Root canal sealers induce cytotoxicity and necrosis

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There are three types of the root canal sealers commonly used in clinical applications. They are calcium hydroxide base (Sealapex), zinc oxide–eugenol base (Canals), and epoxy-resin base (AH Plus). Elutable substances and degradation products from root canal sealers may gain access to periodontal tissue in a number of ways. The purpose of the present study was to evaluate the biologic effects of the root canal sealers on human oral cancer cell line (OC2). The tetrazolium bromide (MTT) assay was to evaluate the cell's survival rate. The DNA electrophoresis was used to evaluate the OC2 cell's DNA damage. The results demonstrated that the above root canal sealers' survival rates are in dose-dependent increase ($p < 0.05$). The toxicity of fresh mix group is higher than that of the mixed after 24 h group. DNA fragmentation assay of sealer treated OC2 cells shows a smear layer pattern on the electrophoresis gel. There is no DNA damage found. The toxicity that regulated the cell death is not by the apoptic change of cells.

Introduction

Adverse effects of dental materials may play an important role in endodontic failures in many cases in which no major fault in treatment can be identified [1]. Since most dental materials release small amounts of various substances into their physiological environment, the potential genotoxicity, mutagenicity, and carcinogenicity of dental materials must be determined so that appropriate regulations can be applied to their use. Previous studies have shown that dental materials may induce local and systemic adverse effects due to the release of extractable monomers and/or other inorganic and organic ingredients [2].

There are a number of root canal sealers currently available that are based on quite different formulae (Table I). That is epoxy-resin sealer such as AH Plus (Dentsply, DeTrey Co., Surrey, UK), the calcium hydroxide-based materials include Sealapex (Kerr Co., MI, USA), and the zinc oxide–eugenol based sealer such as Canals (Showa Co., Tokyo, Japan).

Eugenol is known to be an antioxidant and anti-inflammatory agent. However, it has been demonstrated that zinc oxide–eugenol were moderately to severely toxic in implantation studies. Severe inflammation was found in injected subcutaneous connective tissue of rabbit [3]. Moderate inflammation was observed in apical and periapical tissue of baboons after a two-year observation period [1].

Calcium hydroxide-based compounds are thought to meet most of the requirements of endodontic treatment. Calcium hydroxide-based root canal sealer showed mild to moderate tissue irritating activities [3, 4]. Several studies examining toxicity inflammatory response demonstrated generally a mild inflammatory reaction, with an influx of foreign body giant cells [5–7].

The resin-based root canal sealer AH 26 caused severe tissue irritation after observation periods of a few days in various biocompatibility studies, however, mild tissue reactions were usually reported from long-term investigation [4, 8, 9]. The fresh and set specimens of the epoxy resin-based root canal sealer AH 26 may induce strong cytotoxic effects. These experimental observations have been confirmed by some clinical case reports [7, 9, 10]. Recently, a new resin-based sealer called AH plus has been developed to avoid this problem. According to the manufacturers, AH plus is biocompatible and does not release formaldehyde. The growth inhibition test with primary human periodontal ligament fibroblasts and permanent 3T3 monolayers showed that AH plus caused only a slight or no cellular injuries [11]. Our previous

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TABLE I The composition of three types of root canal sealers

Sealapex	Calcium oxide, barium sulfate, zinc oxide, subMicron silica, tatinium oxide, zinc stearate, Methyl toluene sulfonamide, poly(methyl methyl saliate) resin, methyl salicylate, pigament
Canals	Powder: Zinc oxide, $BaSO_4$, Bi-subcarbonate, Rosin. Liquid: Eugenol oil, olive oil
AH Plus	Paste A: Epoxy resin, calcium tungstate, zirconium oxide, aerosil, iron oxide. Paste B: Adamantane amine, calcium tungstate, zirconium oxide, Aerosil, N,N′-dibenzyl-5-oxanonane-diamine-1,9 TCD-diamine, silicone oil.

studies have demonstrated that AH plus existed dose dependent toxicity on hepatocytes by LDH assay and toxic to human oral cancer cell line (OC2) by (MTT) test [12, 13].

The exposing cell to the exogenous agent brings about two kinds of changes. One is the cell changing into apoptosis (programmed cell death) pathway and the other is necrosis (reproductive cell death). The induction of the cell toxicity may induce rapidly by a variety of chemical and physical stimuli, including oxidative stress, tumor promoters, and DNA damage agents [14–19]. In addition, many other types of apoptotic stimuli also result in the generation of intracellular reactive oxygen intermediates or are associated with evidence of oxidative stress [20, 21]. The former, our study has demonstrated that some root canal sealers can cause the oxidative stress in hepatocytes [22]. Very little information is available on the root canal sealers' toxic mechanism. The purpose of the present study was to evaluate the cytologic effect of root canal sealers and its mechanism of cellular toxicity.

Materials and methods

Test materials and sample preparation

The various root canal sealers that were tested in the present study are listed in Table I. The materials were mixed according to the manufacturers' instructions. Samples that were tested for cytotoxicity were prepared as follows: the fresh mix group was defined as freshly mixed materials filled in glass rings (3 mm height, 5 mm diameter) and immediately eluted in 10 ml of cell culture medium at 37 °C for 24 h in a 5% CO_2 air atmosphere. The after-24 h mix group was defined as freshly mixed materials were filled in glass rings (3 mm height, 5 mm diameter) and set for 24 h at 37 °C in a humidified chamber. The test specimens were then eluted in 10 ml of cell culture medium at 37 °C for 24 h in a 5% CO_2 air atmosphere. With serial dilution by culture medium, the final concentrations of the root canal sealers were as follows 0.02, 0.1, 0.5, 2.5, and 12.5 mg/100 µl.

Survival rate of root canal sealer materials

The human oral cancer cell line (OC2) was routinely cultivated in DMEM (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St Louis, MO, USA) at 37 °C in an air atmosphere containing 5% CO_2 [13, 23]. Single cell suspension of OC2 cells was obtained from monolayer cell cultures close to confluent after trypsinization. Cell numbers were determined by hemocytometer counting and 10^4 cells/well were seeded into 96 well plates. The cells were then incubated for 24 h in a humidified atmosphere of air and 5% CO_2 at 37 °C. Cell cultures were exposed to 200 µl aliquots of serially diluted eluates. Eight wells were used for each single eluate concentration in each of the three independent experiments. Exposure of cell cultures was stopped by discarding the exposure medium after 24 h. Viable cells in both treated and untreated cell cultures were stained with the formazan dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) (1 mg/ml) (Sigma Chemical Co., St Louis, MO, USA) dissolved in 200 µl culture medium as described [21]. After 3 h at 37 °C, the MTT solution was discarded and formazan crystals were solubilized with 200 µl of DMSO. Optical densities were measured at 570 nm in a multiwell spectrophotometer (Hitachi, Tokyo, Japan).

Data presented in Tables II to IV are mean values ($\pm$ SD) calculated from 24 measurements in three independent experiments as described. Statistical analysis was conducted using the SAS software (SAS Institute, Gary, NC, USA) by one-way analysis of variance. A value of $p < 0.05$ was considered statistically significant.

TABLE II The MTT assay of the OC2 cell line treated with calcium hydroxide-based root canal sealer (Sealapex)

Concentration (mg/100 µl)	Survival rate (%)			
	Base	Catalyst	Fresh mix	After 24 h mix
0.02	99.6 $\pm$ 1.3	102.2 $\pm$ 0.3	64.7 $\pm$ 0.4	82.7 $\pm$ 1.6
0.1	86.0 $\pm$ 0.5	80.0 $\pm$ 1.7	41.7 $\pm$ 0.5	71.2 $\pm$ 1.4
0.5	74.5 $\pm$ 0.3	56.4 $\pm$ 0.5	33.4 $\pm$ 0.6	63.7 $\pm$ 1.2
2.5	57.5 $\pm$ 0.4	49.3 $\pm$ 0.5	28.7 $\pm$ 0.2	49.6 $\pm$ 0.3
12.5	8.7 $\pm$ 1.1	44.2 $\pm$ 0.1	20.7 $\pm$ 0.2	33.6 $\pm$ 0.8
$p < 0.05$	Yes	Yes	Yes	Yes
TC50 (mg/100 µl)	1.61	3.04	0.07	1.96

The present of survival rate is mean $\pm$ SD. TC50 means that concentration inhibited 50% cell survival.

TABLE III The MTT assay of the OC2 cell line treated with zinc oxide-eugenol based root canal sealer (Canals)

Concentration (mg/100 µl)	Survival rate (%)			
	Liquid	Powder	Fresh mix	After 24 h mix
0.02	89.8 ± 3.5	88.0 ± 2.4	79.5 ± 0.7	82.8 ± 0.7
0.1	41.7 ± 0.7	63.3 ± 0.3	19.6 ± 0.6	64.0 ± 1.1
0.5	12.1 ± 1.8	38.5 ± 0.03	6.6 ± 0.3	57.9 ± 1.0
2.5	4.8 ± 1.8	17.3 ± 0.2	5.9 ± 0.1	50.5 ± 0.5
12.5	1.1 ± 0.7	3.2 ± 0.2	3.6 ± 0.6	8.2 ± 1.2
$p < 0.05$	Yes	Yes	Yes	Yes
TC50 (mg/100 µl)	0.11	0.25	0.04	0.65

TABLE IV The MTT assay of the OC2 cell line treated with resin based root canal sealer (AH plus)

Concentration (mg/100 µl)	Survival rate (%)			
	Paste A	Paste B	Paste A + Paste B (Fresh mix)	Paste A + Paste B (After 24 h mix)
0.02	103.3 ± 1.8	76.0 ± 1.0	93.6 ± 0.7	98.4 ± 1.1
0.1	49.4 ± 0.5	69.8 ± 0.5	63.2 ± 0.6	69.0 ± 0.8
0.5	38.8 ± 0.4	54.5 ± 0.1	31.2 ± 0.8	34.0 ± 0.2
2.5	14.2 ± 0.1	49.0 ± 0.3	26.5 ± 0.4	32.0 ± 0.3
12.5	8.6 ± 0.2	48.0 ± 0.5	22.3 ± 0.8	21.8 ± 0.5
$p < 0.05$	Yes	Yes	Yes	Yes
TC50 (mg/100 µl)	0.30	3.60	0.40	0.55

DNA fragmentation assay

Following the method of Fady *et al*. [25], cells were lysed in 10 mM Tris-HCL (pH 7.5), 100 mM EDTA, 0.5% SDS, and 100 µg/ml of proteinase K for 18 h at 37 °C. DNA was then extracted twice with phenol, protein C inhibitor (PCI), and choloroform, precipitated in ethanol, centrifuged (30 min at 10 000 speed gram), and resuspended in TE buffer containing 100 µg/ml of RNase for 1 h at 37 °C. Following one more extraction in phenol, PCI, and chloroform and precipitation in 70% ethanol, DNA was suspended in TE buffer and 5–10 µg DNA per lane was electrophoresed in a 1% agarose gel for 2 h at 45 V. The gels were visualized with ethidium bromide.

Results

Survival rate of the root canal sealers

The survival rate and 50% toxic concentration of the root canal sealers were shown in Table II to IV. The root canal sealers existed statistical dose dependent increase toxicity ($p < 0.05$).

The fresh mix group of calcium hydroxide-based root canal sealer (Sealapex) showed the lowest TC50 (0.07 mg/100 µl). The TC50 values of 24 h mixing group were 30-fold higher than that of 1 h mixing group (Table II).

The fresh mix group of zinc oxide–eugenol based root canal sealer (Canals) showed the lowest TC50 (0.04 mg/100 µl). The 24 h mixing group had higher TC50 value (0.65 mg/100 µl) (Table III).

The epoxy resin-based root canal sealer (AH Plus) paste A showed the lowest TC50 (0.30 mg/100 µl). The fresh mix and after 24 h mix groups showed similar TC50 (Table IV).

DNA fragmentation

Electrophoresis of DNA obtained from OC2 cells treated with one-fifth TC50 value root canal sealers are shown in Figs. 1 and 2. The fresh mix group with short time treatment showed that DNA cleavage as smear patterns on electrophoresis gel (Fig. 1). The fresh mix group with 12 or 24 h treatment revealed same smear layer (Fig. 2)

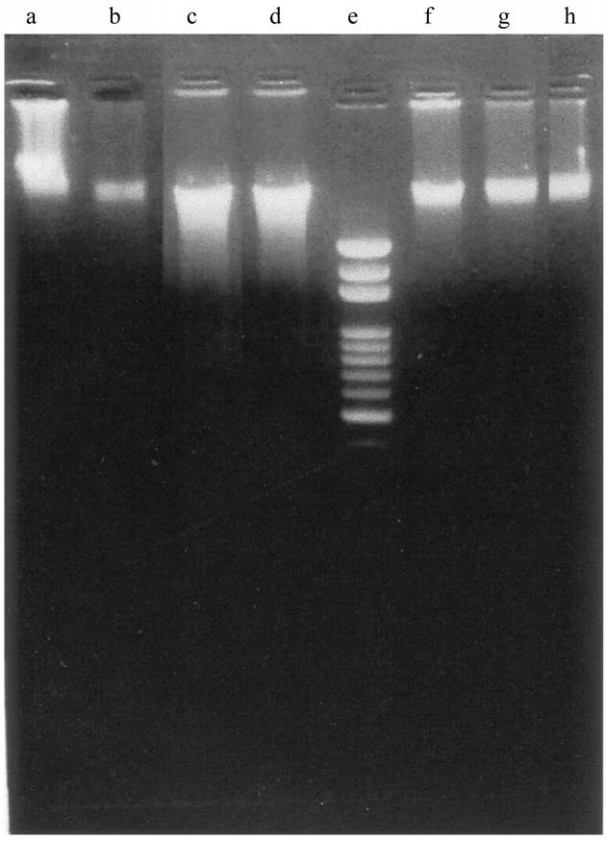

Figure 1 The DNA fragmentation assay of the root canal sealers treated on the OC2 cells.
Lanes a–e is the 1/5TC50 of root canal sealers treated on OC2 cells for 1 h. Lanes f–h is the 1/5TC50 of root canal sealers treated on OC2 cells for 6 h. a: DMSO, b: Canals, c: Sealapex, d: AH plus, e: reference ladder, f: DMSO, g: Canals, g: Sealapex, h: AH plus.

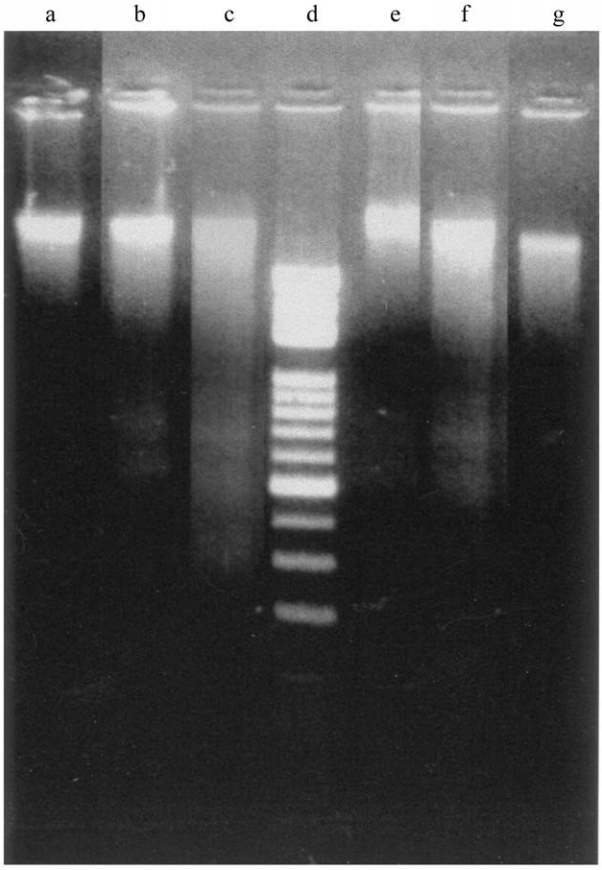

Figure 2 The DNA fragmentation assay of the root canal sealers treated on the OC2 cells.
Lanes a–e is the 1/5TC50 of root canal sealers treated on OC2 cells for 12 h. Lanes a–e is the 1/5TC50 of root canal sealers treated on OC2 cells for 24 h. a: Canals, b: Sealapex, c: AH plus, d: reference ladder, e: Canals, f: Sealapex, g: AH plus.

Discussion

Sealapex is a calcium hydroxide type sealer, in use since the early 1980s. It has been used in endodontics for a number of years to repair root perforations, halt root resorption, control exudates in problem teeth, and stimulate development of root formation. The calcium hydroxide-based root canal sealer of fresh mix group exist the lowest TC50 value. That is, the highest cytotoxicity is found in fresh mix group. It is apriorism that the toxicity is result from high pH value of fresh mix Sealapex. The previous study found that calcium hydroxide-based sealers (Sealapex and Tubli seal) were cultured with human gingival fibroblasts for three weeks. After a cytotoxic reaction at the beginning of the trial the fibroblasts showed almost complete recovery with sealers between the 5th and 9th day of culturing and remained this way during the rest of the testing period [26].

The zinc oxide–eugenol sealers are used in general practice for root canal sealer. Eugenol (4-allyl-2-methoxyphenol, a natural substance derived from the oil of cloves) was previously found to be genotoxic in several *in vitro* systems including the Ames test [27]. Furthermore, a zinc oxide–eugenol based sealer was found to be cytotoxic, and this action was ascribed to the eugenol component [28]. The diffusion of eugenol from zinc oxide–eugenol sealers appears to depend on hydrolysis of the eugenol component [29]. The present study confirmed these previous studies, showing that all zinc oxide–eugenol-containing sealers tested induced dose-dependent increases in cytotoxicity (Table III).

The epoxy resin-based root canal sealer AH Plus, according to the manufacturer, described that AH Plus is the new product that has the advantageous properties of AH 26, but preserves the chemical property of the epoxy amine better so that material no longer releases formaldehyde. Due to the complex chemical composition (Table I), numerous substances may be released form AH plus into the adjacent tissues and might thus induce local and/or systemic adverse effect, including cytotoxicity or genotoxicity [30, 31]. These results are identical to our present study. Present study shows that the TC50 value of paste A and fresh mixed AH Plus were low. The paste A and fresh mix AH Plus are more toxic than other components. The epoxy resin-based sealer toxicity is statistical dose-dependent increase (Table IV). We inferred that the cause of toxicity is from the epoxy-resin component of the AH Plus sealer. Contrary to our experiment, Leyhausen *et al*. indicate that the new epoxy resin-based sealer AH Plus is cytocompatible with 3T3 (Swiss albino mouse fibroblast) or human periodontal ligament fibroblast cell [32]. The different results may come from the different conditions of the experiment.

Usually, exposing cell to the drug brings about two kinds of changes. One is the cell changing into apoptosis (programmed cell death) pathway and the other is necrosis (reproductive cell death). The morphological changes associated with necrosis are cell swelling, rupture of membranes, and lysis of organized structure. In contrast, during the process of apoptosis, condensation, and fragmentation of the cytoplasm and nucleus occur in the target cells while normal organelle structure is maintained. Several studies indicate that apoptosis can be induced by exposing cells to exogenous oxidants [17–19, 33, 34]. In addition, many other types of apoptotic stimuli also result in generation of intracellular reactive oxygen intermediates (ROIs) or are associated with evidence of oxidative stress. Therefore, the investigator has hypothesized that the stimulated generation of ROIs participates in pathways of apoptosis common to many different types of triggers [35]. The previous study demonstrated that the root canal sealers existed the oxidative stress on the cultured cells. From present result, DNA fragmentation assay showed no DNA ladder formation. There is no apoptic body found in microscope observation. The difference in toxicity patterns may be related to the different cells and the degree of setting.

The present study was used the one-fifth concentrations of the TC50. The reason is low concentrations of oxidants can induce cytostasis without affecting viability or target cell lysis not associated with characteristic apoptotic morphology or DNA fragmentation. In some instances, oxidant induced cell death mediated by pathways of necrosis can be contrasted with those of apoptosis, and intensity of the oxidative stress may determine which pathway is triggered [19, 36, 37]. The present result is similar with above previous findings. The target cells appear not to die through apoptotic pathway.

Conclusion

The results of the present study indicate that root canal sealer can cause the cytotoxicity on OC2 cells, but not have significant effect on DNA of OC2 cells. The change of the cell toxicity is not from the apoptotic change but necrosis of the cell. This result could be investigated further including by kinase analysis.

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