Eugenol Toxicity in Human Dental Pulp Fibroblasts of Primary Teeth

Maria Escobar-García*/ Karen Rodríguez-Contreras**/ Socorro Ruiz-Rodríguez ***/ Mauricio Pierdant-Pérez ****/ Bernardino Cerda-Cristerna *****/ Amaury Pozos-Guillén ******

Objective: *The aim of the study was to determine the eugenol concentrations at which toxicity occurs in human dental pulp fibroblasts of primary teeth.* ***Study design:*** *Samples of primary dental pulp tissue were taken. Tissue samples were seeded by means of explant technique and used in the 4th-5th pass. Single Cell Gel Electrophoresis (Comet), phenazine MeThoSulfate (MTS), LIVE/DEAD® Cell Viability/Toxicity and trypan blue assays for evaluation of the cytotoxicity of increasing concentrations of eugenol (0.06 to 810 μM) were performed.* ***Results:*** *The results of toxicity tests showed toxic effects on dental pulp fibroblasts, even at very low concentrations of eugenol (0.06 μM). Very low concentrations of eugenol produce high toxicity in human dental pulp fibroblasts.* ***Conclusions:*** *All of the concentrations of eugenol that we evaluated produced high toxicity in human dental pulp fibroblasts of primary teeth.*

Key words: *Toxicity, eugenol, fibroblasts.*

INTRODUCTION

The use of any dental biomaterial is to maintain maximal tissue vitality and to prevent cytotoxic reactions. A variety of methodologies evaluated the biological characteristics of the materials both *in vivo* and *in vitro*, thus establishing potential adverse effects prior to clinical use.[1] In Pediatric Dentistry, among the materials used in dental treatments, eugenol is among the most common; in combination with Zinc Oxide (ZOE), it is used as root canal sealer in cases of pulpectomies,[2,3] as temporary filling material, and as a pulp sedative in cases of pulpotomies.[4] Furthermore, eugenol combined with thymol or carvacrol is able to inhibit the growth of fungal pathogenic organisms; therefore, it is used in treating oral infectious diseases.[5–7]

Eugenol (4-Allyl-1,2-dimethoxyphenol) is a natural phenolic substance and has several uses, including in food flavorings, non-alcoholic beverages, insect repellents, and it possesses antifungal properties.[5,8] It is a pale yellow oily liquid extracted from certain essential oils, especially clove, nutmeg, cinnamon, and bay leaf.[9] Its chemical structure is similar to that of the safroles and estragoles, which are recognized as carcinogens.[10] Biological and biochemical mechanisms by which the compounds of the family of alkenylbenzenes, such as eugenol, cause mutagenesis or carcinogenesis are not well ascertained to date.[11]

There are different mechanisms of action proposed in an attempt to understand the toxicity of eugenol; the following which may include some of these mechanisms: (i) alteration of ionic homeostasis; (ii) specific lesions of the cell plasma membrane[12,13] and (iii) generation of Oxidative stress (OS).[14,15] Studies in human platelets have shown that eugenol has antiplatelet activity, which is due, at least in part, to inhibition of the formation of thromboxane A2-dependent Cyclooxygenase (COX).[16]

Toxicity tests are commonly used *in vitro* to assess the biocompatibility of dental materials. These methods involve observation of cell growth inhibition, membrane permeability testing, enzymatic activity testing, or cell death.[17] DNA integrity is fundamental to the health and proper functioning of body's appearance; however, genetic material is susceptible to damage by numerous agents and/or processes,[18,19] in which the genotoxicity testing have great importance.[20,21]

The aim of the study was to determine the eugenol concentrations at which toxicity occurs in human dental pulp fibroblasts of primary teeth.

From the San Luis Potosi University, San Luis Potosi, SLP. Mexico.
*Maria Escobar-García, PhD, Basic Sciencies Laboratory, Faculty of Dentistry.
** Karen Rodríguez-Contreras, DDS, Pediatric Dentistry Postgraduated Program, Faculty of Dentistry.
*** Socorro Ruiz-Rodríguez, DDS, MS, Pediatric Dentistry Postgraduated Program, Faculty of Dentistry.
**** Mauricio Pierdant-Pérez, MD, MS, Clinical Epidemiology Department, Faculty of Medicine.
***** Bernardino Cerda-Cristerna, DDS, PhD, Faculty of Dentistry, Río Blanco. Universidad Veracruzana, Región Córdoba-Río Blanco.
****** Amaury Pozos-Guillén, DDS, PhD, Basic Sciencies Laboratory, and Pediatric Dentistry Postgraduated Program, Faculty of Dentistry.

Send all correspondence to

Amaury Pozos Guillén; Facultad de Estomatología, Universidad Autónoma de San Luis Potosí. Av. Dr. Manuel Nava #2, Zona Universitaria, CP 78290, San Luis Potosí, SLP, México.
Phone: 52 444 8262357 X 5134
E-mail: apozos@uaslp.mx

MATERIALS AND METHOD

Study design

The study was conducted in two phases. The first was a clinical phase in which pulp tissue samples of 15 primary teeth, donated voluntarily by patients attending the pediatric dental clinic, were taken. The parents or guardians of the participating children previously signed an informed consent agreeing for their children to participate voluntarily in the study, which was approved by the Committee of Ethics in Research of the Faculty of Dentistry. The second phase comprised a one-time laboratory appointment at which pulp tissue samples were obtained. Teeth obtained from patients were previously disinfected with 2% Chlorhexidine gluconate (IndiSpense® Refill, Ultradent Products, Inc., USA). Pulp chamber tissue was removed by opening with a micrometer and diamond disc. The extracted pulp tissue was placed in a 2-mL microtube containing transport medium (Phosphate-buffered solution [PBS]) with 3% of antibiotics (1,000 U/mL penicillin, 1 mg/mL streptomycin, and amphotericin B 2.5 mg/μL), labeled, and stored at 4°C for a period of 6-12 h for subsequent processing in the laboratory.

Cell culture

The samples were washed with sterile PBS and incubated for 4-6 h with 2 mg/mL collagenase 1; after this time, the pulp tissue was dissected into parts of approximately 1-3 mm per explant and plated on 25 mL cell box cultures with 3 mL of culture medium (Dulbecco's Modified Eagle's Medium [DMEM]-D6046; Sigma-Aldrich BioSciences, St. Louis, MO, USA), supplemented with 10% Fetal bovine serum (FBS) and 1% antibiotic, incubated at 37°C in an atmosphere of 95% humidity and 5% CO_2, with a change of medium every third day. Cells were used on the 4th-5th pass once they reached 80% confluence.[22,23] When a confluence of >80% was observed, subcultures were performed. The monolayers of cells adhered to the culture dishes were detached with the aid of Trypsin EDTA 0.25% 1X solution (Gibco, Life Technologies, USA) to assess cell count and viability. This began with an equal number of cells (20,000) for each experiment.

Preparation of eugenol

Different concentrations of eugenol were prepared from a 2.0-mM stock solution in Dimethyl sulfoxide (DMSO; Sigma-Aldrich Bio-Sciences, USA). Then, 13 concentrations (ranging from 0.06–810 mM) were prepared in PBS1X.

Alkaline Comet assay

The standard alkaline version was carried out[20] on fibroblasts with their respective controls. The slides were observed under a confocal laser scanning microscope (CLSM) (model DMI4000B; Leica Microsystems, Wetzlar, Germany). Counting the cells grouped according to damage level was performed at obtain arbitrary units.[18,19] One hundred microliters (μL) of cell suspension (20,000) in contact with different concentrations of eugenol was measured and incubated at 37°C in an atmosphere of 95% humidity and 5% CO_2 for a 1-h period, after which time 10 μL of the cell suspension with eugenol was out and 75 μL of low-melting-point agarose at 0.5% in PBS at 37°C was added. This mixture was homogenized with the aid of automatic pipette-cleaned slides pretreated with agarose 0.5% normal-melting-point agarose. Finally the slide was covered with a final layer of low-melting-point agarose. The slides were maintained at 4°C between agarose layers, this ensuring solidification. The slides were placed in lysis buffer prepared extemporaneously (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100) incubated for 1 h at 4°C. After this, the coverslips were transferred into the alkaline solution of unwinding DNA (300 mM NaOH, 1 mM EDTA, pH 13) and maintained under conditions of darkness at 4°C for 30 min. After that time, the slides were subjected to electrophoresis in the same solution at the same temperature and also under conditions of darkness for a 30-min period at 25V and 300 mA. Slides were washed 3 times with a 0.4 M Tris pH 7.5 solutions to neutralize the alkalinity, fixed with 100% ethanol, and allowed to dry; the slides were finally stained with ethidium bromide solution. Three slides per concentration were read, and 100 cells were counted randomly. The experiments were performed in duplicate.

Cell proliferation assay (MTS)

CellTiter 96® AQueous Non-Radioactive Cell Proliferation is a colorimetric assay used to measure the number of viable cells in proliferation or chemosensitive cells. This product consists of Tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium] engaging MTS reagent (phenazine MeThoSulfate) PMS. The MTS is bioreduced to formazan within the cells. Once the cell cultures are found at 80–90% confluence, they were detached with trypsin; 50,000 cells were plated on 100 μl of culture medium in 96-well microplates and these were incubated during a 1-h period at 37°C, 5% CO_2, and 95% humidity. The cells were in contact with 13 different concentrations of eugenol (ranging from 0.06 to 810 mM) and were incubated for additional 1 h under the same conditions of temperature, humidity, and CO_2 percentage. A sufficient amount of working solution was prepared for each 2-mL rate of MTS solution, 100 μL PMS solution immediately prior to use (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corporation). Twenty microliters of this working solution was taken and placed into individual wells containing cells and eugenol at different concentrations. The microplate was incubated for a 4-h period at 37°C, 5% CO_2, and 95% humidity and read on a microplate reader (Thermo Scientific FC Multiskan®, Vantaa, Finland) at 490 nm. All tests were compared with control cells untreated with eugenol, and all dilutions were tested in triplicate, reporting the average values of three readings.

LIVE/DEAD® test

Two- or 3-day cell cultures were performed (the time at which they reached appropriate density fibroblasts) in 24-well culture boxes, on which we previously placed circular, 13-mm-diameter coverslips. After completion of this time, the cells were washed with PBS to remove the esterase present in the FBS used to enrich the culture medium in which the cells were grown, treated (with different concentrations of eugenol), and incubated for a 1-h period at 37°C with 5% CO_2 and 95% humidity. The LIVE/DEAD® working solution at a concentration of 2 μM and 4 uM calcein EthD-1 in PBS was prepared as directed by the manufacturer (Life Technologies; LIVE/DEAD® Viability/Cytotoxicity kit; Life Technologies, USA). Of this solution, 100 μL was added directly to the cells, which were incubated for a 45-min period at room temperature. At the end of this time, the cells were washed with PBS, and the

coverslip-stained cells were removed and observed in the CLMS. Thirteen increasing concentrations of eugenol (ranging from 0.06 to 810 μM) in fibroblasts were applied and compared with a negative control. Tests were performed in triplicate.

Trypan blue assay

Once the cells were exposed to different eugenol concentrations, and put into contact with trypan blue at 2% at a 1:10 dilution, this dilution was loaded into the hemocytometer, and the counting of live and dead cells was performed in the quadrants of the four outer chambers. The data was recorded and calculations were made to determine the percentage of dead and live cells for each treatment to which the cells were exposed.

Statistical analysis

Differences between control and experimental groups were statistically analyzed by the comparison of several groups. One-way analysis of variance (ANOVA) tests were considered statistically significant with a value of $p < 0.05$. The SigmaPlot ver. 11.0 statistical software package was used.

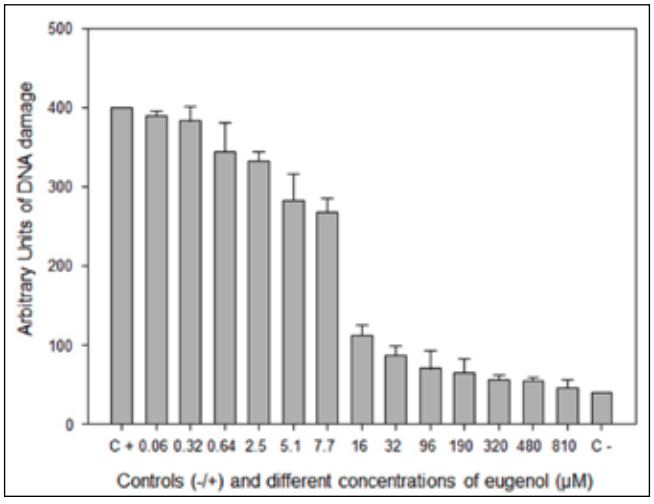
RESULTS

Seventy percent of the proliferated cell cultures exhibited a cell viability of >95%. These conditions facilitated the conduction of different toxicity tests.

Comet assay

This assay was performed in dental pulp fibroblasts treated with different eugenol concentrations for a 1-h period at 37°C, 5% CO_2, and 95% humidity. Genetic damage of treated cells was higher with eugenol compared with untreated DNA-damaged cells. DNA damage was clearly observed in the initial eugenol concentrations (range, 0.06-5.1 μM), because after the 6th eugenol concentration tested (7.7 μM), DNA damage decreased until it disappeared in the last three eugenol concentrations (ranging from 320 to 818 μM). Damage to DNA is expressed in arbitrary units (AU) (Figure 1), and the level of damage ranged from level 0 to level 4 (Figure 2).

Figure 1. Genotoxicity assay: Arbitrary units (AU) of DNA damage in human fibroblasts treated with eugenol at different concentrations.



MTS assay

MTS assay results are depicted in figure 3. This figure demonstrates a clear reduction in cell viability dependent on the eugenol concentration (0.06-320 μM) when compared with the control group corresponding to a cell culture without eugenol. The cytotoxic effect was observed from the first concentration with a decrease in cell viability. Statistical significant difference when comparing different concentrations of eugenol ($p < 0.05$) was found.

LIVE/DEAD® assay

This assay was performed in triplicate and the same phenomenon was observed in each test. The results are illustrated in figure 4: bright green fluorescence was observed in living cells corresponding to control group, while red fluorescence (dead cells) was observed from the group with the lowest eugenol concentration (0.06-μM) group, indicating that even at this low concentration, eugenol possesses cytotoxic activity.

Trypan blue assay

Results are presented in figure 5. No viable cells were observed from the lowest eugenol concentration of eugenol, which may indicate the damage that this can cause to the chemical compound in the cell membrane on allowing the treated cells to penetration of the stain, while that of an intact cell repels the dye, this translating directly into live and dead cells, and in exact agreement with the LIVE/DEAD® assay (Figure 4), where this phenomenon is repeated from the lowest concentrations of eugenol.

DISCUSSION

It is well documented that sealing the root canal can cause adverse local and/or systemic effects in periradicular tissue and alveolar bone due to the release of extractable monomer and/or other inorganic and organic ingredients. Root canal sealants could not only cause degradation of the tissue beneath the endodontic sealant, but also could delay wound healing.[24]

Although eugenol is known for its various properties, such as antibacterial, anesthetic, analgesic, and anti-inflammatory, among others, these properties may be useful in certain dental treatments.[25] Studies have been inconclusive in terms of whether eugenol causes health problems,[26] as cytotoxicity assays have been conducted whose results could be different according to the cell lines employed, especially on comparing cell lines of animal and human origin.[27]

Cells are very selective in terms of the compounds that pass through their membrane. In a viable cell, trypan blue is not absorbed; however, this dye penetrates the membrane of dead cells. Consequently, under a microscope, dead cells are exhibited with a distinctive deep-blue, and live cells are excluded from staining; therefore this staining method is utilized to assess cell viability and, as can be observed, eugenol kills some cells even at low concentrations. This is consistent with the study of Correa and Samara, in which they also found that eugenol, compared with other dental sealants, such as AH-Plus and Sealapex, is more cytotoxic.[28,29]

Also, the U.S. National Institute of Health (NIH), through the U.S. Department of Health and Human Services (HHS), the U.S. Public Health Service (USPHS), and the U.S. National Toxicology Program (NTP) issued an official report on carcinogenesis in which eugenol, in addition to other substances among chemicals classified as toxic and as potential inducers of tumor formation, was strongly

Figure 2. Single Cell Gel Electrophoresis (Comet) assay: Confocal laser scanning microscope (CLMS) images after staining with ethidium bromide. A) Damage level 0, negative control. (0-100 Arbitrary units [AU]) equivalent to cells in culture without any treatment; B) Damage level 1, cells treated with eugenol (101-200 AU); C) Damage level 2, cells treated with eugenol (201-300 AU); D) Damage level 3, cells treated with eugenol (301-400 AU); E) Damage level 4, positive control cells treated with H_2O_2 (400 AU).

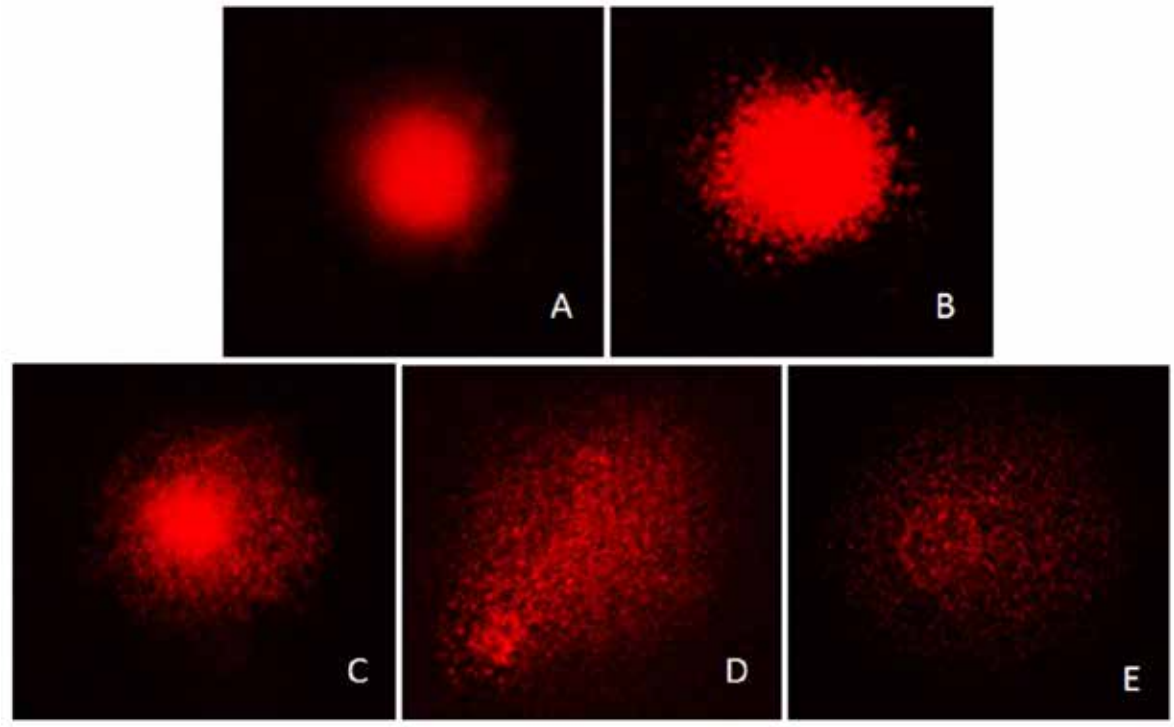


Figure 3. Cellular proliferation assay: Formazan absorbance (490 nm) in the control groups (+/-) and different concentrations of eugenol (range, 0.06-320 µM).

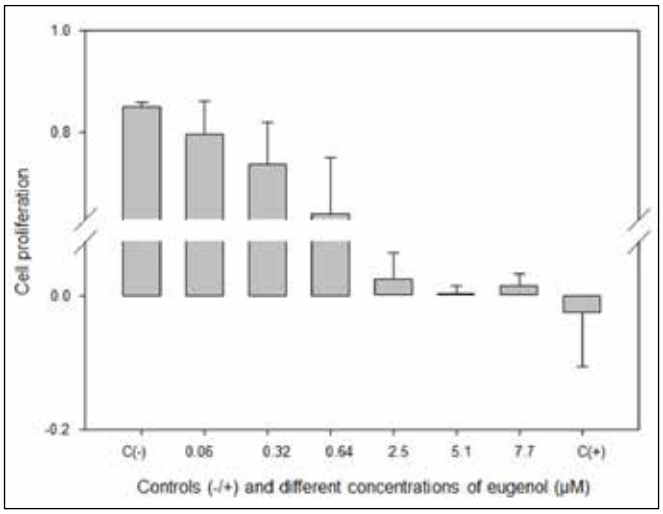


Figure 4. LIVE/DEAD® assay: Confocal laser scanning microscope (CLMS) images after staining with ethidium AM and Ethidium homoDimer (EthD-1) calcein. Treated with various concentrations of eugenol. A) Negative control cells in culture without any treatment; B) Positive control cells treated with H_2O_2; C), D), E), and F), cells treated with different concentrations of eugenol.

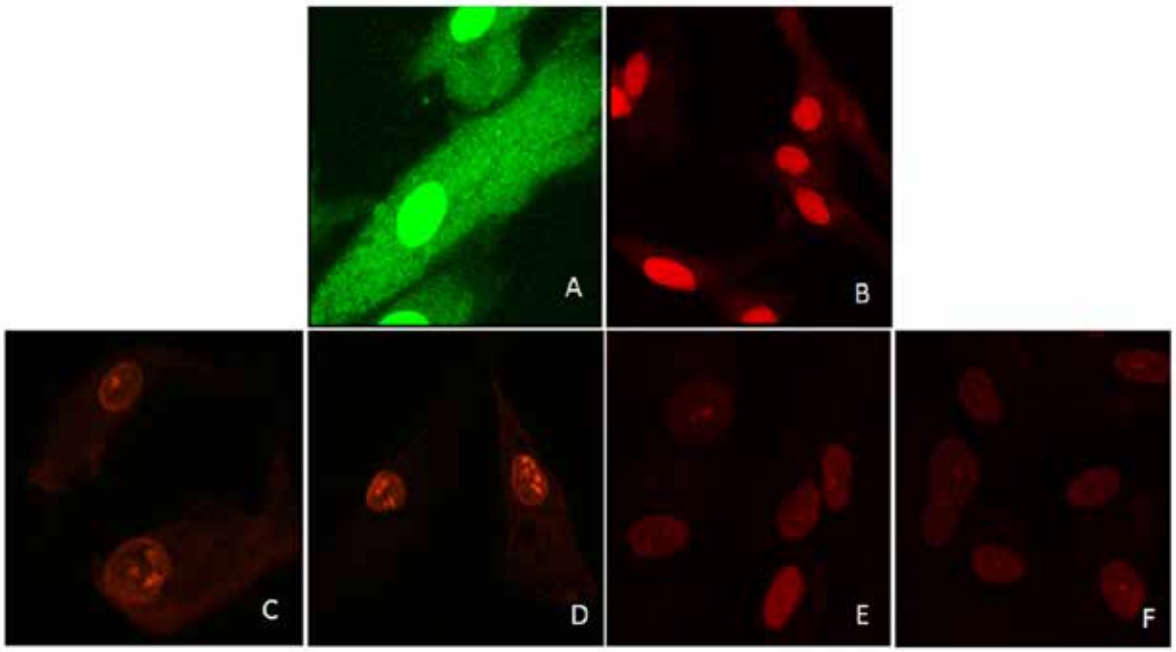
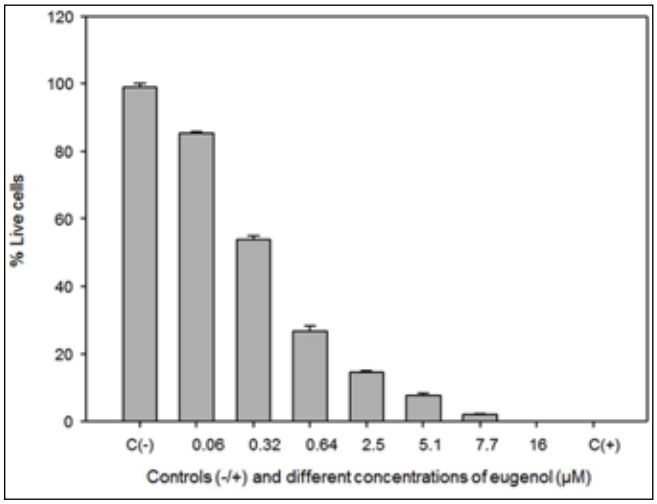


Figure 5. Viability assay: percentage of dead and live cells after staining with trypan blue in the control groups (+/-) and different concentrations of eugenol (range, 0.06-320 µM).



unrecognized as a carcinogen in humans, but was recognized as a carcinogen in laboratory animals.[6,7,30] Other studies have been reported on rabbits, where skin, liver, kidney, and brain samples were analyzed, and results were obtained of severe local toxicity at the application site. Damage severity was proportional to exposure time, dose, and concentration.[7,31]

Cell cultures *in vitro* comprise a tool used for testing cytotoxicity and genotoxicity induced within different materials, which include the processes employed in endodontics, such as sealants, filling material, and intracanal medication irrigants.[32]

Living cells are characterized by active intracellular esterase, which converts nonfluorescent calcein AM nonfluorescent into an intensely green fluorescent molecule; this molecule is retained by the living cell, while Ethidium homoDimer 1 (EthD-1) enters cells with damaged membrane and binds nucleic acids to produce a bright red fluorescent color. EthD-1 does not enter cells with intact membranes; eugenol, even at low concentrations, is capable of damaging the cell membrane of fibroblasts, demonstrated by EthD-1 penetration into the nucleus of fibroblasts treated with varying concentrations of eugenol. Likewise, the conversion of the MTS salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium] into soluble formazan is given by the dehydrogenase enzyme found in metabolically active cells. The amount of formazan produced is directly proportional to the number of viable cells in culture. Our results showed a statistically significant reduction of cell viability dependent of eugenol concentration.

Without neglecting the consideration of the therapeutic effects of eugenol, such as its being anesthetic, analgesic, antibacterial, and anti-inflammatory, which can be useful and the various contradictions involved in toxic and therapeutic effects mentioned previously,

it is important to analyze in detail, and in cytotoxicity and genotoxicity studies, these types of compounds in order to provide evidence to clarify, at least in part, the effects of eugenol. This is because, if we consider that one of the main uses of eugenol is when it is mixed with ZOE in the specialty of pediatric dentistry, as expected, the age of patients who arrive for care in this field is mainly between 5 and 10 years, which renders the exposed population more vulnerable to any cytotoxic effect that eugenol could trigger. If we add that the treatment zone and application of eugenol is the mouth area, it is clear that this facilitates eugenol intake by swallowing, inhalation due to proximity to the upper airways, and absorption due to the characteristics of high-diffusion mucous. All of these factors increase the risk of developing adverse effects, such as those reported in poisoning by ingestion of clove oil, ranging from central nervous system (CNS) depression, acidosis, hypoglycemia, hematuria, proteinuria, and increased transaminases.[33]

It has been shown that the elevation of eugenol induces cytosolic Ca_2 + in yeasts, and prokaryotes cause hepatotoxicity through a process that involving the reduction and loss of the protective effect of antioxidant intracellular glutathione transferase, with subsequent hepatocellular injury.[15] This damage may be due in part to the xenobiotic molecule bioactivan and to its carcinogenic metabolite (1'hydroxymethyleugenol) via cytochrome P450 enzymes such as (CYP1A2, CYP2A6, and CYP2C9) and sulfotransferases.[34,35]

On the other hand, the existence is noteworthy of the large interindividual variability rendered by the possible presence of polymorphisms in CYP450 enzymes (CYP1A2, CYP2A6, and CYP2C9) responsible for metabolism in the liver of these compounds, which would further increase the vulnerability of the treated population. This is due to that, although polymorphisms are known to exist in these enzymes,[36] to date there has been no studies to clarify the presence of polymorphisms in CYP and the effects of treatment with eugenol in children.

Because the genotoxic effect of eugenol was evaluated in this study, it was interesting to observe that the higher the concentration of eugenol, the lower the DNA damage. This can be explained by the high toxicity of eugenol, which causes damage to the cellular machinery, acting as a fixative that prevents it from reaching level that triggers a genotoxic effect without observing DNA damage in concentrations >320 μM avoiding the drawing of draw regarding the genotoxicity produced in dental pulp fibroblasts. This leaves the door open to future studies in which we clarified the molecular level for affecting genes involved in eugenol protection genome as P53; in addition, this is also important because eugenol affects the expression of genes involved in apoptosis and inflammatory processes that are of high importance in the development of chronic degenerative diseases and cancer processes.[37] With the findings in this study, we can state that eugenol is highly toxic when applied directly on human dental pulp fibroblasts.

Eugenol, even at low concentrations, produces a toxic effect when applied to the isolated pulp of human tissue fibroblasts, based on exposure to increasing eugenol concentrations, this confirmed by performing different tests for toxicity. Considering the property of eugenol as a highly lipophilic compound, we can conclude that the average life of eugenol is long, which would facilitate its accumulation, thus perhaps increasing toxicity after consecutive eugenol administrations within a given period of time.[38,39]

Commercially available sealants are based on ZOE, and may be cytotoxic due to the content of eugenol. In Paediatric Dentistry, it is very important that, as novel root canal sealers are developed, to undergo rigorous cytotoxicity testing prior to introduction into clinical use. It is also of the utmost importance for interested health professionals know the significance of this type of evidence in new products.

CONCLUSIONS

All of the concentrations of eugenol that we evaluated produced high toxicity in human dental pulp fibroblasts. The eugenol presented dose-dependent genotoxicity and cytotoxicity, although the possible setting effect of eugenol cells from the 7.7-mM concentration in which a genotoxic effect was not observed is clear.

ACKNOWLEDGMENTS

Karen Rodriguez-Contreras is a CONACYT fellow (448697). This work was supported partially by C15-FAI-04-101.101 grant.

REFERENCES

1. Edwards V, Markovic E, Matisons J, Young F. Development of an in vitro reproductive screening assay for novel pharmaceutical compounds. Biotechnol Appl Biochem; 5: 163–171, 2008.
2. Tannure PN, Azevedo CP, Barcelos R, Gleiser R, Primo LG. Long-term outcomes of primary tooth pulpectomy with and without smear layer removal: a randomized split-mouth clinical trial. Pediatr Dent; 33: 316–320, 2011.
3. Lin B, Zhao Y, Yang J, Wang W, Ge LH. Effects of zinc oxide-eugenol and calcium hydroxide/ iodoform on delaying root resorption in primary molars without successors. Dent Mater J; 33: 471–475, 2014.
4. Hui-Derksen EK, Chen CF, Majewski R, Tootla RG, Boynton JR. Retrospective record review: reinforced zinc oxide-eugenol pulpotomy: a retrospective study. Pediatr Dent; 35: 43–46, 2013.
5. Markowitz K, Moynihan M, Liu M. Biologic properties of eugenol and zinc oxide-eugenol. Oral Surg Oral Med Oral Pathol; 73: 729–737, 1992.
6. Mutoh N, Tani-Ishii N. A biocompatible model for evaluation of the responses of rat periapical tissue to a new zinc oxide-eugenol sealer. Dent Mater J; 30: 176–182, 2011.
7. Vera J, Siqueira JF Jr, Ricucci D, Loghin S, Fernández N, Flores B, Cruz Ag. One- versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod; 38: 1040–1052, 2012.
8. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils- a review. Food Chem Toxicol; 46: 446–475, 2008.
9. Devi KP, Sakthivel R, Nisha SA, Suganthy N, Pandian SK. Eugenol alters the integrity of cell membrane and acts against the nosocomial pathogen Proteus mirabilis. Arch Pharm Res; 36: 282–292, 2013.
10. Sipe HJ Jr, Lardinois OM, Mason RP. Free radical metabolism of methyleugenol and related compounds. Chem Res Toxicol; 27: 483–489, 2014.
11. Ding W, Levy DD, Bishop ME, Lyn-Cook Lascelles E, Kulkarni R, Chang CW, Aidoo A, Manjanatha MG. Methyleugenol genotoxicity in the Fischer 344 rat using the Comet assay and pathway-focused gene expression profiling. Toxicol Sci; 123: 103–112, 2011.
12. Zore GB, Thakre AD, Jadhav S, Karuppayil SM. Terpenoids inhibit Candida albicans growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine; 18: 1181–1190, 2011.
13. Darvishi E, Omidi M, Bushehri AA, Golshani A, Smith ML. The antifungal eugenol perturbs dual aromatic and branched-chain amino acid permeases in the cytoplasmic membrane of yeast. PLoS One; 8: e76028, 2013.
14. Khan A, Ahmad A, Akhtar F, Yousuf S, Xess I, Khan LA, Manzoor N. Induction of oxidative stress as a possible mechanism of the antifungal action of three phenylpropanoids. FEMS Yeast Res; 11: 114–122, 2011.
15. Roberts SK, McAinsh M, Cantopher H, Sandison S. Calcium dependence of eugenol tolerance and toxicity in Saccharomyces cerevisiae. PLoS One; 9: e102712, 2014.
16. Raghavendra RH, Naidu KA. Spice active principles as the inhibitors of human platelet aggregation and thromboxane biosynthesis. Prostaglandins Leukot Essent Fatty Acids; 81: 73–78, 2009.
17. Bakopoulou A, Papadopoulos T, Garefis P. Molecular toxicology of substances released from resin-based dental restorative materials. Int J Mol Sci; 10: 3861–3899, 2009.
18. Slyskova J, Langie SA, Collins AR, Vodicka P. Functional evaluation of DNA repair in human biopsies and their relation to other cellular biomarkers. Front Genet; 5: 116, 2014.
19. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Molecular Biotech; 26: 249–261, 2004.
20. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res; 175: 184–191, 1988.
21. Singh NP, Penn PE, Pendergrass WR, Wolf NS. White light-mediated DNA strand breaks in lens epithelial cells. Exp Eye Res; 75: 555–560, 2002.
22. Morandini AC, Sipert CR, Ramos-Junior ES, Brozoski DT, Santos CF. Periodontal ligament and gingival fibroblasts participate in the production of TGF-β, interleukin (IL)-8 and IL-10. Braz Oral Res; 25: 157–162, 2011.
23. Sipert C, Morandini A, Modena K, Dionísio T, Machado M, Oliveira S, Campanelli AP, Santos CF. CCL3 and CXCL12 production in vitro by dental pulp fibroblasts from permanent and deciduous teeth stimulated by Porphyromonas gingivalis LPS. J Appl Oral Sci; 21: 99–105, 2013.
24. Yilmaz Z, Dogan AL, Ozdemir O, Serper A. Evaluation of the cytotoxicity of different root canal sealers on L929 cell line by MTT assay. Dent Mater J; 31: 1028–1032, 2012.
25. Shih YH, Lin DJ, Chang KW, Hsia SM, Ko SY, Lee SY, Hsue SS, Wang TH, Chen YL, Shieh TM. Evaluation physical characteristics and comparison antimicrobial and anti-inflammation potentials of dental root canal sealers containing hinokitiol in vitro. PLoS One; 9: e94941, 2014.
26. Fujisawa S, Atsumi T, Kidoma H. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Toxicology; 177: 39–54, 2002.
27. Kwon J, Illeperuma R, Kim J, Kim K, Kim K. Cytotoxicity evaluation of zinc oxide-eugenol and non-eugenol cements using different fibroblast cell lines. Acta Odont Scand; 72: 64–70, 2013.
28. Correa GT, Veranio GA, Silva LE, Hirata Junior R, Coil JM, Scelza MF. Cytotoxicity evaluation of two root canal sealers and a commercial calcium hydroxide paste on THP1 cell line by trypan blue assay. J Appl Oral Sci; 17: 457–461, 2009.
29. Samara A, Sarri Y, Stravopodis D, Tzanetakis GN, Kontakiotis EG, Anastasiadou E. A comparative study of the effects of three root-end filling materials on proliferation and adherence of human periodontal ligament fibroblasts. J Endod; 37: 865–870, 2011.
30. Mutoh N, Satoh T, Watabe H, Tani-Ishii N. Evaluation of the biocompatibility of resin-based root canal sealers in rat periapical tissue. Dent Mater J; 32: 413–419, 2013.
31. Shen Q, Li W, Li W. The effect of clove oil on the transdermal delivery of Ibuprofen in the rabbit by in vitro and in vivo methods. Drug Dev Ind Pharm; 33: 1369–1374, 2007.
32. Silva EJ, Accorsi-Mendonça T, Almeida JF, Ferraz CC, Gomes BP, Zaia AA. Evaluation of cytotoxicity and up-regulation of gelatinases in human fibroblast cells by four root canal sealers. Int Endod J; 45: 49–56, 2012.
33. Goulet F, Vachon P, Hélie P. Evaluation of the toxicity of eugenol at anesthetic doses in African clawed frogs (Xenopus laevis). Toxicol Pathol; 39: 471–477, 2011.
34. Gardner I, Wakazono H, Bergin P, de Waziers I, Beaune P, Kenna JG, Caldwell J. Cytochrome P450 mediated bioactivation of methyleugenol to 1'-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes. Carcinogenesis; 18: 1775–1783, 1997.
35. Jeurissen SM, Bogaards JJ, Boersma MG, ter Horst JP, Awad HM, Fiamegos YC, van Beek TA, Alink GM, Sudhölter EJ, Cnubben NH, Rietjens IM. Human cytochrome p450 enzymes of importance for the bioactivation of methyleugenol to the proximate carcinogen 1'-hydroxymethyleugenol. Chem Res Toxicol; 19: 111–116, 2006.
36. Jetten MJ, Claessen SM, Dejong CH, Lahoz A, Castell JV, van Delft JH, Kleinjans JC. Interindividual variation in response to xenobiotic exposure established in precision-cut human liver slices. Toxicology; 2: 61–69, 2014.
37. Wang S, Chen F, Tang L. IL-32 promotes breast cancer cell growth and invasiveness. Oncol Lett; 9: 305–307, 2015.
38. Liao TT, Wang L, Jia RW, Fu XH, Chua H. Lipophilic organic pollutants induce changes in phospholipid and membrane protein composition leading to Vero cell morphological change. J Environ Sci Health B; 49: 760–768, 2014.
39. Tsakalozou E, Adane ED, Liang Y, Arnold SM, Leggas M. Protracted dosing of the lipophilic camptothecin analogue AR-67 in non-small cell lung cancer xenografts and humans. Cancer Chemother Pharmacol; 74: 45–54, 2014.