The effect of AH 26 and AH Plus on MCF-7 breast cancer cell proliferation *in vitro*

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Abstract

Pulgar R, Segura-Egea JJ, Fernández MF, Serna A, Olea N. The effect of AH 26 and AH Plus on MCF-7 breast cancer cell proliferation *in vitro*. *International Endodontic Journal*, **35**, 551–556, 2002.

Aim The purpose of this study was to determine whether AH 26 and AH Plus have *in vitro* oestrogenic effects.

Methodology MCF-7 breast cancer cells were trypsinized and plated in 24-well plates at initial concentrations of 10 000 cells per well in 5% FBS in DME. Cells were allowed to attach for 24 h; then the seeding medium was replaced with 10% CDHuS-supplemented phenol red-free DME. Different concentrations of the test compound were added to sample wells (AH 26 and AH Plus at 1/100 to 1/1 000 000 dilutions), 10 pM estradiol-17β to positive control, and no substance to negative control (hormone-free control cells). The assay was stopped after 144 h.

Results AH 26-powder induced MCF-7 cell proliferation in a dose-dependent manner. The cell yield obtained with AH 26-powder at 1/100 dilution sample was 2.5-fold greater than in control cultures. The sample prepared with mixed AH 26 paste/powder 1 : 1 also induced MCF-7 cell proliferation, but showed less potency than AH 26-powder alone. The cell yield obtained with AH 26 paste/powder at 1/100 dilution sample was 1.9-fold greater than in control cultures. AH Plus did not show *in vitro* oestrogenic effect.

Conclusions AH 26 showed *in vitro* oestrogenic effect, but not AH Plus. The endodontist must consider the possible oestrogenic effect of AH 26, as well as the cytotoxic effects of root filling materials, and avoid the leakage of sealer through the apex during root canal treatment.

Keywords: bisphenol A, endodontic sealer, oestrogenicity, root canal treatment.

Received 25 June 2001; accepted 16 November 2001

Introduction

AH 26 and AH Plus (Dentsply DeTrey, Konstanz, Germany) are two of the most commonly used root canals sealers, both are epoxy resin-based materials. According to the manufacturer, AH Plus will not release formaldehyde, although AH 26 does (Huang *et al.* 2000). The potential leakage of endodontic sealers into periapical tissues during root canal filling requires that these materials are neither cytotoxic, genotoxic, mutagenic nor associated with any other negative biologic effects.

The cytotoxicity, genotoxicity, and mutagenicity of the epoxy resin-based root canal sealers have been studied *in vitro* by means of several tests. Miletic *et al.* (2000) reported the high cytotoxicity of AH Plus and AH 26 after determining the number of viable cells using a light microscopy. Cohen *et al.* (2000) demonstrated the cytotoxic effect of both AH 26 and AH Plus using the agar diffusion test. Similar results were obtained by Huang *et al.* (2000) in cultures of rat hepatocytes. The cytotoxicity of AH Plus when tested using an *in vitro* culture of human gingival fibroblasts was no longer detectable after 4 h, whereas the cytotoxic effect of AH 26 remained at a high level until 5 weeks (Azar *et al.* 2000). Both AH 26 and AH Plus have been showed to be mutagenic toward strains TA of Salmonella immediately after mixing until 1 month after polymerization (Jukic *et al.* 2000). On the contrary, studies using the growth inhibition test with primary human periodontal ligament

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fibroblasts and permanent 3T3 monolayers, the procaryotic umu test, the eucaryotic DNA synthesis inhibition test, and the Ames test, did not find cytotoxicity or mutagenicity with AH Plus (Leyhausen *et al.* 1999).

The monomer 2,2-bis[4-(2-hydroxy-3-methacrylyloxypropoxy)phenyl]-propane (bisGMA), prepared from bisphenol A (BPA) and glycidyl methacrylate (GMA), is the major ingredient of the epoxy-resin based root canal sealers AH 26 and AH Plus (Peutzfeldt 1997). Furthermore, diglycidyl ether of bisphenol A (BADGE) is also an ingredient of AH 26 (Leonardo 1994). The potential impact that the BPA may have on the biocompatibility of endodontic sealers with oral tissues has been of great concern. Pulp studies have shown lack of significant pulpal irritation after the placement of properly sealed resin composite filling containing BPA (Cox *et al.* 1987). Regeneration of a part of the periodontal ligament has been observed in periapical tissue of rat molars after the application of light-cured composite resin as retrofillings (Maeda *et al.* 1999). Furthermore, root-end sealing of mandibular molars with dentine-bonded resin composite containing BPA showed complete apical healing in 92% of cases (Rud *et al.* 2001). On the contrary, it has been demonstrated that the resin composites used as retrograde root filling materials reduce the proliferation of gingival fibroblasts and rat sarcoma cells (Peltola *et al.* 1992). Moreover, it has been shown that BPA, at low concentrations, increased spleen cell proliferation to concanavalin A (Jontell *et al.* 1995) and inhibits macrophage adhesion to plastic surfaces (Segura *et al.* 1999).

Olea *et al.* (1996) showed the oestrogenic effect of BPA and BADGE in the MCF-7 breast cancer proliferation assay, and this was confirmed by Hashimoto & Nakamura (2000). Thus, the use of epoxy resin-based materials has been questioned on the basis that bisGMA-based composites and sealants leak oestrogenic monomers, such as BPA and BADGE, into the environment (Pulgar *et al.* 2000).

However, no study has been performed to analyze the potential oestrogenicity of epoxy resin-based endodontic sealers. In this paper the oestrogenic activity of AH 26 and AH Plus are investigated using the MCF-7 based proliferation bioassay (Villalobos *et al.* 1995).

Materials and methods

Sample preparation

Six samples of AH 26 and AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany; lot nos. 9706001368 and 9907000915, respectively) were prepared as follows: (1) paste A – AH Plus (epoxy resin and others); (2) paste B – AH Plus (amine and others); (3) mixed AH Plus pastes A/B 1 : 1; (4) paste AH 26; (5) powder AH 26; and (6) mixed AH 26 paste/powder 1 : 1. To prepare samples, 50 mg of each one of the six products were dissolved to saturation in 5 mL ethanol. Then, dilutions (1/100; 1/1000; 1/10 000; 1/100 000; and 1/1 000 000) were prepared in ethanol and assayed in the proliferation assay.

Cell line and culture conditions

Cloned MCF-7 human breast cancer cells were grown for routine maintenance in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (FBS, BioWithaker, Brussels, Belgium) in an atmosphere of 5% CO_2/95% air under saturating humidity at 37 °C. The cells were subcultivated at weekly intervals using a mixture of 0.05% trypsin and 0.01 EDTA (Sigma, St. Louis, MO, USA).

Charcoal-dextran treatment of serum to remove sex steroids

Plasma-derived human serum was prepared from outdated plasma by adding calcium chloride to a final concentration of 30 mM to facilitate clot formation. Sex steroids were removed from serum by charcoal-dextran stripping. Briefly, a suspension of 5% charcoal (Norit A, Sigma Chemical Co, St. Louis, MO, USA) with 0.5% dextran T-70 (Pharmacia-LKB, Uppsala, Sweden) was prepared. Aliquots of the charcoal-dextran suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1000 × *g* for 10 min Supernatants were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal–serum mixture was maintained in suspension by rolling at 6 cycles min^{-1} at 37 °C for 1 h. The suspension was centrifuged at 1000 × *g* for 20 min, and the supernatant was then filtered through a 0.20-μm filter (Millipore). Charcoal dextran-treated human serum (CDHuS) was stored at −20 °C until needed.

Cell proliferation experiments

Estradiol-17α (Sigma, St. Louis, MO, USA) was stored as a 1-mM stock solution in ethanol at −20 °C. The final ethanol concentration in the culture medium did not exceed 0.1%; this concentration did not affect cell yields. MCF-7 cells were used in the test of oestrogenicity according to a technique slightly modified (Villalobos *et al.* 1995) from that originally described by Soto *et al.*

(1992). Briefly, cells were trypsinized and plated in 24-well plates (Limbro, McLean, VA, USA) at initial concentrations of 10 000 cells per well in 5% FBS in DME. Cells were allowed to attach for 24 h, then the seeding medium was replaced with 10% CDHuS-supplemented phenol red-free DME.

Different concentrations of the test compound were added to sample wells, 10 pM estradiol-17β to positive control, and no substance to negative control (hormone-free control cells). The assay was stopped after 144 h by removing medium from wells, fixing the cells and staining them with sulforhodamine-B (SRB). The cells were treated with cold 10% trichloracetic acid and incubated at 4 °C for 30 min, washed five times with tap water and left to dry. Trichloroacetic-fixed cells were stained for 10 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 N.M. Linearity of the SRB assay with cell number was verified prior to cell growth experiments. Mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. MCF-7 cell proliferation (fold-over control) was calculated as the ratio between the cell yield obtained with the substance tested (AH 26, AH Plus, or estradiol-17β) and the proliferation of hormone-free control cells (control).

Statistical analysis

All values were expressed as the mean ± SD of four separate experiments performed in triplicate. Mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. Differences between the samples of endodontic sealers and estradiol-17β group were assessed by analysis of variance. A value of $P < 0.05$ was regarded as statistically significant.

Results

The addition of 10 pM estradiol-17β to CDHuS-supplemented medium increased the numbers of MCF-7 cells in culture 4.7 ± 0.2–fold over control (Table 1) ($P < 0.01$). In the absence of estradiol-17β (control) cells proliferated minimally.

The six samples of endodontic sealers prepared as described previously were assayed. Results are shown in Table 1. The three samples prepared with AH Plus (1)

Table 1 Cell proliferation of MCF-7 cells in the presence of different substances.

Substance	Concentration/ dilution factor	Cell proliferation (fold-over control)	*P* (vs. control)
Estradiol-17β	10 pM	4.71 ±0.23	<0.001
Paste A – AH Plus	1/100000	0.99 ± 0.08	>0.05
Paste A – AH Plus	1/10000	1.11 ±0.12	>0.05
Paste A – AH Plus	1/1000	1.13 ± 0.12	>0.05
Paste A – AH Plus	1/100	Toxic effect	
Paste B – AH Plus	1/100000	1.21 ±0.14	>0.05
Paste B – AH Plus	1/10000	0.95 ± 0.08	>0.05
Paste B – AH Plus	1/1000	Toxic effect	
Mixed AH Plus	1/100000	1.03 ± 0.08	>0.05
Mixed AH Plus	1/10000	1.06 ± 0.07	>0.05
Mixed AH Plus	1/1000	Toxic effect	
Paste AH 26	1/100000	0.95 ± 0.09	>0.05
Paste AH 26	1/10000	1.11 ±0.08	>0.05
Paste AH 26	1/1000	Toxic effect	
Powder AH 26	1/100000	1.02 ± 0.07	>0.05
Powder AH 26	1/10000	1.23 ± 0.14	>0.05
Powder AH 26	1/1000	2.15 ± 0.19	<0.05
Powder AH 26	1/100	2.53 ± 0.21	<0.05
Mixed AH 26	1/100000	0.95 ± 0.14	>0.05
Mixed AH 26	1/10000	1.53 ± 0.16	<0.05
Mixed AH 26	1/1000	1.24 ± 0.19	>0.05
Mixed AH 26	1/100	1.92 ± 0.17	<0.05

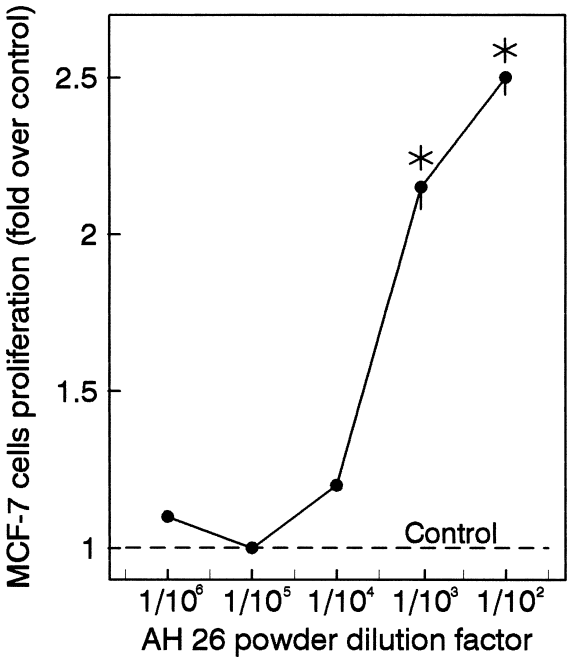

Figure 1 Oestrogenic effect of AH 26-powder. Values significantly different from control, $^{*}P < 0.05$.

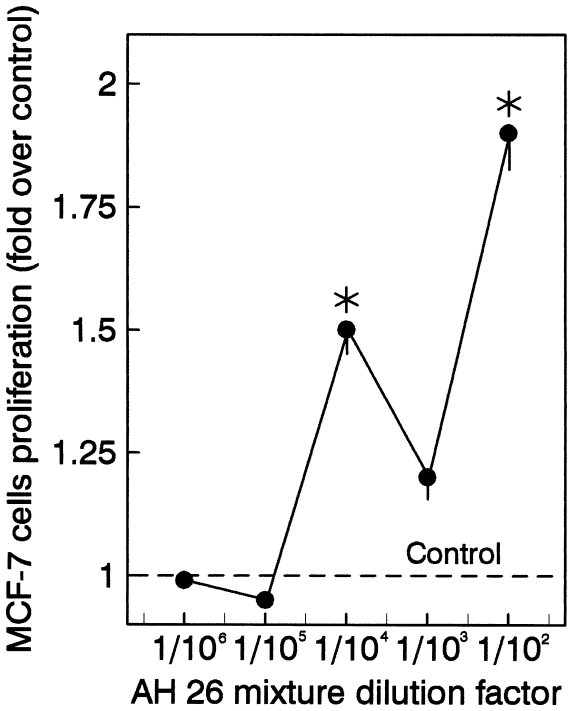

Figure 2 Oestrogenic effect of AH 26-mixture. Values significantly different from control, $^{*}P < 0.05$.

paste A (epoxy resin and others); (2) paste B (amine and others); and (3) mixed AH Plus pastes A/B 1 : 1, did not induce MCF-7 cell proliferation ($P > 0.05$). Cell toxicity was observed at 1/100 dilution of paste A – AH Plus, and at 1/1000 dilution of paste B-AH Plus and mixed AH Plus.

On the contrary, the sample prepared with powder of AH 26 induced MCF-7 cell proliferation in a dose-dependent manner (Fig. 1). The cell yield obtained with AH 26-powder at 1/100 dilution sample was 2.5-fold greater than in control cultures ($P < 0.05$). The sample prepared with mixed AH 26 paste/powder 1 : 1 also induced MCF-7 cell proliferation ($P < 0.05$) (Fig. 2), but showed less potency than AH 26-powder alone. The cell yield obtained with AH 26 paste/powder at 1/100 dilution sample was 1.9-fold greater than in control cultures ($P < 0.05$). The paste of the endodontic sealer AH 26 alone did not stimulate MCF-7 cell proliferation ($P > 0.05$), and caused cell toxicity at 1/1000 dilution (Table 1).

Discussion

In the present study, the *in vitro* oestrogenic effect of the endodontic sealer AH 26 has been demonstrated. On the contrary, AH Plus did not show an oestrogenic effect.

Adverse influences of endodontic materials on periapical tissues are attributed to either toxic or hypersensitivity reactions. Researchers often fail to ascertain other adverse biological reactions, such as effects upon endocrine or immune functions. This study demonstrated the possible effects of endodontic sealers on endocrine function and this issue must be borne in mind in the future.

It is controversial whether the dental resinous materials containing (bisGMA), which is synthesized from the oestrogenic compound BPA, include unreacted BPA and/or can mimic the effects of natural steroid hormones. However, the present study demonstrated that a bisGMA-based endodontic sealer, such as AH 26, shows an oestrogenic effect *in vitro*.

The leakage into the environment of oestrogenic monomers, such as BPA and BADGE, from bisGMA-based composites and sealants (Pulgar *et al.* 2000) as well as the oestrogenic effect of BPA, a component of AH 26 (Olea *et al.* 1996, Hashimoto & Nakamura 2000, Pulgar *et al.* 2000) has been demonstrated. Thus, although it can be hypothesized that the oestrogenic effect of AH 26 showed in this study is due to its content in BPA, the oestrogenic effect of AH 26 also can be due to other

unknown ingredients of the sealer. Tarumi *et al.* (2000) found that two commercially available sealants showed oestrogenic activity, although none of the tested materials contained BPA.

Cytotoxicity testing of endodontic materials must be viewed as an assessment of hazards, that is the potential of the material to cause periapical problems. In this context, bisGMA-based endodontic sealers are possible hazards to periapical tissues. The risks that these materials will cause periapical toxicity or endocrine effects *in vivo* can be partly estimated by assessing the cytotoxicity or the endocrine activity of the substances which are released from these materials *in vitro* and comparing these cytotoxic concentrations with those concentrations that are present *in vivo*. In the case of AH 26 and BPA the endocrine activity of these substances in current tests, and therefore the risks of periapical or systemic effects, depends on their ability to leak through the apical foramen into periradicular tissues.

Imai & Komabayashi (2000) studied the leaching characteristics of BPA from composite resins. The same authors found that the elution of BPA was rapid during 6-h period, and then declined and continued steadily, suggesting that little or no oestrogenic effect due to long-term elution of BPA from commercial bisGMA-based resins is expected in practice.

Lewis *et al.* (1999), using high-pressure liquid chromatography (HPLC), analyzed 28 different commercially available dental resins for the presence of BPA finding that only one resin, Delton II, had detectable levels of BPA. Likewise, eluates from Delton II were the only samples that elicited a significant proliferative response in two of the MCF-7 sublines tested. These authors concluded that dental resins in general do not represent a significant source of BPA exposure.

However, since there are no realistic estimates of the concentrations levels at which polymerized resin components may occur in the periradicular tissues following root canal treatment, no firm clinical conclusions can be drawn. Pulgar *et al.* (2000) showed the leaching of oestrogenic monomers into the environment by bisGMA-based composites and sealants in concentrations at which biologic effects have been demonstrated in *in vivo* experimental models. Moreover, Kaplan *et al.* (1997) have shown that the endodontic sealer AH 26, containing BADGE, disintegrated and lost 1.22% of mass when stored at 37 °C and a relative humidity above 95% for 45 days. This result suggests that bisGMA based resins used in endodontics such as AH 26 could constitute a chronic source of BPA. Thus, although the results of this study are based on results *in vitro* and do not necessarily confirm any clinical activity, the oestrogenic effect of AH 26 in this work is evident.

AH Plus did not have an oestrogenic effect. Other properties and advantages of AH Plus are that it is eugenol and *para*formaldehyde free, has a rapid setting time, higher radiopacity, improved removability, lower solubility and an acceptable biocompatibility (Whitworth & Boursin 2000, De Almeida *et al.* 2000).

Conclusions

AH 26 showed *in vitro* oestrogenic effect, but not AH Plus. The endodontist must consider the possible oestrogenic effect of AH 26, as well as the cytotoxic effects of root filling materials, in order to avoid the leakage of sealer through the apex during root canal treatment. The choice of AH Plus as endodontic sealer avoids the possibility of oestrogenic activity in the periradicular tissues

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