Comparison of the biocompatibility between 2 endodontic filling materials for primary teeth

CHUNG-WEN CHEN 1  CHIA-TZE KAO 2  TSUI-HSIEN HUANG 1,3

1 Institute of Stomatology, Chung Shan Medical University, Taichung, Taiwan, ROC.
2 Institute of Oral material science, Chung Shan Medical University, Taichung, Taiwan, ROC.
3 Dental Department, Chung Shan Medical University Hospital Taichung, Taiwan, ROC.

The most-popular root canal filling materials for primary teeth are zinc oxide eugenol (ZOE), iodoform paste, and calcium hydroxide. Root canal filling materials need to be biocompatible with the periapical tissue. There are few papers which indicate the mechanism of cellular change of root canal filling materials. The purpose of the present study was to evaluate the cellular changes of a ZOE with formocresol (FC) and Vitapex-treated human osteosarcoma cell line. The ZOE powder mixed with the FC liquid and Vitapex alone were eluted in culture medium. The elution solutions were used to treat the human osteosarcoma cell line. The MTT assay was used to investigate the survival rate of U2OS cells. Western blot assay was used to compare the c-jun N terminal kinase (JNK) expression. One way ANOVA was used to compare the survival difference with p < 0.05 accepted as statistically significant. The result showed that the ZOE sealer was toxic to U2OS cells (p < 0.05). Vitapex was biocompatible with U2OS cells. JNK kinase expression was seen only in the ZOE with FC group. We concluded that ZOE with FC is not biocompatible with U2OS cells, and cell death occurred by apoptosis. Clinically, we suggest that Vitapex is a good choice for a primary tooth root canal filling material.

Key words: endodontic filling material for primary teeth, MTT assay, Western blot analysis.

Endodontic therapy for primary teeth has long been advocated when the criteria for classical pulpotomy treatment can’t be met. Resorption of the filling material is considered one of the requirements of an ideal root-canal medicament for pulpectomies of primary teeth. Resorption of the root canal filling material should occur as the root of the primary tooth is resorbed during exfoliation, permitting normal eruption of the succedaneous tooth. If the material is expressed beyond the apex, it should be resorbable and non-toxic to the periapical tissues and the permanent tooth germ.

The most-popular root canal filling materials for primary teeth are zinc oxide eugenol (ZOE), iodoform paste, and calcium hydroxide. ZOE has been the material of choice for many years. Although this agent showed antibacterial effects against pure cultures of bacteria in several studies, combining it with formocresol (FC) increased its antibacterial effect.

Animal studies using ZOE cement as root canal filling material have reported chronic inflammatory reactions and slow resorption of the material. Etrausquin and Muruzabal used ZOE as a root canal filling material in 141 rats followed for 1 to 90 days. They noted that ZOE irritated the periapical tissues and caused necrosis of the bone and cementum. In addition, they noted that extruded ZOE developed a fibrous capsule that prevented resorption. Gould first reported a 1-visit ZOE pulpectomy study in 1972 in which 39 molars were filled with ZOE. He concluded that 35 of the 39 molars followed for a mean time of 16 months had successfully been treated.

Root canal filling material for primary teeth has
Biocompatibility of filling materials

long been iodoform paste (Kri paste), even though unfavorable responses of periapical tissue to Kri paste and increased cytotoxicity were found\textsuperscript{14,15}. A newer preparation, Vitapex, a mixture of iodoform and calcium hydroxide (Ca(OH)\textsubscript{2}), is now available in North America. A preliminary study suggested that it too is efficacious for pulpectomies in primary teeth\textsuperscript{16}. Vitapex demonstrated inhibitory activity against Streptococcus mutans, Staphylococcus aureus, and Lactobacillus casei. The main ingredients of Vitapex are iodoform at 40.4%, calcium hydroxide at 30.3%, and silicone at 22.4%. When extruded into furcal or apical areas, Vitapex can either diffuse away\textsuperscript{17,18} or be resorbed in part by macrophages\textsuperscript{19,20} in as short a time as 1 or 2 weeks\textsuperscript{17}. Bone regeneration has been clinically\textsuperscript{17,21} and histologically\textsuperscript{21,22} documented after using Vitapex. Calcium hydroxide-based compounds are thought to meet most requirements of endodontic treatment. Calcium hydroxide-based filling materials showed mild to moderate tissue-irritating activities\textsuperscript{23}. Several studies examining toxicity inflammatory response, demonstrated a generally mild inflammatory reaction, with an influx of foreign body giant cells\textsuperscript{24-26}.

Cell growth, division, differentiation, and death are now known to be regulated in part by the mitogen-activated protein kinase (MAPK) pathway\textsuperscript{27,28}. Cellular signal transduction is a 2-step process: first, a signaling molecule is sensed by a receptor at a target cell, and then the receptor is activated. The c-Jun N-terminal kinase (JNK), one of the MAP kinases, is a member of an evolutionarily conserved subfamily of mitogen-activated protein (MAP) kinases. Selective phosphorylation of c-Jun by JNK is determined by a specific docking motif in c-Jun, the delta region, which enables JNK to physically associate with c-Jun. Signals relayed by JNK through c-Jun regulate a range of cellular processes including cell proliferation, tumourigenesis, apoptosis, and embryonic development\textsuperscript{29-32}.

Root canal filling materials for primary teeth have slow resorption rates in tissue. Long-term clinical studies reveal retention rates of material in 28.7% to 73.3% of cases\textsuperscript{33}. Another study found that the retained material altered the paths of eruption of succedaneous teeth in 20% of cases\textsuperscript{34}. The mechanism of the cell reaction after root canal filling material treatment has seldom been reported. The purpose of the present study was to evaluate cellular changes of a ZOE- and Vitapex-treated human osteosarcoma cell line.

**MATERIAL AND METHODS**

**Materials and sample preparation**

The experimental groups containing the 2 root canal filling materials are listed in Table 1. The zinc oxide eugenol materials were mixed in the following rate: ZnO: eugenol: FC of 6 g: 1 ml: 1 ml. The Vitapex was directly injected into the ring. Samples tested for cytotoxicity were prepared as follows: freshly mixed materials were filled in glass rings (2 mm in height, 6 mm in diameter) and allowed to set for 24 h at 37 ℃ in a humidified chamber. One to 3 test specimens were then eluted in 10 ml of cell culture medium at 37 ℃ for 24 h in a 5% CO\textsubscript{2} air atmosphere. The concentration of the test material was diluted by adding different volumes of medium. The culture medium without adding test material served as the control group.

**MTT assay**

The human osteosarcoma cell line (U2OS) was routinely cultivated in McCoy’s medium (Sigma Chemical, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Sigma) at 37 ℃ in an air atmosphere containing 5% CO\textsubscript{2}. Single-cell suspensions of U2OS cells were obtained from monolayer cell cultures close to confluence after trypsinization. Cell numbers were determined by

<table>
<thead>
<tr>
<th>Table 1. Composition of the experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Vitapex (Neo Dental Clinical, Tokyo, Japan)</td>
</tr>
<tr>
<td>Zinc oxide (Pulpdent, Watertown, MA, USA)</td>
</tr>
</tbody>
</table>

hemocytometric counting, and $10^4$ cells/well were seeded into 96-well plates. 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 2-, 6-, and 10-$\mu$l aliquots of serially diluted eluates. Five wells were used for each eluate concentration in each of 3 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 MTT (1 mg/ml) (Sigma) dissolved in 200 $\mu$l culture medium as described$^{35-37}$. After 3 h at 37 °C, the MTT solution was discarded, and formazan crystals were solubilized with 200 $\mu$l of DMSO. Optical densities were measured at 570 nm in a multiwell spectrophotometer (Hitachi, Tokyo, Japan). The survival rate was calculated as Survival $\% = \text{absorbance of the treated sample} / \text{absorbance of the medium} \times 100\%$. Results were compared using the one-way analysis of variance (ANOVA). Differences in treatment means were analyzed by the Student-Newman-Keul’s test and were considered to be significant at p < 0.05.

Western blot analysis

From the MTT test of the root canal filling material, 4 $\mu$l of Vitapex and 4 $\mu$l of the ZnO group were used to detect kinase expression$^{38}$. U2OS cells treated for 1 hour were washed once with cold PBS. Five million cells were lysed in 50 $\mu$l of lysis buffer (1% Triton X-100, 0.5% NP40, 10 mM EGTA, 0.2 mM Na$_3$VO$_4$, 0.2 mM NaF, and 0.2 mM PMSF). Cell lysates were cleared at 15,000 rpm for 15 minutes at 4 °C. Twenty-five micrograms of protein from each sample was boiled for 5 min in 1X SDS gel-loading buffer (125 mM Tris (pH 6.8), 5% glycerol, 28 mM SDS, 1% beta-mercaptoethanol, and 0.006% bromophenol blue). Proteins were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature in 3% BSA, 5% nonfat dried milk, 10 $\mu$m Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20. After 4 washes in TBS-T buffer, the membrane was incubated with 0.5 $\mu$g/ml rabbit JNK antibody for overnight. After four more washes, the membrane was overlayed with a second antibody (rabbit anti-JNK antibody) for 1 hour, then washed with TBS-T buffer for 20 minutes.

RESULTS

The survival rate of the Vitapex group showed

![Figure 1. Survival rate (%) of Vitapex-treated U2OS cell.](image-url)
no statistical difference (p > 0.05) with control group (Figure 1). The survival rate of the ZOE with FC group showed a dose dependent decrease (p < 0.05) (Figure 2).

The morphology of U2OS cell treated with the filling materials is shown in Figure 3. Cell membranes in the ZOE group were not intact and contained vesicles. The morphology of the Vitapex group showed that cells were the same as those of the control group.

JNK expression was shown in ZOE-treated U2OS cells. The control group and Vitapex group exhibited no JNK band expression according to the Western blot analysis (Figure 4).

**DISCUSSION**

Because of the anatomy of primary roots and furcal areas, it is difficult to avoid extrusion of sealer beyond the root canals in all pulpectomy cases. Vitapex contains calcium hydroxide and iodoform. In 1991, iodoform paste was advocated as a pulpectomy filler in primary teeth due to its resorbability and disinfectant properties\(^{33}\). Result of the present study show that the Vitapex is more biocompatible with U2OS cells than is the ZOE group. So, the use of ZnO+eugenol+FC as endodontic filling material might cause damage to periapical tissue. This result is similar to that of Erausquin and Muruzaba’s study which demonstrated ZOE to be highly toxic to periapical tissues in rats, causing necrosis of the hard tissues it contacted\(^{39}\). A previous report showed that long-term clinical studies revealed retention rates of the material in 28.7% to 73.3% of cases\(^{34,35}\). It was also shown that the retained material altered the paths of eruption of succedaneous teeth\(^{34,35}\). From the present results, the biocompatibility of the root canal filling material may be the main factor affecting alteration of the eruption path.

Traditionally, zinc oxide eugenol has been the most-commonly used root canal filling material for primary teeth. It has been used in combination with silver nitrate, formocresol, or iodoform, all of which have fixative properties. 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\(^{40}\). Furthermore, a zinc oxide-eugenol- based sealer was found to be cytotoxic, and this action was ascribed to the eugenol component\(^{40}\).

Since the traditional ZOE with FC is toxic to U2OS cells, cellular changes after U2OS cell contact with ZOE were discovered. JNK was expressed in the ZOE group but not in the control or Vitapex groups (Figure 4). This means that in ZOE-treated U2OS cell,

![Figure 2. Survival rate (%) of U2OS cells treated with ZOE with FC cement. *: Statistically significant difference at p < 0.05.](image-url)
its toxic mechanism may be through the JNK pathway. Signals relayed by JNK through c-Jun regulate a range of cellular processes including cell proliferation, tumourigenesis, apoptosis, and embryonic development\(^29-32\). The toxicity of ZOE might cause cell apoptosis rather than necrosis. This finding has not been shown in any previous study.

Although the success rates of ZOE root fillings range from 68.7% to 86.1%, overfilling with ZOE resulted in a much lower-success rate (41%)\(^42\). Vitapex, used as a root canal filling material for pulpectomy treatment for primary teeth is resorbed extraradicularly and intraradicularly without apparent ill effects, and has been proven to be clinically and radiographically successful\(^43\). From the present study, we suggest that Vitapex can be a good choice of an endodontic filling material for primary teeth.

![Figure 3. Morphology of the U2OS cells treated with test materials. A. Control group. B. ZOE with FC group. C. Vitapex group. (magnification, 10X)](image)

![Figure 4. JNK expression by Western blot analysis. The JNK band was expressed in the ZOE with FC group but not in control on Vitapex groups.](image)
REFERENCES

38. Huang TH, Ding SJ, Hsu TC, Kao CT. Effects of mineral