No Evidence for DNA Double-strand Breaks Caused by Endodontic Sealers

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Abstract

Introduction: On extrusion, endodontic sealers might come into close contact with the periapical tissues for long periods. The objective of this study was to test possible mutagenicity of resin-based endodontic sealers by evaluating their potential to induce DNA double-strand breaks (DSBs). **Methods:** Human gingival fibroblasts were exposed to subtoxic concentrations of eluates from 1 epoxy resin–based endodontic sealer (AH Plus Jet) and 2 methacrylate-based endodontic sealers (EndoRez and Real Seal). As control, Calcicur, a $Ca(OH)_2$-based sealer, was used. The γ-H2AX immunofluorescence assay was used to microscopically detect DNA DSBs, and a custom algorithm was developed to quantify them. **Results:** The cytotoxicity of the 24-hour eluates could be ranked in the following order: AH Plus Jet > Real Seal > EndoRez >> Calcicur. The γ-H2AX assay revealed that 1.3%–4.3% of the cell nucleus was occupied by foci when the cells were exposed to the eluates of the endodontic sealers. This was not significantly different from the negative control group in which the cells had been exposed to medium (2.1%). **Conclusions:** No indications for increased risk of genotoxicity of resin-based root canal sealers caused by the induction of DNA DSBs were found in this study. *(J Endod 2012;38:636–641)*

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0099-2399/$ - see front matter

doi:10.1016/j.joen.2011.12.037

Key Words

DNA double-strand breaks, endodontic sealers, epoxy resin, gamma H2AX assay, methacrylate-based, resin-based, root canal sealer

Endodontic sealers have been developed for intracanal use but might unintentionally be extruded over the foramen apicale and come into contact with the periapical tissues. The literature provides ample evidence that this might induce a mild or serious transient inflammation reaction (1, 2). Clinically, this might be evident as percussion and/or pressure pain of the treated tooth. Even if the extruded sealer will degrade over time, the periapical tissues might remain in contact with the root canal sealer and its degradation products for a long period (3). Even in cases without extrusion, eluates might irritate the periapical tissues. This makes good biocompatibility of root canal sealers primordial.

The genotoxic potential of both epoxy resin–based and methacrylate-based root canal sealers has raised some concern (4). Both kinds of sealer are frequently used clinically because of their improved solubility and good treatment outcome (5). Epoxy resin sealers might release small amounts of bisphenol A diglycidyl ether and formaldehyde, which are considered carcinogens. In addition, previous research by using the Ames (6) and micronucleus tests (7) already pointed toward weak mutagenicity for epoxy resin–based sealers. However, there is no consensus in literature, because other researchers could not find DNA damage (8, 9). Regarding methacrylate-based sealers, recent research revealed that methacrylate monomers in relatively high concentrations might induce DNA double-strand breaks (DSBs) (10).

The objective of this study was to evaluate the genotoxic potential of resin-based (epoxy or methacrylate) root canal sealers to induce DNA DSBs by using the γ-H2AX assay, which detects the phosphorylated (γ) H2AX histone (11). This modified type of the H2AX histone has been shown to form in cases of chromosomal integrity loss caused by DNA DSBs (12). The null hypothesis to test was that endodontic sealers do not induce DNA DSBs.

Materials and Methods

Cell Culture

Human gingival fibroblasts (HGFs) were obtained from Provitro (cat-no. 1210412; Berlin, Germany). The HGFs (passage 8–10) were cultured in 175-cm^2 cell-culture flasks at a constant atmosphere of 5% CO_2 and 100% humidity and a temperature of 37°C. Quantum 333 medium for fibroblasts with L-glutamine (PAA Laboratories, Cölbe, Germany) supplemented with 1% antibiotic/antimycotic solution (10,000 U/mL penicillin, 25 mg/mL streptomycin sulfate, 25 μg/mL amphoterecin B; PAA Laboratories) was used. Experiments were performed with confluent cell monolayers with a density of 80%–90%.

Eluates of Endodontic Sealers

Two methacrylate-based endodontic sealers (EndoRez, Ultradent, South Jordan, UT; Real Seal, SybronEndo, Orange, CA) and 1 epoxy resin–based endodontic sealer (AH Plus Jet, Dentsply DeTrey, Konstanz, Germany) were included (Table 1).

Calcium hydroxide sealer (Calcicur, Voco, Cuxhaven, Germany) was chosen as control.

The 24-hour eluates of each endodontic sealer were obtained by applying each material in a 24-well plate strictly according to the instructions of the manufacturer and subsequently by filling the wells with 300 μL of medium. Each well with sealer represented an exposed surface area of 2 cm^2, which corresponded to 0.2–0.25 g of sealer on average (Table 1). To simulate the clinical situation of endodontic sealer extruded over the apex, the sealers were not light-cured (in the case of EndoREZ and Real Seal). After 24 hours at 37°C in the incubator (dark environment), the medium was carefully removed, filter sterilized (Acrodisc 0.2 μm; Pall Corporation, Port Washington, NY), and diluted with medium by 3, 10, 30, 100, or 300.

XTT Viability Assay

To determine the half-maximum effect concentrations (EC50) and subtoxic concentrations of the eluates, the cytotoxicity of the eluates was tested by means of the XTT test. The HGFs were seeded in 96-well microtiter plates at a concentration of 20,000 cells per well (100 μL per well). After 24 hours of incubation, the medium was removed and replaced by the undiluted and diluted eluates. After 24 hours, the cells were washed, and a mixture of XTT (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling agent (in RPMI without phenol red) and electron-coupling reagent (PMS [N-methyldibenzopyrazine methyl sulfate] in phosphate-buffered saline [PBS]) was added according to the recommendations of the manufacturer (Cell proliferation kit; Roche Diagnostic, Penzberg, Germany) 4 hours before photometric analysis. The XTT test assesses the metabolic activity of the cells by detection of formazan, which is a cleavage product from the tetrazolium salt XTT. Quantification of the formazan production was performed photospectrometrically at a wavelength of 450 nm by using a microtiter plate reader (Victor 3; Perkin Elmer Las, Jügesheim, Germany). As negative and positive controls, the cells were exposed to medium or to 1% Triton X-100 in medium, respectively. The formazan production in percentage was calculated relative to the negative and positive controls. Each eluate was tested in quadruplicate per test, and the XTT test was repeated 4 times. The EC50 values were calculated by plotting the viability results onto a dose-effect sigmoidal curve.

γ-H2AX Immunofluorescence

Mutagenicity of the eluates was assessed by the so-called γ-H2AX focus assay, which has been described before (10). Briefly, HGF cells were seeded on HCL-roughened coverslips in a 24-well microtiter plate (70,000 cells per well). After incubation overnight, the cells were exposed to subtoxic concentrations of the eluates, corresponding to 1/3 EC50 and 1/10 EC50. In the negative control, medium was added, whereas in the positive control, the cells were exposed to 1 mmol/L H_2O_2 in medium for 10 minutes (strong oxidizing effect). After 6 hours of exposure to the eluates (according to the Organization for Economic Co-operation and Development [OECD] guidelines for chemical testing), the cells followed a protocol of washing with PBS, fixation with 4% paraformaldehyde in PBS for 10 minutes at 4°C, permeabilization of the cell membrane by a solution of Triton X-100 (0.1% sodium citrate, 0.1% Triton X-100), and immunofluorescence staining. The latter step encompassed treatment of the cells with blocking buffer (Dako, Hamburg, Germany), overnight exposure to mouse monoclonal anti-γ-H2AX (Millipore, Billerica, MA), washing with PBS, and treatment with a fluorescence-labeled secondary mouse antibody (Fluorolink Cy3 Goat anti mouse; GE Healthcare, Munich, Germany). After staining of the nuclei with SYBR Green I (1:50,000), the coverslips were fixed on a glass slide by a small drop of Prolong Antifade mixed with DAPI (Dako, Carpinteria, CA) and kept in the freezer until microscopic analysis. Dichrome (red and green) fluorescence images (1024 × 1024 pixels) of the HGFs were taken by using a Zeiss Axioplan 2 imaging fluorescence microscope (Carl Zeiss, Jena, Germany) at a magnification of 63×.

To quantify the foci in a standardized way, a custom algorithm was developed. First, the foci were discriminated by Adobe Photoshop CS2 (Adobe, San Jose, CA) by using the difference in intensity of the foci in the red channel and the background of the cells. The cells were selected beforehand by using the green channel. Second, the surface area and that of the cells were measured by means of Image J (National Institutes of Health, Bethesda, MD). The percentage of surface per total cell surface area

TABLE 1. Overview of the Experimental Groups

Endodontic sealer	Manufacturer	Classification	Composition*	Average weight of 2 cm^2 sealer (g)
EndoREZ	Ultradent, South Jordan, UT	Methacrylate-based	UDMA, zinc oxide, barium sulfate, resins, pigments	0.21 ± 0.05
Real Seal	SybronEndo, Orange, CA	Methacrylate-based	UDMA, PEGDMA, Bis-EMA, BisGMA, silane-heated barium borosilicate glasses, barium sulfate, silica, calcium hydroxide, bismuth oxychloride with amines, peroxide, photoinitiator, stabilizers, pigment	0.26 ± 0.06
AH Plus Jet	Dentsply DeTrey, Konstanz, Germany	Epoxy resin–based	Paste A: diepoxide, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: 1-adamantane amine, N,N-dibenzyl-5-oxa-nonandiamine-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosil silica, silicone oil	0.20 ± 0.03
Calcicur	Voco, Cuxhaven, Germany	$Ca(OH)_2$ sealer	45% $Ca(OH)_2$, radio-opaquer, thickeners	0.21 ± 0.06

Bis-EMA, ethoxylated bisphenol A dimethacrylate; BisGMA, bisphenol A diglycidyl dimethacrylate; PEGDMA, polyethylene glycol dimethacrylate; TCD-diamine, (3(4),8(9)-Bis-(aminomethyl)- tricyclo [5.2.1.02,6]decane; UDMA, urethane dimethacrylate.

*Composition according to information provided by the manufacturers.