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ORIGINAL ARTICLE

Cytologic effects of primary tooth endodontic filling materials

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Background/purpose: Primary tooth endodontic filling materials should be biocompatible with periodontal tissue. The purpose of this study was to analyze the biologic effects of different endodontic filling materials for primary teeth on a human osteosarcoma cell line (U2OS).
Materials and methods: Experimental groups comprised different mixes of endodontic filling materials: zinc oxide-eugenol (ZnOE)+formocresol (FC); calcium hydroxide [$Ca(OH)_2$]+FC; $Ca(OH)_2$+iodoform+deionized water; $Ca(OH)_2$+iodoform+camphorated parachlorophenol (CPC); $Ca(OH)_2$+CPC; and Vitapex. These were prepared and used to fill special glass rings, which were subsequently eluted in 10 mL of cell culture medium at 37°C in a 5% carbon dioxide-in-air atmosphere for 24 hours. Cell culture medium alone was used as the control group. A DNA fragmentation assay was performed to determine the genotoxicity of each mix of materials. The level of cyclooxygenase (COX)-2 protein expression, the extent of dental material-elicited inflammation of U2OS cells, and the degree of mitogen-activated protein (MAP) kinase expression were determined using Western blot analysis.
Results: The results revealed that no DNA breakage was apparent after U2OS cells were treated with the various materials. COX-2 band expression dramatically declined in the ZnOE+FC group compared with the control group, although high levels of expression of the COX-2 band were noted for the $Ca(OH)_2$+FC and $Ca(OH)_2$+iodoform+CPC groups. Band levels of extracellular signal-regulated kinase (ERK-1 and ERK-2) expression declined in the ZnOE+FC and $Ca(OH)_2$+CPC groups compared with the control group. p53 and caspase-3 protein bands appeared in all experimental groups.
Conclusion: The cytotoxic mechanism of endodontic filling materials on U2OS cells was induced by means of activation of the p53 and caspase-3 apoptosis signaling pathways.

Introduction

Primary tooth endodontic filling materials are required to be resorbable and nontoxic to periapical tissues and the permanent tooth germ. According to reports by manufacturers of these materials, many different kinds of filling materials matching such requirements are currently available on the

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market. Such materials include calcium hydroxide [$Ca(OH)_2$], zinc oxide (ZnO), ZnO-eugenol (ZnOE) either with or without the incorporation of formocresol (FC), and iodoform pastes, such as Kri-1 paste (a mixture of iodoform and camphorated parachlorophenol [CPC]) and Vitapex paste [a mixture of 40.4% iodoform, 30.3% $Ca(OH)_2$, and 22.4% silicone]. The respective manufacturers all claim that these materials are biocompatible.

The use of ZnOE or ZnO to fill root canals of primary teeth was first described by Sweet in 1930. Such agents have been demonstrated to have antibacterial effects against pure cultures of certain bacteria, as reported by a number of studies.[1,2] However, Prashar et al.[3] reported that clove oil was highly cytotoxic to human skin cells at concentrations as low as 0.03% (v/v) with up to 73% of this effect being attributable to eugenol. Eugenol and FC components have been demonstrated to be toxic to cultured mammalian cells.[3,4] Similar cytotoxicity results were found in our previous work, and the addition of eugenol or FC to different endodontic filling materials revealed different degrees of toxicity toward a human osteosarcoma cell line (U2OS).[5]

The results of an animal study indicated that purified eugenol elicited less tissue necrosis and inflammation at all different exposure levels and for all different exposure times than was the case for commercially available eugenol. The degree of inflammation elicited by various ZnOE mixtures was strongly influenced by the quantity of free eugenol present in the mixture.[6] Eugenol compounds have various biologic effects including both antioxidant and anti-inflammatory activities.[7–10]

In 1997, Fuks et al.[11] compared the pulpal responses of baboon teeth to ferric sulfate and FC. Biologic outcomes of both tested chemical agents appeared to be equivalent 6 weeks after exposure to these materials, with 60% of the teeth from each test group showing mild inflammation. Cotes et al.[12] confirmed a similar inflammatory response of rat teeth after exposure to ferric sulfate and FC. Two studies of histologic impacts of FC upon dental pulp following pulpotomy showed chronic inflammation and necrosis of the radicular pulp,[13,14] while other animal models revealed the systemic distribution of FC from the pulpotomy site[15] and allergic and/or mutagenic properties of formaldehyde.[16]

The eukaryotic transcription factor nuclear factor (NF)-κB plays a primary role in general inflammation and the immune response.[17] NF-κB is a critical regulator of cyclooxygenase (COX)-2 expression in many different cell lines.[17,18] The intracellular signaling cascades controlling NF-κB activation are reported to be highly complex and involve a distinct set of kinases. Of the potential protein kinases involved in the activation of NF-κB, the activity of mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase/stress-activated protein kinase signaling pathways, have been well characterized.[19,20]

Our purpose was to analyze the biologic effects of different endodontic filling materials upon primary teeth. We attempted to evaluate the genotoxicity of these dental materials, their ability to stimulate an inflammatory reaction in test cells, and the possible mechanisms of their action in a U2OS.

Materials and methods

Materials and sample preparation

Following our previous investigation,[5] the experimental group comprised six different endodontic filling material formulations, as listed in Table 1. These different endodontic filling material formulations were: (1) 6 g ZnO + 1 mL eugenol + 1 mL FC; (2) 6 g $Ca(OH)_2$ + 1 mL FC; (3) 6 g $Ca(OH)_2$ + 0.6 g iodoform + 2 mL deionized water; (4) 6 g $Ca(OH)_2$ + 0.6 g iodoform + 2 mL CPC; (5) 6 g $Ca(OH)_2$ + 2 mL CPC; and (6) Vitapex (Neo Dental Chemical, Tokyo, Japan), a non-mixing type of material which was tested as

Table 1. Experimental groups and the composition of the experimental root filling materials

	Material	Composition	Dilute solution	Immersion time (hr)
Control group	Medium	McCoy's medium	NA	NA
Group 2	Vitapex [$Ca(OH)_2$ + iodoform + silicone oil]	0.5 g	1 mL McCoy's medium	24
Group 3	ZnOE + FC	6 g/1 mL/1 mL	1 mL McCoy's medium	24
Group 4	$Ca(OH)_2$ + FC	6 g/1 mL	1 mL McCoy's medium	24
Group 5	$Ca(OH)_2$ + iodoform	6 g/0.6 g	1 mL McCoy's medium	24
Group 6	$Ca(OH)_2$ + iodoform + CPC	6 g/0.6 g/2 mL	1 mL McCoy's medium	24
Group 7	$Ca(OH)_2$ + CPC	6 g/2 mL	1 mL McCoy's medium	24

$Ca(OH)_2$ = calcium hydroxide; ZnOE = zinc oxide-eugenol; FC = formocresol; CPC = camphorated parachlorophenol.

supplied by the manufacturer. Freshly mixed materials were placed in glass rings (2 mm in height, 6 mm in diameter) and allowed to set for 24 hours at 37°C in a humidified chamber. Five samples of each endodontic filling material were then eluted in 10 mL of cell culture medium for 24 hours at 37°C, in a 5% carbon dioxide-in-air atmosphere. Test materials were diluted by adding an appropriate volume of culture medium to achieve a final concentration of 5 μL/mL. Culture medium with no additional experimental material served as the control group.

DNA fragmentation assay

The DNA fragmentation assay was based on the method of Fady et al.[21] as described previously. Following treatment with selected endodontic filling materials, U2OS cells were lysed in 10mM Tris-HCl (pH 7.5), 100mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), and 100 μg/mL of proteinase K for a period of 18 hours at 37°C. DNA was then extracted twice with phenol/chloroform/isoamyl alcohol, precipitated in ethanol, centrifuged (30 minutes at 10,000*g*), and re-suspended in Tris-EDTA buffer containing ribonuclease at a concentration of 100 μg/mL for a period of 1 hour at 37°C. Following a second extraction in phenol/chloroform/isoamyl alcohol, and precipitation in 70% ethanol, the DNA was suspended in Tris-EDTA buffer, and 5–10 μg of DNA per lane was electrophoresed in a 1% agarose gel for 2 hours at a potential difference of 45 V. Gels were subsequently visualized following treatment with ethidium bromide.

Inflammatory protein and COX-2 protein evaluation

Using Western blot analysis, cell lysates derived from U2OS cell cultures were collected. In this assay, lipopolysaccharide was added to U2OS cells as a positive control. McCoy's medium was used as a negative control. The protein assay was performed as described in our previous study.[22] Briefly, U2OS cells were solubilized in SDS-solubilization buffer (1mM $MgCl_2$, 50mM Tris-HCl, 5mM EDTA, pH 7.5, 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride [PMSF], and 1mM *N*-ethylmaleimide) for 30 minutes on ice. Following this, cell lysates were centrifuged at 12,000*g* and 4°C, and the protein concentrations were determined using Bradford reagent; bovine serum albumin (BSA) was used as the standard. Equivalent amounts of total protein per sample of cell extracts were run on a 10% SDS polyacrylamide gel electrophoresis (PAGE) assay and immediately transferred to nitrocellulose membranes. The membranes were then blocked with phosphate-buffered saline (PBS) containing 3% BSA for 2 hours, rinsed, and incubated with the primary anti-COX-2 antibody diluted 1:1000 in PBS containing 0.05% Tween 20 for a period of 2 hours. Following three washes with Tween 20 for 10 minutes each, the membranes were incubated for 1 hour with a biotinylated secondary antibody (polyclonal anti-rabbit immunoglobulin G [IgG] for COX-2) diluted 1:2000 in the same buffer used for washing. Then membranes were washed again as described above and treated with a 1:2000 streptavidin-peroxidase solution for 30 minutes. A β-actin antibody was used as the control for Western blotting. Following a series of three further washing steps, the extent of the immunologic reactions which had taken place was determined by diaminobenzidine. The relative intensities of the obtained bands were determined with a densitometer (AlphaImager 2000, Alpha Innotech, San Leandro, CA, USA).

Analysis of MAP kinases: ERK, p53, and caspase-3

After U2OS cells had been exposed to endodontic filling materials, culture plates were washed once with cold PBS. Five million U2OS cells were collected and lysed in 50 μL of lysis buffer (1% Triton X-100, 0.5% NP40, 10mM EGTA, 0.2mM Na_3VO_4, 0.2mM NaF, and 0.2mM PMSF) for a period of 30 minutes. Cell lysates were cleared at 15,000*g* for a period of 15 minutes at 4°C. Twenty-five micrograms of protein from each sample was collected and boiled for 5 minutes in 1×SDS gel-loading buffer (125mM Tris, pH 6.8, 5% glycerol, 28mM SDS, 1% β-mercaptoethanol, and 0.006% bromophenol blue). Proteins were separated by 12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 1 hour at room temperature in 3% BSA, 5% nonfat dried milk, 10mM Tris (pH 7.5), 100mM NaCl, and 0.1% Tween 20. Following four washes in TBS-T buffer (10mM Tris, pH 7.5, 70mM NaCl, and 0.1% Tween), the membranes were incubated with 0.5 μg/mL rabbit antibody overnight. Following four further washes with TBS-T buffer, the membrane was overlain with a second antibody for 1 hour, and then washed with TBS-T buffer for 20 minutes. The resultant films were scanned and quantified using a densitometer and the SCION image program (Scion, Frederick, MD, USA).

Results

Cell morphology

After treating U2OS cells with different endodontic filling materials for different time periods, there were changes in U2OS cell morphology, as presented in Fig. 1. U2OS cells in the ZnOE+FC group

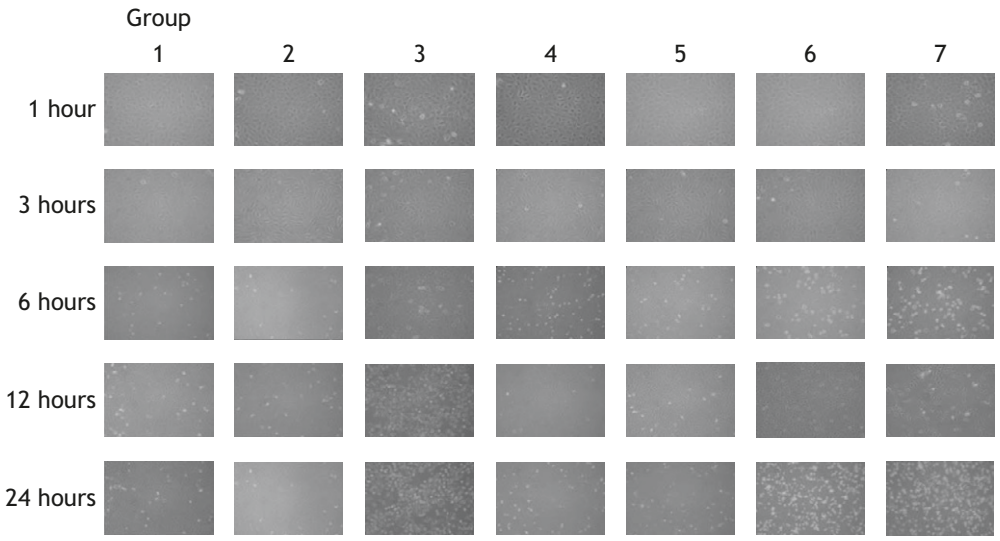

Fig. 1 U2OS cell morphologic changes following treatment with different formulations of endodontic filling materials at different time intervals (1, 3, 6, 12 and 24 hours of exposure to the test dental materials). Lane 1 represents the control group, lane 2 the Vitapex group, lane 3 the zinc oxide-eugenol (ZnOE)+formocresol (FC) group, lane 4 the $Ca(OH)_2$+FC group, lane 5 the $Ca(OH)_2$+iodoform group, lane 6 the $Ca(OH)_2$+iodoform+camphorated parachlorophenol (CPC) group, and lane 7 the $Ca(OH)_2$+CPC group.

appeared to have become condensed, revealing cell separation and a decline in cell number after treatment for 12 hours. Cell death followed treatment of cells with $Ca(OH)_2$ +iodoform+CPC for 24 hours. Virtually all cells in the $Ca(OH)_2$ +CPC group became somewhat star-shaped and quite condensed following 12 hours of treatment; and following 24 hours of treatment, significant cell separation was apparent. For the remaining experimental groups (groups in lanes 1, 2, 4 and 5 of Fig. 1), cell morphology post-treatment appeared to be normal with no significant changes in cell growth.

DNA fragmentation assay

No DNA-ladder bands appeared following the gel assay in any experimental group or the control group (Fig. 2). The DNA-breakage assay for U2OS cells revealed no ladders after treatment with the various dental materials, suggesting that after exposure to those dental materials, there was no evidence of any DNA fragmentation.

COX-2 protein expression

The strength of U2OS cell COX-2 protein expression (in order from high to low expression) for the six experimental groups in Fig. 3 was: lane 6>lane 4>lane 5>lane 7>lane 2>lane 3. The level of COX-2 band expression in U2OS cells from the ZnOE+FC group was virtually zero, but COX-2 expression in the $Ca(OH)_2$+FC and $Ca(OH)_2$+iodoform+CPC groups was quite high (Fig. 3).

MAP kinase expression

ERK (ERK-1, and ERK-2) kinase expression in U2OS cells from the ZnOE+FC and $Ca(OH)_2$+CPC groups was substantially lower than that in the control group. p53 and caspase-3 protein bands appeared in the Western blot assay of all experimental groups (Fig. 4).

Discussion

Endodontic filling materials and genotoxicity

The results of the DNA-fragmentation study showed that none of the tested formulations of endodontic filling materials were genotoxic to cultured U2OS cells (Fig. 2). Similar results were reported in a study by Ribeiro et al.[23] Their findings suggested that FC, paramonochlorophenol and $Ca(OH)_2$ do not promote DNA damage in L5178Y mouse lymphoma cells.

Although the eugenol and FC components of the tested endodontic filling materials were previously shown to be toxic agents in cultured mammalian cells,[3,4] these materials do not appear to be genotoxic to L5178Y mouse lymphoma cells or U2OS cells.[23] The lack of genotoxicity of FC on cell lines might be related to the material containing cresol, because cresol is a chemical inhibitor of reactive oxygen species synthesis.[24]

Eugenol was found to be genotoxic to a cultured human hepatoma cell line (Hep G2) in an *in vitro*

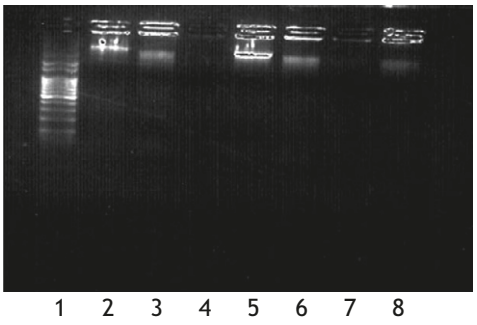

Fig. 2 DNA fragmentation assay results for U2OS cells treated with different formulations of pulpectomy materials. Lane 1 represents the marker, lane 2 the control group, lane 3 the Vitapex group, lane 4 the zinc oxide-eugenol (ZnOE)+formocresol (FC) group, lane 5 the $Ca(OH)_2$+FC group, lane 6 the $Ca(OH)_2$+iodoform group, lane 7 the $Ca(OH)_2$+iodoform+camphorated parachlorophenol (CPC) group, and lane 8 the $Ca(OH)_2$+CPC group.

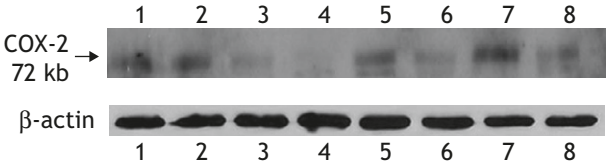

Fig. 3 Inflammation protein cyclooxygenase (COX)-2 expression subsequent to U2OS cells being treated with different formulations of pulpectomy materials, as revealed by Western blot analysis. Lane 1 represents the control group, lane 2 the lipopolysaccharide group, lane 3 the Vitapex group, lane 4 the zinc oxide-eugenol (ZnOE)+formocresol (FC) group, lane 5 the $Ca(OH)_2$+FC group, lane 6 the $Ca(OH)_2$+iodoform group, lane 7 the $Ca(OH)_2$+iodoform+camphorated parachlorophenol (CPC) group, and lane 8 the $Ca(OH)_2$+CPC group.

study.[25] A similar finding was reported by Maralhas et al.[26] who studied the effects of eugenol upon V79 cells using a chromosomal-aberration analysis. In contrast to those results, our results demonstrated no DNA fragmentation in U2OS cells.

$Ca(OH)_2$ did not exhibit genotoxicity toward astrocytes, as reported in our previous comet assay.[27] Similarly, $Ca(OH)_2$ did not induce DNA damage in mammalian cells.[23] Analogously, our results suggest that $Ca(OH)_2$-containing endodontic filling materials were not genotoxic in U2OS cells.

A study by Chang et al.[28] showed that CPC was toxic to cultured human pulp fibroblasts, but was incapable of inducing genotoxicity towards such cells. Here, test groups 6 and 7 contained CPC but did not appear to elicit any genotoxicity toward cultured U2OS cells.

Iodine, the main component of iodoform, induced morphologic transformation[29] and sister chromatid exchanges but not unscheduled DNA synthesis (UDS)

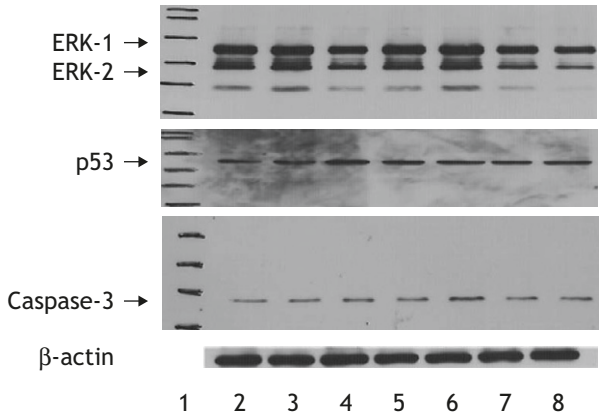

Fig. 4 MAP kinase expression of U2OS cells treated with different formulations of pulpectomy materials for 24 hours. Lane 1 represents the marker, lane 2 the control group, lane 3 the Vitapex group, lane 4 the zinc oxide-eugenol (ZnOE)+formocresol (FC) group, lane 5 the $Ca(OH)_2$+FC group, lane 6 the $Ca(OH)_2$+iodoform group, lane 7 the $Ca(OH)_2$+iodoform+camphorated parachlorophenol (CPC) group, and lane 8 the $Ca(OH)_2$+CPC group.

in Syrian hamster embryo cells.[30] Iodoform-containing dental materials (experimental groups 2, 5 and 6) did not appear to elicit any DNA fragmentation in cultured U2OS cells.

Endodontic filling materials induce U2OS-cell COX-2 protein expression

No COX-2 protein expression following exposure of U2OS cells to ZnO+eugenol+FC or to Vitapex was apparent (Fig. 3). This suggests that these types of dental material do not elicit U2OS cell inflammation. This is consistent with the results of a rat-tooth study by Cotes et al.[12] They suggested from the histology of the pulpal healing process after a pulpotomy in rat teeth that FC combined with ZnOE showed the smallest pulpal inflammatory response. Furthermore, purified eugenol elicited less necrosis and inflammation than commercial eugenol, as was reported in an animal study.[6] In this study, test group 3 produced little inflammation, which might be due to the purified eugenol content.

Apart from experimental group 3, all other test groups contained a $Ca(OH)_2$ component. Levels of COX-2 protein expressed by U2OS cells varied between the different study groups. Similar results were reported in a study by Nelson Filho et al.,[31] who demonstrated that when mast cells were exposed to all $Ca(OH)_2$-containing formulations, there was some degree of inflammatory response. Pitts et al.[32] found that after placing material in the apex of a tooth, giant cells but no inflammatory cells were found adjacent to any remaining $Ca(OH)_2$ particles. Segura et al.[33] found that $Ca(OH)_2$ inhibited macrophage function and reduced the inflammatory

reaction in periapical tissue and in dental pulp when it was used in root canal therapy or in direct pulp capping and pulpotomy, respectively.

Iodoform paste is bactericidal to microorganisms in the root canal and loses only 20% of its potency over a 10-year period. Kawakami et al.[34] found that Vitapex showed no necrotizing effect in rat subcutaneous tissue implantation. Similar results were noted in the present study; COX-2 protein expression did not occur in U2OS cells from the Vitapex group. Hence it would appear that Vitapex does not induce U2OS cell inflammation.

CPC is a tissue irritant.[35] Llamas et al.[36] reported that two different phenolic compounds inhibited adherence of rat peritoneal macrophages to the surface of proximate plastic tubes, suggesting that CPC may alter certain macrophage functions. We revealed that materials containing CPC elicited enhanced COX-2 protein expression by U2OS cells.

COX-2 expression related to MAP kinase expressions

No previous investigations relating to the effects on signaling pathways of cells stimulated with certain endodontic filling materials appear to have been published. Thus, it would be both appropriate and interesting to investigate the process of MAP kinase expression of cells exposed to various primary tooth endodontic filling materials.

When certain biomaterials come into contact with cells, the materials activate kinase signaling pathways of the cell. Here, levels of expression of ERK, p53 and caspase-3 protein kinase were evaluated following treatment with various endodontic filling materials. Significant ERK expression by U2OS cells was noted for all test groups. Thus, we can conclude that the materials used stimulated U2OS cell proliferation and differentiation.

Both p53 and caspase-3 proteins were expressed by U2OS cells in all test groups. Although we previously demonstrated that these test materials elicited various levels of cytotoxicity,[5] we again note that the specific cell death mechanism responsible for such cytotoxicity is still unknown. The specific cell death mechanism may be related to activation of p53 and/or caspase-3 protein kinase in U2OS cells. After exposure to certain endodontic filling materials, U2OS cells may undergo apoptosis and proceed through certain mechanisms which ultimately result in cell death. Similar results were reported by Kitamura et al.[37] who concluded that all $Ca(OH)_2$-containing dental capping agents were able to actively induce apoptosis during pulp wound healing.

Here, we observed no test material-elicited genotoxicity in any of the experimental groups. Furthermore, we noted that p53 induced COX-2 protein expression in all experimental groups except test group 2. It was previously proposed that COX-2 is the ultimate downstream target of p53, and that COX-2 activation is mediated by p53's induction of heparin-binding epidermal growth factor-like growth factor, which subsequently activates the Ras/Raf/MAPK pathway.[38] In 2005, Choi et al.[39] reported that p53 induces COX-2 expression, and COX-2 inhibits p53- and genotoxic stress-induced apoptosis. Furthermore, it was also reported that COX-2 inhibits DNA damage-induced apoptosis through direct regulation of p53 function.

Conclusion

Here, we demonstrate that the tested endodontic filling materials were not genotoxic toward U2OS cells, but they caused COX-2 inflammatory protein expression in all tested groups except for the ZnOE+FC group. Furthermore, the study also showed that the cytotoxic mechanism following U2OS cell exposure to the tested endodontic filling materials was induction of cell apoptosis.

References

1. Tobias RS, Browne RM, Wilson CA. Antibacterial activity of dental restorative materials. *Int Endod J* 1985;18:161–71.
2. Stevens RH, Grossman LI. Evaluation of the antimicrobial potential of calcium hydroxide as an intracanal medicament. *J Endod* 1983;9:372–4.
3. Prashar A, Locke IC, Evans CS. Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. *Cell Prolif* 2006;39:241–8.
4. Ozaki T. Cytotoxicity of formocresol on cultured mammalian cells. *Shigaku* 1988;75:1027–38. [In Japanese]
5. Huang TH, Ding SJ, Kao CT. Biocompatibility of various formula root filling materials for primary teeth. *J Biomed Mater Res B Appl Biomater* 2007;80:486–90.
6. Webb JG Jr, Bussell NE. A comparison of the inflammatory response produced by commercial eugenol and purified eugenol. *J Dent Res* 1981;60:1724–8.
7. Rao M, Kumar MM, Rao MA. In vitro and in vivo effects of phenolic antioxidants against cisplatin-induced nephrotoxicity. *J Biochem* 1999;125:383–90.
8. Rauscher FM, Sanders RA, Watkins JB 3rd. Effects of isoeugenol on oxidative stress pathways in normal and streptozotocin-induced diabetic rats. *J Biochem Mol Toxicol* 2001;15:159–64.
9. Reddy AC, Lokesh BR. Studies on anti-inflammatory activity of spice principles and dietary n-3 polyunsaturated fatty acids on carrageenan-induced inflammation in rats. *Ann Nutr Metab* 1994;38:349–58.
10. Kim SS, Oh OJ, Min HY, et al. Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells. *Life Sci* 2003;73:337–48.
11. Fuks AB, Eidelman E, Cleaton-Jones P, Michaeli Y. Pulp response to ferric sulfate, diluted formocresol, and IRM in pulpotomized primary baboon teeth. *ASDC J Dent Child* 1997;64:254–9.

12. Cotes O, Boj JR, Canalda C, Carreras M. Pulpal tissue reaction to formocresol vs. ferric sulfate in pulpotomized rat teeth. *J Clin Pediatr Dent* 1997;21:247–53.
13. Berger JE. A review of the erroneously labeled "mummification" techniques of pulp therapy. *Oral Surg Oral Med Oral Pathol* 1972;34:131–44.
14. Røolling I, Lambjerg-Hansen H. Pulp condition of successfully formocresol-treated primary molars. *Scand J Dent Res* 1978;86:267–72.
15. Myers DR, Shoaf HK, Dirksen TR, Pashley DH, Whitford GM, Reynolds KE. Distribution of 14C-formaldehyde after pulpotomy with formocresol. *J Am Dent Assoc* 1978;96:805–13.
16. Judd PL, Kenny DJ. Formocresol concerns: a review. *J Can Dent Assoc* 1987;53:401–4.
17. Kojima M, Morisaki T, Izuhara K, et al. Lipopolysaccharide increases cyclooxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kB activation. *Oncogene* 2000;19:1225–31.
18. D'Acquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M, Carnuccio R. Involvement of NF-kB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett* 1997;418:175–8.
19. Janssen-Heininger YM, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor κB. *Free Radic Biol Med* 2001; 28:1317–27.
20. Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S. Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology* 1997;198:35–49.
21. Fady CM, Gardner A, Jacoby F, et al. Atypical apoptotic death induced in L929 targets by exposure to tumor necrosis factor. *J Interferon Cytokine Res* 1995;15:71–80.
22. Huang TH, Ding SJ, Hsu TC, Kao CT. Effects of mineral trioxide aggregate (MTA) extracts on mitogen-activated protein kinase activity in human osteosarcoma cell line (U2OS). *Biomaterials* 2003;24:3909–13.
23. Ribeiro DA, Marques MEA, Salvadori DMF. Lack of genotoxicity of formocresol, paramonochlorophenol and calcium hydroxide on mammalian cells by comet assay. *J Endod* 2004;30:593–6.
24. Yeung SY, Lan WH, Huang CS, et al. Scavenging property of three cresol isomers against H_2O_2, hypochlorite, superoxide and hydroxyl radicals. *Food Chem Toxicol* 2002;40:1403–13.
25. Rompelberg CJ, Evertz SJ, Bruijntjes-Rozier GC, van den Heuvel PD, Verhagen H. Effect of eugenol on the genotoxicity of established mutagens in the liver. *Food Chem Toxicol* 1996;34:33–42.
26. Maralhas A, Monteiro A, Martins C, et al. Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. *Mutagenesis* 2006;21:199–204.
27. Huang TH, Lee H, Kao CT. Evaluation of the genotoxicity of zinc oxide eugenol-based, calcium hydroxide-based, and epoxy resin-based root canal sealers by comet assay. *J Endod* 2001;27:744–8.
28. Chang YC, Huang FM, Cheng MH, Chou LS, Chou MY. In vitro evaluation of the cytotoxicity and genotoxicity of root canal medicines on human pulp fibroblasts. *J Endod* 1998;24: 604–6.
29. Yamaguchi F, Tsutsui T. Cell-transforming activity of fourteen chemical agents used in dental practice in Syrian hamster embryo cells. *J Pharmacol Sci* 2003;93:497–500.
30. Yamamoto T, Tsutsui T. Assessment of the genotoxicity of chemical substances used in dental practice: studies on the ability of chlorhexidine, glutaraldehyde, hydrogen peroxide, iodine, sodium arsenite, erythrosine B, fuchsin acid and fuchsin basic to induce unscheduled DNA synthesis in cultured Syrian hamster embryo cells. *Odontology* 2000;87: 587–96. [In Japanese, English abstract]
31. Nelson Filho P, Silva LA, Leonardo MR, Utrilla LS, Figueiredo F. Connective tissue responses to calcium hydroxide-based root canal medicaments. *Int Endod J* 1999;32:303–11.
32. Pitts DL, Jones JE, Oswald RJ. A histological comparison of calcium hydroxide plugs and dentin plugs used for the control of Gutta-percha root canal filling material. *J Endod* 1984;10:283–93.
33. Segura JJ, Llamas R, Rubio-Manzanares AJ, Jimenez-Planas A, Guerrero JM, Calvo JR. Calcium hydroxide inhibits substrate adherence capacity of macrophages. *J Endod* 1997;23: 444–7.
34. Kawakami T, Nakamura C, Hasegawa H, Eda S. Fate of ^{45}Ca-labeled calcium hydroxide in a root canal filling paste embedded in rat subcutaneous tissues. *J Endod* 1987;13: 220–3.
35. Spangberg LSW. Intracanal medication. In: Ingle JI, Backland LK, eds. *Endodontics*, 4th ed. Baltimore: Williams & Wilkins, 1994:627–40.
36. Llamas R, Segura JJ, Jiménez-Rubio A, Jiménez-Planas A. In vitro effect of parachlorophenol and camphorated parachlorophenol on macrophages. *J Endod* 1997;23: 728–30.
37. Kitamura C, Ogawa Y, Morotomi T, Terashita M. Differential induction of apoptosis by capping agents during pulp wound healing. *J Endod* 2003;29:41–3.
38. Han JA, Kim JI, Ongusaha PP, et al. p53-mediated induction of Cox-2 counteracts p53- or genotoxic stress-induced apoptosis. *EMBO J* 2002;21:5635–44.
39. Choi EM, Heo JI, Oh JY, et al. COX-2 regulates p53 activity and inhibits DNA damage-induced apoptosis. *Biochem Biophys Res Commun* 2005;328:1107–12.