



Evaluation of the genotoxicity of zinc oxide-eugenol cement to *Allium cepa* L.

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ABSTRACT. Dental materials can induce local and systemic effects. The *Allium cepa* assay was used to evaluate the genotoxicity and/or cytotoxicity of zinc oxide and eugenol (ZOE) at different proportions. The ZOE solution was tested at the concentration of 1 drop of eugenol (in each drop of liquid, the approximate concentration of eugenol is 85%) and 1 portion of zinc oxide cement (treatment I), and twice the concentration of eugenol (treatment II). Treated roots appeared to be yellowish-brown, fewer in number, thicker and less turgid compared with the control, suggesting a cytotoxic activity of ZOE. A significant difference was found in the root size between the control and treatment II. This treatment reduced by 79% the size of the root compared with the control, and the mitotic index was 66%, indicating a 22.4% reduction relative to the control, which in turn evidenced the cytotoxicity of ZOE. The significant increase in anaphase bridges suggests a genotoxic effect.

Keywords: cytotoxicity, micronucleus (MN), dental materials, mutagenesis.

Avaliação da genotoxicidade do cimento de óxido de zinco e eugenol em *Allium cepa* L.

RESUMO. Materiais dentários podem induzir diversos efeitos, tanto local quanto sistêmico. Objetivou-se avaliar a ocorrência de danos genéticos e de alterações nucleares degenerativas indicativas de genotoxicidade e citotoxicidade em células de cebola em mitose, expostas a óxido de zinco e eugenol (ZOE) em diferentes proporções. Os bulbos foram postos a germinar, com a parte inferior mergulhada na solução de ZOE + água destilada. A solução de ZOE foi testada na concentração de uma gota de eugenol (aproximadamente 85%) e 1 porção do cimento de óxido de zinco (tratamento I) e o dobro da concentração do eugenol (tratamento II). As raízes do controle apresentaram coloração esbranquiçada e eram túrgidas, enquanto as raízes dos tratamentos se mostraram de cor amarela – amarronzada, em menor quantidade, mais grossas e menos túrgidas, sugerindo ação citotóxica de ZOE. Houve diferença significativa do tamanho das raízes entre o controle e o tratamento II. O tratamento II reduziu 79% do tamanho da raiz e o índice mitótico foi 66%. Isto mostra redução de 22,4% quando comparado com o controle, evidenciando a ação citotóxica de ZOE. O aumento significativo de pontes anafásicas sugere efeito genotóxico.

Palavras-chave: citotoxicidade, micronúcleo (MN), materiais odontológicos, mutagênese.

Introduction

In the last century, dentistry has acquired great technological advances that sought promptness for the professionals of this area and comfort for the patient. Nevertheless, taking a simple restoration as an example, it is necessary to utilize various chemical elements that, depending on the concentration used, can affect human health and are also capable of causing injury to genetic material (MUNERATO et al., 2005; REZENDE et al., 2011). One possible consequence of a prolonged exposure to these elements is cancer. Currently, there are a large number of studies regarding the toxicological profile of some materials.

Eugenol (EUG) is an aromatic compound generally found in cloves, mainly from *Syzygium aromaticum* (L.) Merr & L. M. Perry. It has a remarkable analgesic effect, which makes it to be used for the treatment of toothaches (HUME; MASSEY, 1990; ROMPELBERG et al., 1996; VIDAL et al., 2008). Together with zinc oxide, referred to as zinc oxide-eugenol, it is also greatly used in temporary dental fillings (HUME; MASSEY, 1990). Despite the beneficial effect of this material, the possibility of genotoxic effects cannot be ignored since genotoxicity is one of the side effects of chemical products (MUNERATO et al., 2005; REZENDE et al., 2011). Considering that most dental materials release small quantities of

various substances into their physiological environment (pulp, oral cavity), an appropriate regulation should ensure that genotoxicity is eliminated or at least reduced.

There is a great chance of causing cell mutations, such as: micronuclei, nucleoplasmic bridges, nuclear buds, among others (HUANG et al., 2002; REZENDE et al., 2011). Micronucleus test, sister chromatid exchange test and genotoxicity analysis are performed to detect the toxicity of elements (GALINDO; MOREIRA, 2009). The micronucleus test is of great applicability since it is an easy, fast and low-cost technique. The micronucleus test is one of the methods available for the evaluation of spontaneous or induced chromosome damage. Micronuclei are free corpuscles that measure from 1/16 to 1/3 of the size of the nucleus, with a shape from round to oval and are usually found beside the main nucleus, to which they are similar in terms of shape and color (FENECH et al., 2003; VRAL et al., 2011). The micronucleus originates from a late chromosome region or irregular migrations during anaphase (CELIK et al., 2003).

Nucleoplasmic bridges are also indicators of genotoxicity and result from the toxic activity of substances. Bridges are observed between two nuclei that are not too close or overlaid (FENECH et al., 2003), and originate from dicentric chromosomes whose centromeres are pulled to opposite poles during anaphase. An increase in the incidence of cytogenetic changes is a sign of relevant exposure, indicating genetic changes in cells (FENECH, 2000).

Plants can be used as biosensors of genetic toxicity of environmental pollutants (RANK et al., 2002; MIŠÍK et al., 2011). *Allium cepa* is a sensitive biological tool to rapidly detect genotoxic contaminants (KALCHEVA et al., 2009). There is a relationship between the root apical meristem of *A. cepa* and the testing systems in mammals: *in vitro* and *in vivo*, in this way, onion is an alternative system to evaluate the genotoxicity of chemical substances (AMARAL et al., 2007; LEME; MARIN-MORALES, 2008; KURÁS et al., 2009). The advantage of an *in vitro* test is the possibility of having a control and the test can be easily and economically reproduced (FISKEJO, 1985). *Allium cepa* is widely used due to its low-cost, is easily obtained and handled, being required simple methodology for slide preparation and evaluation of chromosomes (AMARAL et al., 2007).

In this context, the objective of this study was to evaluate the occurrence of genetic damage and

degenerative nuclear changes indicating genotoxicity and cytotoxicity to the cells of onion (*A. cepa*) in mitosis and those that have been exposed to zinc oxide-eugenol in different proportions (powder/liquid).

Material and methods

Small, non-germinated and healthy onions of the same origin were used. Bulbs were left to germinate on appropriate plastic containers, with the lower part (plate) dipped in a zinc oxide-eugenol solution and distilled water. The experiment comprised six bulbs, and the control (distilled water).

Substances tested were used in the normal and recommended concentrations, one drop of eugenol and one portion/measure of zinc oxide cement, and twice the concentration of eugenol and one portion/measure of zinc oxide. In each drop of liquid, the approximate concentration of eugenol is 85%. The remainder is composed of vegetable oils (usually olive oil) and small traces of other substances to control the reaction of setting of the material, such as acetic acid (FERRACANE, 2001).

Each *A. cepa* testing system was aerated in a cultivation chamber during seven days with an air compressor. The system was maintained at room temperature and covered with a carton during three and a half days, and then the carton was removed. At the end of the seventh day at 10 a.m., four root tips of each bulb were cut (root apical meristem). These were put in ten drops of 2% acetic orcein stain (GUERRA; SOUZA, 2002) + one drop of 1N HCl, buckling was performed for a short time (1 to 2 minutes), just to allow hydrolysis and staining of the cell. The material was crushed and taken to a binocular microscope (*Leica*), cells were counted and the aberrations were photographed.

Analysis and assessment of mutagenicity and toxicity

The interpretation of the slides was done according to the following parameters:

Mitotic Index (MI): the number of cells in mitosis (prophase + prometaphase + metaphase + anaphase + telophase) were divided by the total number of cells (interphase + mitosis) and multiplied by 100 (PIRES et al., 2001);

Anomalies in the Mitotic Cycle (Anaphase - Telophase):

- anaphase and telophase bridges: dicentric chromosomes or exchanges;
- chromosomes or free fragments;
- micronuclei: 1000 cells were analyzed per root and 4000 cells per bulb, and micronuclei that

resulted from abnormal divisions were identified;

Toxicity was evaluated by macroscopic parameters, like length of the roots, turgidity and hardening of the root tips, color alterations and thickening (tumors).

Statistical analysis

Values of root size were compared with RC50.

The statistical analysis of the root sizes and nuclear anomalies were carried out using the Scott-Knott test.

Results and discussion

The reason for using a plant to test a chemical, ZOE, which is used in human dental treatment, is because *Allium cepa* is a useful organism in biomonitoring of toxic substances. Therefore, the onion is an alternative system for carrying out genotoxicity and cytotoxicity tests and is also promising in terms of reduced experimentation in live animals (AMARAL et al., 2007; BAGATINI et al., 2007; KOVALCHUK et al., 1998; KURÁS et al., 2009; LEME, MARIN-MORALES, 2008). Also, advantages arise when using *A. cepa* as a testing organism since it is possible to control the assembled system, the system provided data in a fast and economical manner, and it is very advantageous in the evaluation of the toxic effects of chemical materials (AMARAL et al., 2007; ANTONSIEWICZ, 1990; BROWNE, 1988).

The mutagenic effects of the substance tested could be analyzed through macroscopic parameters, like growth and shape of the root, and also by cytological parameters, such as the type and frequencies of chromosomal aberrations and abnormal cell divisions (KOVALCHUK et al., 1998). The control presented a whitish color while the roots from the two treatments were yellowish-brownish. Despite the small number, the roots from the two treatments were thicker compared to the control. There was a difference in turgidity, the roots from the treatments were soft, while those from the control were hardened. In some onions, the roots did not develop and the root growth region was covered by a viscous gelatinous substance (Figure 1A and B). These controls characters were also observed by Bakare et al. (2009). These variations between treatments and control indicate a cytotoxic potential of the analyzed substance. The study of Cortes et al. (1990) also found an inhibition of the root development in onion bulbs exposed to different concentrations of radiation.

A significant variation was detected in the root size, as demonstrated by the Scott-Knott test.

Between the onions of treatments I and II and the control onions, there were significant differences as to the size of the roots (Table 1). In treatment I, the mean value was 1.75 cm, while in treatment II the mean was 0.67 cm and in the control it was 3.0 cm (Figure 1). Growth is the most important parameter and is easily transformed into indices, like RC50. In the present work, treatment II had a reduction of 79% in the size of the roots compared with the control, a value higher than RC50, which indicates the cytotoxicity of the cement. The treatment I presented a reduction of 41.67% compared with the control, a value close to 50%, but non-significant (Table 1). However, the Scott-Knott test pointed out a significant difference in the root size between treatment I and the control, showing that the treatment was also cytotoxic. There was also a large difference between the root size from treatment I and II, treatment II was reduced by 64% in comparison to the treatment I.

Table 1. Root growth inhibition (macroscopic analysis).

Treatment	Average root length for each bulb (range in cm)	Mean \pm SE	Growth in % of control	Percent inhibition (RC ₅₀)
T I	0.9-1.2	1.75 \pm 0.03	35.830	41.67%
T II	0.6-0.8	0.67 \pm 0.02	22.500	79%**
C	2.0-4.0	3.00 \pm 0.09*	-	-

*Values are significantly different from the control and both treatments at $p < 0.05$ (Scott-Knott); **RC₅₀ Values of treatment II are significantly different from the control. TI (treatment I); TII (treatment II); C (control).

Several studies showed the cytotoxic potential of the zinc oxide-eugenol. Hume (1984) supported that the cytotoxicity of ZOE is due to eugenol. Chong et al. (1994) stated that ZOE is cytotoxic and that its cytotoxicity varies according to the different formulations of ZOE. Senne et al. (2009) evaluated the cytotoxicity of three endodontic cements and found that those based on zinc oxide and eugenol were highly cytotoxic, similar results were observed by Chang et al. (2000) and Vasconcelos et al. (2008). The study developed by Ho et al. (2006) sought to elucidate the toxic effects of eugenol in cell culture of human osteoblasts, and discovered the cytotoxic mechanism of action of eugenol; the authors observed the inhibition of growth and proliferation of cells tested and the high toxicity of eugenol to periapical tissues. Other effects of the zinc oxide-eugenol cement are irritation of tissues and inflammatory action (SCARPARO et al., 2009; YESILSOY et al., 1988). REZENDE et al. (2011) observed the genotoxicity of dental materials through the micronucleus (MN) test, in the buccal cells of children. This mentioned study evaluated three types of dental materials; ZOE based cement, monomers, and their combination, and observed

that treatments using monomer + cement-based materials significantly increased the number of nuclear anomalies.

Huang et al. (2002) verified that endodontic cements with zinc oxide-eugenol were the most cytotoxic and related it to the fact that cements tend to dissolve in aqueous medium for a prolonged time, increasing the cytotoxicity. The cytotoxicity is mainly associated with the action of eugenol which is released from the hydrolysis of zinc oxide-eugenol (CHONG et al., 1994). The fresher the zinc oxide-eugenol, the greater the toxic potential of this dental material (CHONG et al., 1994). The wetter the ZOE, the greater the capacity to release eugenol through hydrolysis (HUME, 1984). In the system tested, the preparation of zinc oxide-eugenol was always in direct contact with distilled water, where intense hydrolysis occurred, which means that eugenol was released in great amounts. Due to this reason, it was possible to observe the cytotoxic effects of the material.

Many materials, particularly the valves of root canals as ZOE, remain in contact with vital tissues for a long period, and the possible action of these materials is the cellular aggression by chemical, physical and mechanical elements; being respiration one of the first cell systems affected. Some aggressor agents, particularly chemicals, block important enzyme systems involved in protein synthesis and/or generation of ATP; other lead to the generation of harmful intracellular products or, still, act directly destroying vital structural components of the cell (SENNE et al., 2009).

In all the slides, it was possible to observe different phases of mitosis; prophase, prometaphase, metaphase, anaphase, telophase and interphase. The mitotic index was 87% in treatment I, 66% in treatment II and 85% in the control (Table 2). In treatment II, there was a 22.4% reduction of the MI when compared with the control. Antonsiewicz (1990) confirms that a 22% reduction in the MI compared with the negative control can cause lethal effects in the organism, which can be caused by the cytotoxic effect of the tested substance. In the present work, the treatment II caused toxic effects to the organism. The MI of treatment I was 2.3% greater than the control, which indicates that the amount of the substance used in treatment I was not enough to produce changes in the MI. Variables that were not controlled have to be considered, for example the genetic variation of the onions and its consequences and the amount and quality of the fertilizer. In studies carried out by Kovalchuk et al. (1998), they also found a reduction in the mitotic index of the onion root meristem. The higher the levels of radioactive contamination of the soil, the greater the chromosomal aberrations, and the smaller the MI.

Table 2. Chromosomal aberrations (microscopic analysis)

Treatment	N. of dividing cells	Mitotic index(MI)	Late chromosomes average	Micronuclei average	Nuclear buds average	Anaphase bridges average
T I	870	87%	1.42	0.90	0.95	0.90
T II	660	66%	1.24	0.77	0.71	1.05*
C	852	85%	1.32	0.82	0.76	0.74

*Significant result at $p < 0.05$ (Scott-Knott). The analysis of variance was performed with the square root transformation + 0.5. TI (treatment I); TII (treatment II); C (control).

The control slides presented no chromosomal aberrations or micronuclei formation. The cells corresponding to treatments I and II presented the following chromosomal aberrations: anaphase bridges (Figure 1C-E), chromosome breakages (Figure 1F), micronuclei (Figure 1G) and late chromosomes. For these aberrations, only the anaphase bridges were statistically significant in treatment II (Table 2). This result indicates a probable genotoxicity of the zinc oxide-eugenol. Nucleoplasmic bridges were observed in cells exposed to clastogenic agents (FENECH et al., 2003). Since these cells originate from dicentric chromosomes in which the centromeres are pulled to the opposite poles (FENECH, 2000), there is a traction force during this process and the chromosome can suffer breakage in any point, which can form a chromosomal fragment that can be transformed into MN (VRAL et al., 2011). This can also occur in independent cells with different morphological characteristics of the chromosome conjunction since one of the cells can remain with a larger part of the broken chromosome, and this part could link to any other part of the chromosomes and modify their morphology, or even remain as a chromosome in the chromosome set.

The micronucleus test allows identifying an increase in the frequency of mutations in cells when are exposed to a variety of genotoxic agents (CARVALHO et al., 2002; THOMAS et al., 2011). Several studies have proven the efficacy of this test as an indicator of cytogenetic damage (GALINDO; MOREIRA, 2009; RAMIREZ et al., 1999; REIS et al., 2002; THOMAS et al., 2011). Biological markers may reflect doses of exposure to carcinogens and their interaction with macromolecules, as the DNA (REIS et al., 2002; VRAL et al., 2011; BONASSI et al., 2011). It is not yet established a relationship between the frequency of micronuclei and the development of cancer, but the detection of micronuclei demonstrates the exposure of cells to chemical agents (RAMIREZ et al., 1999; REIS et al., 2002). The validation of the micronucleus (MN) as marker of phenotypic susceptibility to cancer has received decisive support

by the work of Bonassi et al. (2011). The authors showed increased frequency of tobacco carcinogen-

induced MN, nuclear buds and especially nucleoplasmic bridges in cancer patients.

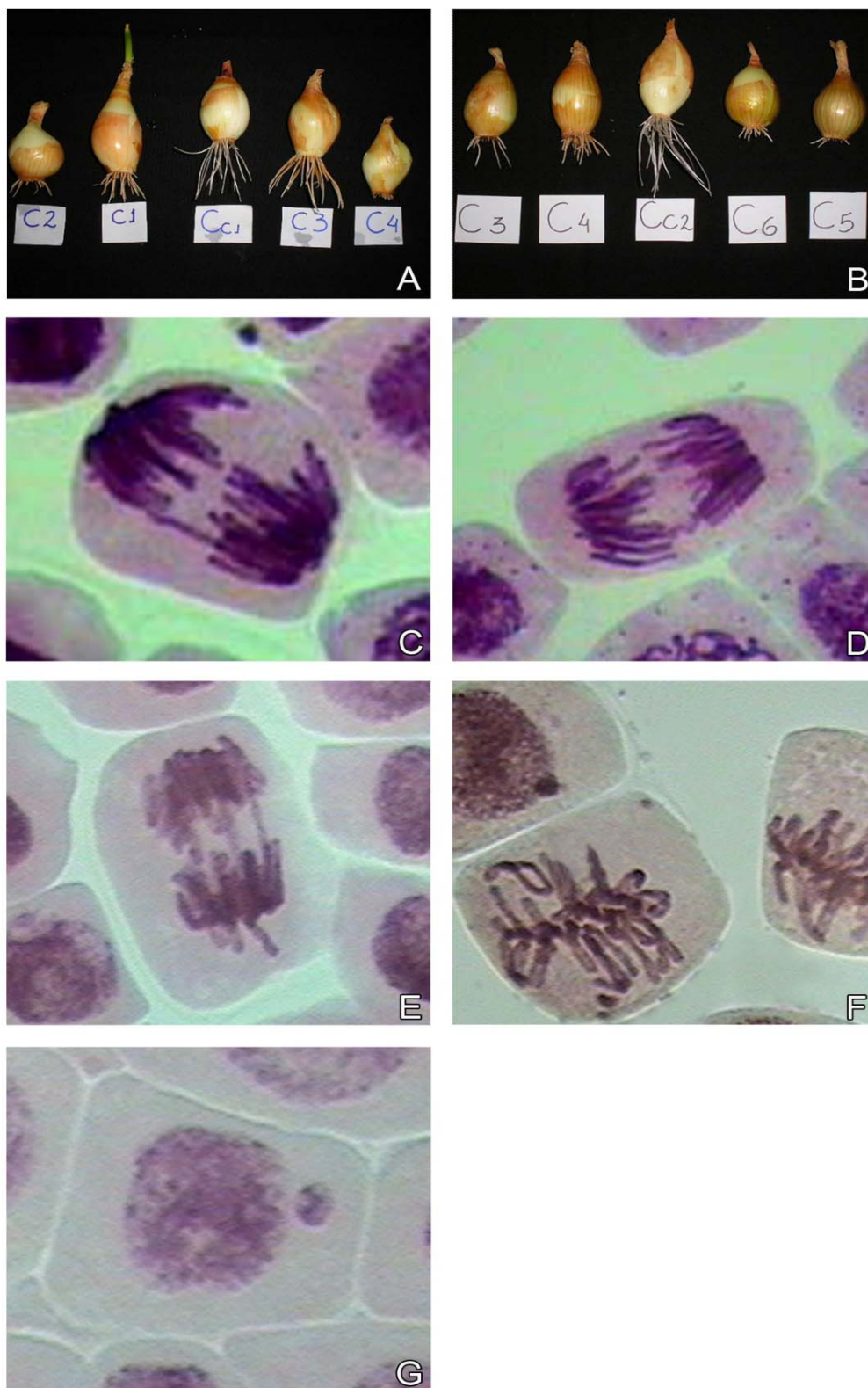


Figure 1. (A) Treatment 1: control (Cc1), treated onions (C1, C2, C3, C4), after 6 days. (B) Treatment 2: control (Cc2), treated onions (C3, C4, C5 e C6), after 6 days. (C-E) Anaphase bridges. (F) Chromosomal breakages. (G) Micronucleus.

Cells of *A. cepa* are equivalent to epithelial tissue, have similar mitotic characteristics and are sensitive to the activity of chemical agents, representing an alternative system for testing genotoxicity and cytotoxicity (KOVALCHUK et al., 1998). As our work demonstrates the cytotoxic effect of the zinc oxide-eugenol cement, it can also be compared with other studies in animal cells named in the text. Despite the possible cytotoxic effect of this material in the oral mucosa, it is still very used in dental clinics. Many other products which do not have the eugenol, such the ones based on calcium hydroxide (HUANG et al., 2002), have already been launched on the market, and these are more indicated for use in dental treatments with least risk of toxicity.

Conclusion

In summary, changes in size, thickness and color of the treated roots were caused by the cytotoxic activity of the zinc oxide-eugenol cement, in the concentration of one or two drops of eugenol (85% per drop) for each portion of zinc oxide cement. Chromosomal aberrations, such as anaphase bridges, suggest the genotoxic effect of this substance, in the concentration of two drops of eugenol for each portion of zinc oxide.

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