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Mechanisms of cytotoxicity of eugenol in human osteoblastic cells *in vitro*

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Abstract

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Aim To evaluate the mechanisms of cytotoxicity of eugenol in human osteoblastic cells *in vitro*.

Methodology Cytotoxicity and cell proliferation assays were performed to elucidate the toxic effects of eugenol on the human osteoblastic cell line U2OS. Furthermore, the effects of antioxidants catalase (scavenger of H_2O_2), superoxide dismutase (SOD, an extracellular superoxide free radical scavenger) and *N*-acetyl-*L*-cysteine (NAC, a cell-permeable glutathione precursor) were added to discover the possible mechanisms of eugenol-induced cytotoxicity. Paired Student's *t*-test was applied for the statistical analysis of the results.

Results Eugenol demonstrated a cytotoxic effect to U2OS cells in a dose-dependent manner ($P < 0.05$). The 50% inhibition concentration of eugenol was approximately 0.75 mmol L^{-1}. Eugenol also inhibited cell proliferation during a 4-day culture period ($P < 0.05$). Addition of NAC extracellularly protected the cells from eugenol-induced cytotoxicity ($P < 0.05$). Neither, SOD nor catalase provided any protective effects on eugenol-induced cytotoxicity ($P > 0.05$).

Conclusions The levels of eugenol tested inhibited growth and proliferation of U2OS cells. Eugenol has significant potential for periapical toxicity. These inhibitory effects were associated with glutathione levels.

Keywords: antioxidants, cytotoxicity, eugenol, glutathione, ZOE-based root canal sealers.

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Introduction

Eugenol (4-allyl-2-methoxyphenol) is an extract of clove oil widely used in dentistry as a therapeutic agent, most commonly as a component of zinc oxide-eugenol (ZOE) cement applied as a base or temporary dressing to dentine, or as a root canal sealer. Several studies have been reported on the influence of ZOE-based root canal sealers on tissues from the viewpoint of histopathology (Holland 1994, Tepel *et al.* 1994). Several cytotoxicity tests using cultured cells have also been performed (Tai *et al.* 2001, Huang *et al.* 2002,Tai *et al.* 2002) which revealed that ZOE-based sealers irritated periapical tissue *in vivo* and exhibited cytotoxic effects *ex vivo*. Eugenol that leaches out of ZOE-based root canal sealers, specifically from setting material extruded into the periapical region, may participate in the development of periapical inflammation or the continuation of a preexisting periapical lesion (Lindqvist & Otteskog 1981, Leonardo *et al.* 1999).

Numerous studies have indicated that eugenol is cytotoxic to mouse fibroblast cell line L929 (Hume 1984), rat hepatocytes (Thompson *et al.* 1991), pulp cells (Hume 1984, Kasugai *et al.* 1991, Chang *et al.* 2000) and oral mucosal fibroblasts (Jeng *et al.* 1994) *in vitro*. Eugenol was also found to cause injury to rat oral mucosa membranes *in vivo* (Kozam & Mantell 1978). However, the effects of eugenol on human

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osteoblasts have not been investigated. The potential toxicological implications of eugenol still remain to be elucidated.

Osteoblasts are significantly represented in the periapical tissues. Eugenol leached from ZOE-based root canal sealers will come into contact with, or in close proximity to the periapical tissues. Little is known about whether chemical interactions can affect the cytotoxic effects of eugenol on the human osteoblastic cell line U2OS. Antioxidants are substances that, when existing at low concentrations compared with those of an oxidizable substrate, significantly delay or prevents oxidation of that substrate (Halliwell 1997). To gain further insight into the toxic mechanisms of eugenol and to find agents for further chemoprevention, antioxidants catalase (scavenger of H_2O_2), superoxide dismutase (SOD, an extracellular superoxide free radical scavenger) and *N*-acetyl-*L*-cysteine (NAC, a cell-permeable glutathione precursor) were used for elucidation of the mechanisms of eugenol-induced cytotoxicity in the present study.

Materials and methods

Eugenol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). [Methyl-^{3}H]thymidine was obtained from Amersham International Plc (Amersham, Buckinghamshire, UK). All culture materials were obtained from GIBCO (Grand Island, NY, USA). Eugenol was first dissolved in ethanol and then diluted with the culture medium. The final concentration of solvent in the medium did not exceed 0.25% (v/v). At these concentrations, the solvents used were not toxic to cells. The concentrations of eugenol used in this study were 0.001–2 mmol L^{-1}.

Cell culture

U2OS cells (American Tissue Type Collection HTB 96), derived from human osteogenic sarcoma, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 μg mL^{-1} of streptomycin and 100 mg mL^{-1} of penicillin at 37 °C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2 (Huang & Chang 2005). Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediamine-tetraacetic acid (EDTA) for 5 min and aliquots of separated cells were subcultured. The cells were subcultured at 1 : 4 splits every 3 days.

Cytotoxicity assay

A simple colorimetric assay developed by Mosmann (1983), as a test for cell proliferation and survival, has been adapted for the measurement of cytotoxicity. MTT solution was prepared as 1 mg mL^{-1} in complete medium just before use. Cells were diluted in fresh complete medium and seeded in 96-well plates (2×10^4 cells per well). After overnight attachment, cells were treated with various concentrations of eugenol for 20 h, then 50 μL MTT dye was added to each well. Plates were incubated in a CO_2 incubator for 4 h. Optical density was determined by eluting the dye with dimethyl sulphoxide and the spectrophotometric absorbance measured at 550 nm using a spectrophotometer (Hitachi, Tokyo, Japan).

Cell proliferation assay

[Methyl-^{3}H]thymidine incorporation into cellular DNA was used as a measure of cell proliferation. The cells were plated at an initial density of 5×10^4 cells per well into six-well culture plates. After overnight attachment, [methyl-^{3}H]-thymidine (0.5 μCi mL^{-1}) was added and cells were exposed to various concentrations of eugenol for 4 days. The concentrations of eugenol used for cell proliferation were lower than those for cytotoxicity assay to evaluate whether at non-lethal doses it could affect cellular DNA synthesis. Finally, the radioactive medium was discarded and cells were washed three times with 5% trichloroacetic acid at 4 °C. Cells were solubilized with 1 mL of 0.1 N NaOH for 15 min at room temperature. Aliquots of the cell lysates were counted in a liquid scintillation counter (Packard model 2100TR; Packard, Downers Grove, NJ, USA).

Effects of antioxidants on eugenol-induced cytotoxicity

Various antioxidants (SOD 100 μg mL^{-1}; catalase 50 μg mL^{-1}; NAC 1 mmol L^{-1}) without cytotoxic concentrations were also added to wells to test their protective effects. U2OS cells were exposed to antioxidants for 1 h prior to the addition of the 50% inhibition concentration (IC_{50}) of eugenol and co-incubation for 24 h. Cytotoxicity was judged by MTT colorimetric assay as described previously. The percentage of the dehydrogenase activity at each concentration, compared with that of the control, was calculated from the absorbance values.

Statistical analysis

All assays were repeated three times to ensure reproducibility. The significance of the results obtained from control and treated groups was statistically analysed by paired Student's *t*-test.

Results

Eugenol demonstrated a cytotoxic effect on the human osteoblastic cell line U2OS (Fig. 1). Eugenol reduced the activity of dehydrogenase of cells over a 24-h culture period in a dose-dependent manner ($P < 0.05$). The 50% inhibition concentration of eugenol was approximately 0.75 mmol L^{-1}.

Cell proliferation using [^{3}H]thymidine incorporation into cellular DNA is shown in Fig. 2. Eugenol at the concentration of 0.01 mmol L^{-1} inhibited cell proliferation, as shown by the [^{3}H]thymidine incorporation. The effect of inhibition was dose-dependent during 4-days of culture period ($P < 0.05$). Eugenol was cytotoxic at a concentration level higher than 0.01 mmol L^{-1}. Elevating the eugenol concentration to 1 mmol L^{-1} almost completely inhibited DNA synthesis (Fig. 2).

The three antioxidants were added to investigate whether they could protect cells against cytotoxicity induced by eugenol. The combined effects of 0.75 mmol L^{-1} eugenol and three antioxidants on U2OS cells by MTT assay are shown in Fig. 3. Addition of NAC extracellularly protected the cells from eugenol-induced cytotoxicity ($P < 0.05$). Eugenol caused about 50% of cell death over the 24 h incubation period. Co-incubation with 1 mmol L^{-1} NAC increased the cell viability up to 79% ($P < 0.05$). Neither SOD nor catalase showed any protective effects on eugenol-induced cytotoxicity ($P > 0.05$).

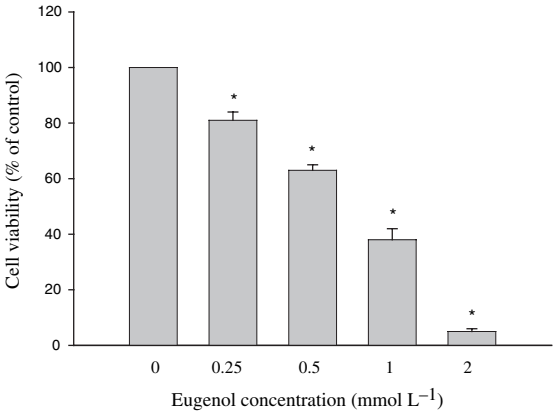

Figure 1 Effect of eugenol on human U2OS cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Percentage of absorbance at each concentration, compared with that of control was calculated. Each point and bar represent a mean ± SD. *Significant differences from control values with $P < 0.05$.

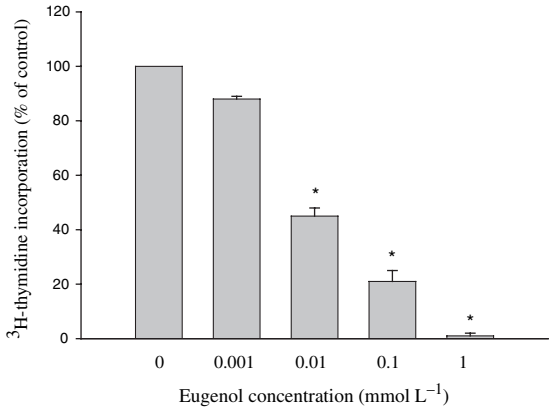

Figure 2 Effect of various concentrations of eugenol on cell proliferation of human U2OS cells. Results are expressed as a percentage of [methyl-^{3}H]thymidine incorporation relative to untreated control. Data are shown as mean ± SD (bars). *Significant differences from control values with $P < 0.05$.

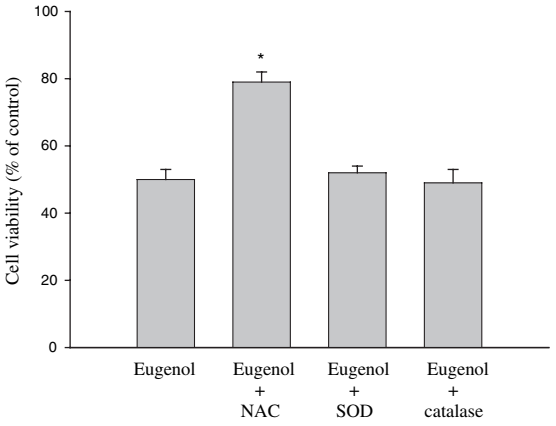

Figure 3 Effects of antioxidants on 0.75 mmol L^{-1} eugenol-induced cytotoxicity to U2OS cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Percentage of cell viability after incubation with eugenol with or without antioxidants, compared with that of control was calculated. Each point and bar represent a mean ± SD. *Significant differences from eugenol alone with $P < 0.05$.

Discussion

Many types of cells have been applied to evaluate the cytotoxicity of eugenol. The effects of eugenol on human osteoblasts have not been well investigated. The human osteoblastic cell line U2OS was used in this study because these cells express the osteoblast phenotype (Nelissen *et al.* 2000). In addition, the selection of a permanent cell line was desirable as they are easily maintained in culture. Furthermore, donor biopsy variability was eliminated and greater reproducibility was possible.

Previous studies have reported that eugenol is a cytotoxic agent to mouse fibroblast cell line L929 (Hume 1984), rat hepatocytes (Thompson *et al.* 1991), pulp cells (Hume 1984, Kasugai *et al.* 1991, Chang *et al.* 2000) and oral mucosal fibroblasts (Jeng *et al.* 1994) *in vitro*. In addition, eugenol may injure rat oral mucous membranes *in vivo* (Kozam & Mantell 1978). In this study, eugenol was shown to inhibit cell growth and proliferation of U2OS cells in a dose-dependent manner. The cytotoxic nature of eugenol was clearly shown.

The present study has focused on the effects of eugenol and the role it could play in periapical tissue breakdown via its direct effects on U2OS cells. In this study, eugenol was found to inhibit cell growth and proliferation of U2OS cells. MacNeil & Somerman (1993) have clearly demonstrated that cell growth, attachment, proliferation and matrix synthesis play an important role in wound healing and tissues regeneration. Therefore, a direct toxic effect on host cells may retard periapical wound healing when eugenol leaches out from the ZOE-based root canal sealers.

The sulphhydryl group containing tripeptide constitutes a first defence intracellular antioxidant (Meister 1994). It is known that glutathione (GSH) plays a role in cellular protection from damage produced by free radicals and electrophiles. When cellular GSH is depleted, cells become extremely prone to oxidative damage. It is well known that NAC, a cell-permeable GSH precursor, has been tested by EUROSCAN as a chemopreventive agent in the prevention of oral leucoplakia with less frequent side-effects (De Vries & De Flora 1978).

In the present study, the cytotoxic effects of eugenol were prevented by the addition of extracellular NAC. These findings indicate that GSH depletion may be one of the mechanisms underlying eugenol-induced cytotoxicity. Such GSH depletion has been described in cultured human oral mucosal fibroblasts after treatment with eugenol (Jeng *et al.* 1994). Previous studies have also demonstrated the formation of GSH conjugates during oxidation of eugenol by microsomal fractions of rat liver and lung (Thompson *et al.* 1990). Taken together, eugenol-induced cytotoxicity is influenced by cellular GSH levels.

In the present study, both catalase (scavenger of H_2O_2) and SOD (an extracellular superoxide free radical scavenger) failed to protect against eugenol-induced cytotoxicity. The reason cannot be determined in the present study. It might be explained by the fact that phenolic compounds have been shown to possess the properties of antioxidant and oxygen free-radical scavengers (Kuehl *et al.* 1977). A free phenolic hydroxyl group is essential for scavenging oxygen free radicals. Thus, the reactive oxygen produced in the cell could be captured by eugenol. In the present study, NAC, but not catalase or SOD, was found to present an effective protection against the cytotoxicity. Taken together, GSH depletion, but not the attack of oxygen free radicals, could be the mechanism for cytotoxicity induced by eugenol.

The cytotoxic effects of eugenol on U2OS cells depend on the exposure dose, frequency and duration. However, it is difficult, if not impossible, to determine how much eugenol acts on periapical tissue after leaching from ZOE-based root canal sealers through the apical foramen. In this study, it was found that the lower concentrations of eugenol could easily reach the effective cytotoxic level on U2OS cells during long-term exposure. It should be emphasized that eugenol has significant potential for periapical toxicity. Care should be taken to reduce the possibility of periapical irritation from inadvertent extrusion of ZOE-based root canal sealers in clinical treatment. It may be possible to add NAC to ZOE-based root canal sealers which would be released into the tissues with eugenol and thus protect periapical tissue against cytotoxicity.

Conclusion

In the present study, eugenol demonstrated a cytotoxic effect on U2OS cells. Eugenol also inhibited cell proliferation during a 4-day culture period. Addition of NAC extracellularly protected the cells from eugenol-induced cytotoxicity. Neither SOD nor catalase showed any protective effects on eugenol-induced cytotoxicity. Taken together, GSH depletion, but not the attack of oxygen free radicals, could be the mechanism for cytotoxicity induced by eugenol. NAC appears as a useful agent in protecting cell damage mediated by eugenol.

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