks with vented cap at 37℃ in a humidified 5% CO_2 containing incubator. Subcultivation was performed on sufficient cultures.

2. Preparation of Test Materials

The commercially available root canal sealers were prepared according to the manufacturer's instructions. Both CAPSEAL I and CAPSEAL II were mixed in 1.5 P/L ratio (g/g). The mixed materials were extracted in cell culture medium (1 g/2 *ml*) immediately after mixing (fresh mixed group), or after 24 h from mixing (24 h set group), for 24 h at 37℃ in a humidified 5% CO_2 containing incubator. Each extracted medium was filter-sterilized through a 0.2 μm filter (Corning Incorporated, Corning, NY, USA).

3. MTT assay

This assay represents the capacity of mitochondrial dehydrogenase in viable cells to convert a yellow water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT: Sigma, St. Louis, MO, USA) into dark blue formazan crystals. 2×10^4 cells in 50 μl culture medium were seeded in flat-bottomed 96-well microplates (Costar, Corning Incorporated, Corning, NY, USA). After overnight attachment, cells were treated with various eluates of sealers (50 μl/well) for 24 h. Then 20 μl MTT solution (5 *mg/ml*) was added to each well and incubated for 4 h at 37℃. 50 μl dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was added to each well to dissolve the formazan precipitate, and the plates incubated for 2 h. Subsequently, the absorbance at 570 nm was measured using a microplate spectrophotometer (PowerWave, Bio-Tek Instruments, Inc., Winooski, VT, USA). Intact cells in 50 μl of culture medium served as a control for cell viability.

B. Genotoxicity test

1. Preparation of Test Materials

The commercially available root canal sealers were prepared according to the manufacturer's instructions. Both CAPSEAL I and CAPSEAL II were mixed in 1.5 P/L ratio (g/g).

The mixed materials were extracted in DMSO (0.1 g/2 *ml*), immediately after mixing (fresh mixed group), or after 24 h from mixing (24 h set group), for 24 h at 37℃ in a humidified 5% CO_2 containing incubator. The quantities assayed for each material were 5, 2.5, 1.25 and 0.62 mg/plate. In case of AH 26 and AH Plus, lower concentrations were tested, down to 0.15 mg/plate.

Negative control was solvent control, DMSO. Chemicals used as positive are listed in Table 2.

2. Ames test

The Ames test was performed as the standard plate incorporation assay on minimal glucose agar (MGA) plates according to Maron and Ames[21].

Two tester strains of *Salmonella typhimurium* TA98 and TA100 were used to detect frame-shift

Table 2. Positive control chemicals

Strain	Control (μg / plate)	
	Without activation (S9)	With activation (S9)
TA98	2-nitrofluorene (1)	2-aminoanthracene (1)
TA100	Sodium azide (1.5)	2-aminoanthracene (1)

and base-pair mutations respectively. The overnight culture of the bacteria was performed in nutrient broth Oxoid No. 2 (Oxoid LTD., Hampshire, England) following the standard plate incorporation assay procedure.

The extracts of test sealers and bacterial broth (0.1 *ml*) were added to 2 *ml* of molten top agar in sequence with vortexing. Then the contents of the test tubes were poured onto the surface of MGA plates. The bacteria were then incubated at 37℃ for 2 days and revertant colonies were counted. The plates were hand-counted but when the plates had above 100 colonies/plate, these were counted automatically (Chemi-Doc, BioRad, Hercules, CA, USA).

The experiments were carried out in the presence and in the absence of a metabolically active microsomal fraction (S9, Moltox, Annapolis, MD, USA) from rat liver. Tests were run in triplicate for each material's dosage.

C. Statistical analysis

The results from the MTT test and Ames test were analyzed using the Kruskal-Wallis test and Mann-Whitney U test.

Ⅲ. Results

A. Cytotoxicity test

CAPSEAL I and CAPSEAL II were less cytotoxic than AH 26, AH Plus, Tubliseal EWT, Pulp Canal Sealer EWT, Sealapex and SARCS II in case of freshly mixed group ($p < 0.01$). The extracts of freshly mixed groups were more toxic than those of 24 h set groups ($p < 0.001$). SARCS II of freshly mixed group showed more cytotoxic effect than other calcium phosphate-based sealers.

AH 26, Tubliseal EWT, Pulp Canal Sealer EWT, and Sealapex of 24 h set groups showed cytotoxic effect ($p < 0.05$). There is no significant difference between CAPSEAL I and CAPSEAL II in both freshly mixed group and 24 h set group. Figure 1 shows the cytotoxic effects of test root canal sealers on L929 fibroblasts.

B. Genotoxicity test

The results of the experiments are presented in Table 3 to 6. Colonies appearing in the absence of a background lawn were survivors of the killing effect of the test chemical and were not counted as revertants. This happened when most of the bacteria on the plate were killed because of the toxic effect of the test chemical, which allowed the survivors to grow into small colonies by using up the available histidine in the top agar.

AH 26 in freshly mixed group was mutagenic to TA98 and TA100 with and without S9 mix. AH Plus extracts also were mutagenic to TA100 with and without S9 mix (Table 3). The extracts of Tubliseal EWT, Pulp Canal Sealer EWT and Sealapex were mutagenic to strain TA100 with S9 mix in case of freshly mixed group. The other sealers in freshly mixed group showed no mutagenic response. Pulp Canal Sealer EWT, Sealapex and SARCS II were toxic to TA98 and TA100 at higher doses (Table 3 and 4). Among the test sealers of 24 h set group, the extracts of SARCS II were mutagenic to TA98 with and without S9 mix and AH 26 showed mutagenic effects to TA98 with S9 mix (Table 5 and 6). There was no statistically significant difference between CAPSEAL I and CAPSEAL II in both freshly mixed group and 24 h set group.

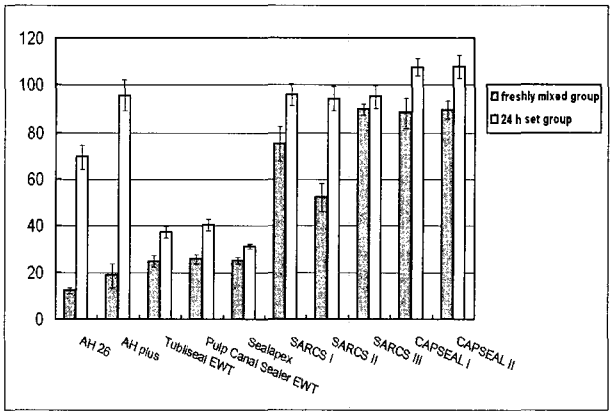

Figure 1. Effect of tested root canal sealers by MTT assay. Percentage of absorbance at each concentration compared with that of control was calculated. Each bar represents a mean ± SD.

Table 3. Mutagenicity of resin-based, ZOE-based and calcium hydroxide-based sealers in freshly mixd group. The number of colonies are the mean values ± SD of triplicates.

Extract (mg/plate)	TA98 Strain				TA100 Strain			
	without S9		with S9		without S9		with S9	
	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.
AH 26								
1.25		Tox	22 ± 1	Neg		Tox		Tox
0.62	12 ± 2	Tox	30 ± 5	Neg		Tox		Tox
0.3	49 ± 5	Mut	52 ± 3	Mut	18 ± 7	Tox	37 ± 9	Tox
0.15	58 ± 3	Mut	51 ± 2	Mut	340 ± 11	Mut	330 ± 8	Mut
AH Plus								
1.25	39 ± 5	Neg	31 ± 2	Neg	127 ± 9	Neg	120 ± 6	Neg
0.62	25 ± 3	Neg	26 ± 4	Neg	295 ± 7	Mut	131 ± 5	Neg
0.3	27 ± 4	Neg	27 ± 3	Neg	192 ± 5	Neg	305 ± 8	Mut
0.15	21 ± 2	Neg	24 ± 2	Neg	121 ± 6	Neg	137 ± 5	Neg
Tubliseal EWT								
5	28 ± 3	Neg	25 ± 3	Neg	125 ± 7	Neg		Tox
2.5	21 ± 5	Neg	23 ± 5	Neg	113 ± 5	Neg	264 ± 15	Mut
1.25	24 ± 4	Neg	24 ± 4	Neg	110 ± 6	Neg	249 ± 9	Mut
0.62	26 ± 4	Neg	27 ± 3	Neg	117 ± 3	Neg	320 ± 8	Mut
PCS EWT								
5		Tox		Tox		Tox		Tox
2.5	23 ± 5	Neg	20 ± 5	Neg		Tox		Tox
1.25	22 ± 3	Neg	22 ± 3	Neg	106 ± 4	Neg		Tox
0.62	27 ± 3	Neg	26 ± 4	Neg	119 ± 3	Neg	277 ± 10	Mut
Sealapex								
5		Tox		Tox	115 ± 3	Neg		Tox
2.5		Tox		Tox	102 ± 5	Neg	53 ± 5	Tox
1.25	22 ± 3	Neg	27 ± 5	Neg	99 ± 9	Neg	54 ± 8	Tox
0.62	20 ± 3	Neg	23 ± 3	Neg	127 ± 7	Neg	312 ± 9	Mut
Negative control	23 ± 5		24 ± 4		120 ± 7		128 ± 9	
Positive control	670 ± 50		2900 ± 80		1226 ± 23		1049 ± 54	

Rever. = revertants; Resp.= response; Tox = toxic; Neg = nonmutagenic and nontoxic; Mut = mutagenic

Table 4. Mutagenicity of calcium phosphatebased sealers in freshly mixd group. The number of colonies are the mean values ± SD of triplicates.

Extract (mg/plate)	TA98 Strain				TA100 Strain			
	without S9		with S9		without S9		with S9	
	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.
SARCS I								
5	18 ± 5	Neg	30 ± 3	Neg	127 ± 4	Neg	132 ± 4	Neg
2.5	29 ± 7	Neg	24 ± 5	Neg	116 ± 5	Neg	156 ± 5	Neg
1.25	30 ± 5	Neg	23 ± 2	Neg	106 ± 4	Neg	256 ± 4	Neg
0.62	23 ± 4	Neg	28 ± 6	Neg	117 ± 3	Neg	203 ± 3	Neg
SARCS II								
5		Tox		Tox		Tox		Tox
2.5		Tox		Tox		Tox	213 ± 12	Neg
1.25		Tox	31 ± 4	Neg	210 ± 9	Neg	220 ± 9	Neg
0.62	26 ± 4	Neg	29 ± 3	Neg	102 ± 3	Neg	225 ± 3	Neg
SARCS III								
5		Tox	25 ± 3	Neg	125 ± 7	Neg	150 ± 3	Neg
2.5		Tox	29 ± 9	Neg	118 ± 5	Neg	123 ± 5	Neg
1.25	28 ± 4	Neg	26 ± 4	Neg	110 ± 6	Neg	230 ± 14	Neg
0.62	25 ± 3	Neg	32 ± 3	Neg	109 ± 5	Neg	217 ± 8	Neg
CAPSEAL I								
5	30 ± 3	Neg	19 ± 3	Neg	102 ± 7	Neg	125 ± 7	Neg
2.5	25 ± 5	Neg	21 ± 2	Neg	100 ± 5	Neg	127 ± 4	Neg
1.25	23 ± 3	Neg	23 ± 8	Neg	121 ± 6	Neg	120 ± 6	Neg
0.62	28 ± 6	Neg	29 ± 3	Neg	117 ± 9	Neg	219 ± 8	Neg
CAPSEAL II								
5	25 ± 3	Neg	25 ± 5	Neg	105 ± 3	Neg		Tox
2.5	21 ± 2	Neg	21 ± 2	Neg	113 ± 5	Neg	126 ± 5	Neg
1.25	26 ± 4	Neg	24 ± 4	Neg	120 ± 4	Neg	136 ± 8	Neg
0.62	29 ± 3	Neg	29 ± 3	Neg	117 ± 3	Neg	227 ± 9	Neg
Negative control	23 ± 5		24 ± 4		120 ± 7		128 ± 9	
Positive control	670 ± 50		2900 ± 80		1226 ± 23		1049 ± 54	

Rever. = revertants; Resp.= response; Tox = toxic; Neg = nonmutagenic and nontoxic; Mut = mutagenic

Table 5. Mutagenicity of resin-based, ZOE-based and calcium hydroxide-based sealers in 24 h set group. The number of colonies are the mean values ± SD of triplicates.

Extract (mg/plate)	TA98 Strain				TA100 Strain			
	without S9		with S9		without S9		with S9	
	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.
AH 26								
1.25		Tox		Tox		Tox		Tox
0.62		Tox		Tox		Tox	131 ± 4	Neg
0.3	32 ± 2	Neg	69 ± 5	Mut	111 ± 6	Neg	129 ± 3	Neg
0.15	30 ± 4	Neg	33 ± 2	Neg	120 ± 5	Neg	136 ± 3	Neg
AH Plus								
1.25	35 ± 4	Neg	39 ± 4	Neg	20 ± 5	Tox	55 ± 7	Tox
0.62	38 ± 3	Neg	31 ± 5	Neg	119 ± 4	Neg	129 ± 4	Neg
0.3	25 ± 5	Neg	29 ± 2	Neg	136 ± 3	Neg	143 ± 5	Neg
0.15	29 ± 7	Neg	33 ± 9	Neg	121 ± 7	Neg	130 ± 7	Neg
Tubliseal EWT								
5	27 ± 3	Neg	22 ± 2	Neg	123 ± 5	Neg	134 ± 3	Neg
2.5	20 ± 6	Neg	32 ± 5	Neg	103 ± 5	Neg	164 ± 5	Neg
1.25	19 ± 4	Neg	25 ± 4	Neg	113 ± 5	Neg	145 ± 4	Neg
0.62	26 ± 4	Neg	35 ± 3	Neg	116 ± 4	Neg	140 ± 6	Neg
PCS EWT								
5	22 ± 3	Neg	29 ± 3	Neg	135 ± 3	Neg	135 ± 3	Neg
2.5	20 ± 2	Neg	21 ± 4	Neg	123 ± 2	Neg	164 ± 5	Neg
1.25	20 ± 3	Neg	22 ± 5	Neg	119 ± 6	Neg	145 ± 4	Neg
0.62	27 ± 3	Neg	30 ± 4	Neg	126 ± 4	Neg	145 ± 3	Neg
Sealapex								
5	24 ± 6	Neg	22 ± 3	Neg		Tox		Tox
2.5	27 ± 3	Neg	30 ± 4	Neg	122 ± 7	Neg	153 ± 5	Neg
1.25	32 ± 3	Neg	25 ± 5	Neg	130 ± 4	Neg	125 ± 3	Neg
0.62	25 ± 3	Neg	27 ± 3	Neg	131 ± 5	Neg	131 ± 9	Neg
Negative control	27 ± 5		30 ± 4		130 ± 7		128 ± 8	
Positive control	675 ± 35		2900 ± 80		1232 ± 14		1149 ± 34	

Rever. = revertants; Resp.= response; Tox = toxic; Neg = nonmutagenic and nontoxic; Mut = mutagenic

Table 6. Mutagenicity of calcium phosphate-based sealers in 24 h set group. The number of colonies are the mean values ± SD of triplicates.

Extract (mg/plate)	TA98 Strain				TA100 Strain			
	without S9		with S9		without S9		with S9	
	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.	Rever.	Resp.
SARCS I								
5	28 ± 2	Neg	30 ± 3	Neg	123 ± 2	Neg	135 ± 3	Neg
2.5	29 ± 4	Neg	32 ± 3	Neg	110 ± 6	Neg	145 ± 4	Neg
1.25	35 ± 2	Neg	25 ± 6	Neg	102 ± 3	Neg	221 ± 4	Neg
0.62	26 ± 2	Neg	29 ± 6	Neg	119 ± 6	Neg	209 ± 7	Neg
SARCS II								
5	123 ± 5	Mut	111 ± 7	Mut	120 ± 4	Neg	120 ± 4	Neg
2.5	90 ± 9	Mut	109 ± 4	Mut	117 ± 9	Neg	102 ± 3	Neg
1.25	40 ± 5	Neg	32 ± 4	Neg	191 ± 5	Neg	215 ± 8	Neg
0.62	21 ± 4	Neg	26 ± 2	Neg	99 ± 4	Neg	219 ± 5	Neg
SARCS III								
5	35 ± 3	Neg	35 ± 3	Neg	115 ± 6	Neg	155 ± 2	Neg
2.5	35 ± 5	Neg	39 ± 7	Neg	117 ± 3	Neg	120 ± 4	Neg
1.25	25 ± 4	Neg	22 ± 4	Neg	121 ± 4	Neg	117 ± 9	Neg
0.62	20 ± 3	Neg	27 ± 3	Neg	118 ± 3	Neg	207 ± 8	Neg
CAPSEAL I								
5	23 ± 2	Neg	20 ± 3	Neg	102 ± 6	Neg	123 ± 5	Neg
2.5	27 ± 5	Neg	20 ± 4	Neg	117 ± 9	Neg	124 ± 7	Neg
1.25	22 ± 3	Neg	33 ± 5	Neg	120 ± 4	Neg	121 ± 5	Neg
0.62	28 ± 5	Neg	30 ± 2	Neg	107 ± 7	Neg	117 ± 9	Neg
CAPSEAL II								
5	29 ± 4	Neg	33 ± 5	Neg	105 ± 5	Neg	120 ± 4	Neg
2.5	29 ± 2	Neg	35 ± 2	Neg	109 ± 6	Neg	125 ± 3	Neg
1.25	28 ± 2	Neg	29 ± 4	Neg	121 ± 3	Neg	115 ± 5	Neg
0.62	20 ± 3	Neg	30 ± 3	Neg	110 ± 2	Neg	102 ± 3	Neg
Negative control	27 ± 5		30 ± 4		130 ± 7		128 ± 8	
Positive control	675 ± 35		2930 ± 70		1232 ± 14		1149 ± 34	

Rever. = revertants; Resp.= response; Tox = toxic; Neg = nonmutagenic and nontoxic; Mut = mutagenic

Ⅳ. Discussion

The MTT assay is a colorimetric method for quantifying viable cell numbers. The methyltetrazolium ring is cleaved by mitochondrial dehydrogenase in viable cells to formazan, which has a blue color and can be measured with a spectrophotometer[17]. The amount of formazan produced is directly proportional to the total viable cell number over wide range of cell numbers. The MTT assay reflects cell numbers at any stage in their growth cycle. Since dead cells are unable to produce the colored formazan product, this assay can be distinguished from dead cells[22]. The advantages of this method are its simplicity, rapidity, and precision, in addition, it does not require radioisotopes.

In this study, the *in vitro* test of newly developed calcium phosphate-based root canal sealers (CAPSEAL I, CAPSEAL II) and other commercially available root canal sealers were compared. Clinically, root canal sealers are inserted into the mouth in a freshly mixed and/or incompletely polymerized stage, but even after the setting period, it is still possible that potentially toxic constituents may be released from the materials by leaching into tissue fluids. For these reasons, in current study, cytotoxicity experiments were performed to estimate the cytotoxic potential of diffusible components of the set sealers. Because of different amounts of reactive substances in the fresh and set states, differences can be seen between the toxicity of fresh and set sealers. In a study in which cytotoxicity of eight root canal sealers were evaluated, Matsumoto *et al.*[23] reported that moderate and strong cytotoxicity was observed in the fresh sealers and definite toxicity was also noted in the set sealers.

Many investigators showed AH 26 had a severe cytotoxic effect[24-27]. These toxic effects of AH 26 could be caused mainly by formaldehyde, which is released primarily during the initial setting reaction[25]. And the toxicity of AH 26 may be related to amines that accelerate epoxy polymerization[4].

Cohen *et al.*[28] reported that AH 26 and AH Plus exhibited severe reactivity by agar diffusion test using L929 cells. Tai *et al.*[29] also showed that AH Plus was found to be a cytotoxic agent on three different cell lines by MTT test.

On the other hand, Koulaouzidou et al[24] showed that AH Plus indeed exhibited a lower cytotoxic potential compared to AH 26.

Beltes *et al.*[30] tested the cytotoxicity of two glass-ionomer root canal sealers. Ketac-Endo exhibited a very low cytotoxicity in all experimental periods. It proved to be a very biocompatible material. In contrast to this study, Willershausen *et al.*[31] reported strong inflammatory reaction for Ketac-Endo, whereas Endion was found to evoke a low increased PGE2 release in all of the cell lines. Kolokuris *et al.*[32] showed mild inflammatory reaction was observed with Ketac-Endo.

Briseno and Willershausen[33] tested the four calcium hydroxide-based sealers using human gingival fibroblast. Sealapex demonstrated a relatively low cytotoxicity after 3 days of culturing. Recently several researchers investigated the biocompatibility of calcium phosphate-based sealers[34]. They concluded that these materials showed mild to moderate inflammatory responses and did not exert any cytotoxic effects.

Leyhausen *et al.*[35] reported that no genotoxic or mutagenic effects were found with AH Plus. In other *in vitro* study, Koulazodou *et al.*[24] reported that AH Plus exhibited a low cytotoxic potential compared with AH 26. However the results of the current study do not correlate with those obtained by Cohen *et al.*[28] who evaluated the toxicity of AH Plus and found severe toxicity. In this study, AH 26 and AH plus were severe cytotoxic especially in state of fresh mixed. The Tubliseal EWT and The Pulp Canal Sealer EWT, ZOE-based sealers, were also toxic. Eugenol could inhibit macrophage function and may influence inflammatory reactions in the periapical tissues[36]. Eugenol liberation from eugenol-containing compounds is initially high just after mixing. Even after the sealer has set, free eugenol is still available for release over an extended period[37,38].

The SARCS II especially revealed the most cytotoxic among the calcium phosphate-based sealers in this MTT assay. This reason may be

due to the polyacrylic acid and iodoform, which it contains. Polyacrylic acid has a low pH and may leak out gradually to the surrounding tissue during the setting process[39]. The CPC containing polyacrylic acid showed an imflammatory response caused by the toxicity of unreacted polyacrylic acid[40]. In addition to polyacrylic acid, the poor cellular response of the SARCS II may be attributed to iodoform because the only difference between the SARCS I and the SARCS II is that the SARCS II contains iodoform. Iodoform-based tooth filling paste reportedly cause considerable tissue necrosis and have higher cytotoxicity than ZOE[41].

New calcium phosphate-based sealers (CAPSEAL I, CAPSEAL II) showed acceptable biocompatibility than the ZOE-based sealer, resin-based sealer and calcium hydroxide-based sealer by MTT assay and Ames test. The CAPSEAL does not have polyacrylic acid. The liquid phase of this is sodium phosphate solution instead of polyacrylic acid. Sodium phosphate is already known to show excellent tissue responses[43]. It has pH7.4 and enhances hydroxyapatite formation compared with polyacrylic acid. The new sealers contain the Portland cement as one component of the powder phase. The Portland cement and mineral trioxide aggregate (MTA) have similar chemical constitutions, except that MTA contains bismuth oxide[44]. Portland cement and white Portland cement have comparable tissue reactions to MTA and no cytotoxic and genotoxic effects[45-46]. CAPSEAL I and CAPSEAL II also had no cytotoxic effects in L-929 fibroblasts and no mutagenic responses in this study

Although further investigation is needed for the more information on the tissue adaptabilities of CAPSEALs, the results from our study suggest that CAPSEALs have the potential to be used in clinical situations.

Ⅴ. Conclusion

The purpose of this study was to compare the cytotoxicity and genotoxicity of new calcium phosphate-based root canal sealers (CAPSEAL I, CAPSEAL II) with commercially available resin-based sealers (AH 26, AH Plus), zinc oxide eugenol-based sealers (Tubliseal EWT, Pulp Canal Sealer EWT), calcium hydroxide-based sealer (Sealapex), and tricalcium phosphate based sealers (Sankin Apatite type I, Sankin Apatite type II, Sankin Apatite type III).

According to this study, the results were as follows:

1. The extracts of freshly mixed group showed higher toxicity than those of 24 h set group in MTT assay ($p < 0.001$).
2. CAPSEAL I and CAPSEAL II were less cytotoxic than AH 26, AH Plus, Tubliseal EWT, Pulp Canal Sealer EWT, Sealapex and SARCS II in freshly mixed group ($p < 0.01$).
3. AH 26 in freshly mixed group showed mutagenicity to TA98 and TA100 with and without S9 mix and AH Plus extracts also were mutagenic to TA100 with and without S9 mix.
4. Tubliseal EWT, Pulp Canal Sealer EWT and Sealapex in freshly mixed group were mutagenic to strain TA100 with S9 mix.
5. Among those of 24 h set groups, the extracts of SARCS II were mutagenic to TA98 with and without S9 mix and AH 26 was shown mutagenic effects to TA98 with S9 mix.
6. No mutagenic effect of CAPSEAL I and CAPSEAL II was detected.
7. There is no statistically significant difference between CAPSEAL I and CAPSEAL II at MTT assay and Ames test in both fresh mixed group and 24 h set group.

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국문초록

신개발 인산칼슘계 근관 봉함재의 세포독성 및 유전독성에 관한 연구

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본 연구의 목적은 기존 상용화된 근관 봉함재인 레진계 봉함재 (AH 26, AH Plus), 산화 아연 유지놀계 봉함재 (Tubliseal EWT, Pulp Canal Sealer EWT), 수산화 칼슘계 봉함재 (Sealapex), 기존의 인산삼칼슘계 봉함재 (Sankin Apatite type I, II, III)와 새로이 개발된 인산칼슘계 근관 봉함재 (CAPSEAL I, CAPSEAL II)의 세포독성과 유전독성을 비교 평가하고자 하였다.

MTT test를 통해 세포독성을 평가하였으며, 미생물을 이용한 복귀돌연변이 시험 (Ames test)으로 유전독성을 평가하였다. 이 연구의 결과는 아래와 같다.

1. 즉시군이 24시간군에 비해 MTT assay에서 세포독성이 높게 나타났다 ($p < 0.001$).
2. 즉시군에서 CAPSEAL I과 CAPSEAL II는 AH 26, AH Plus, Tubliseal EWT, Pulp Canal Sealer EWT, Sealapex와 SARCS II 보다 낮은 세포독성을 보였다 ($p < 0.01$).
3. 즉시군에서 AH 26은 TA98과 TA100에 각각 S9 fraction을 처리한 경우와 그렇지 않은 경우 모두 유전독성을 나타냈으며, AH Plus 또한 TA100에 S9 fraction을 처리한 경우와 그렇지 않은 경우 유전독성을 나타냈다.
4. 즉시군에서 Tubliseal EWT, Pulp Canal Sealer EWT, Sealapex가 TA100 균주에 S9 fraction을 처리하였을 때 유전독성 양성반응이 나타났으며, 그 외의 경우는 모두 음성반응을 나타냈다.
5. 24시간군에서는 SARCS II가 TA98 균주에서 S9 fraction 처리했을 때와 처리하지 않았을 때 모두 유전독성이 나타났고, AH 26은 TA98에 S9 fraction을 처리하였을 때 유전독성이 나타났다. 그 외의 경우는 모두 음성반응을 보였다
6. CAPSEAL I과 CAPSEAL II는 유전독성에서 모두 음성반응을 나타냈다.
7. CAPSEAL I과 CAPSEAL II 두 근관봉함재 간에는 세포독성실험과 유전독성실험에서 즉시군과 24시간군 모두에서 통계학적으로 유의할 만한 차이를 보이지 않았다.

주요어: 근관 봉함재, 세포독성, 유전독성, 생체적합성, 인산칼슘