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EVALUATION OF CYTOTOXICITY OF TWO ENDODONTIC CEMENTS IN A MACROPHAGE CULTURE

AVALIAÇÃO DA CITOTOXICIDADE DE DOIS CIMENTOS ENDODÔNTICOS EM CULTURA DE MACRÓFAGOS

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ABSTRACT

Compared to gutta-percha, the endodontic cements are used in small quantity to seal root canals, but are indispensable to achieve hermetically sealed margins, where its biocompatibility depends on the sum of responses of each cell present in the periapical region. The object of this study was to evaluate the cytotoxicity of two endodontic cements, one based on epoxy resin (Sealer 26) and the other containing zinc oxide eugenol (Endofill) by using cultured peritoneal macrophages from Swiss mice to measure the induced production of nitric oxide. After solidification and pulverization, aliquots of 100µl of suspension containing 18mg/mL of the respective cements were added to 96-well tissue culture plates containing the tissue culture of macrophages at a concentration of $5.0X10^6$ cells/ml. In the positive control group the cell culture was treated with 10mg/mL of lipopolyssaccharide from *Escherichia coli* 026:B6 and the cell culture alone represented the negative control. After 48 hours of incubation, at 37°C, in 5% CO_2, the cultures were placed in an ELISA automatic reader to evaluate the release of nitric oxide. The production of nitric oxide for cement Sealer 26 was between 36.1 and 313.0 µmols, with a mean of 143.82±111.03µmols, while for the Endofill these values were significantly less (p=0.01), varying from 50.8 to 125.7µmols, with a mean of 80.33±28.42 µmols. In the positive and negative control groups the mean release of nitric oxide was of 162.75µmols and 4.42µmols, respectively. There was no significant difference between the positive control group and cement Sealer 26 (p>0.05). Therefore, the cement Sealer 26 caused significantly greater toxicity to the macrophages, possibly due to the components from the epoxy resin and formaldehyde release during polymerization.

Uniterms: Root canal filling materials; Nitric oxide; Macrophages.

RESUMO

Comparativamente à guta-percha, os cimentos endodônticos são utilizados em pequena quantidade nas obturações dos canais radiculares, mas são imprescindíveis para obtenção do hermético selamento marginal, sendo sua biocompatibilidade dependente do somatório das respostas de cada célula presente na região periapical. Por conseguinte, o objetivo deste estudo foi avaliar em cultura de macrófagos peritoneais de seis camundongos Swiss, a citotoxicidade de dois cimentos endodônticos, um à base de resina epóxica (Sealer 26) e outro contendo óxido de zinco e eugenol (Endofill), quanto a indução da produção de óxido nítrico. Após a presa e pulverização, alíquotas de 100µl da suspensão contendo 18mg/mL dos respectivos cimentos foram adicionados em placa de cultura de tecido de 96 poços, contendo a cultura de macrófagos na concentração de $5,0X10^6$ células/ml. Após 48 horas de incubação, a 37°C, em 5% de CO_2, foi feita a leitura em um leitor ELISA automático para se averiguar a liberação de óxido nítrico. Verificou-se, para o cimento Sealer 26, que a produção de óxido nítrico oscilou de 36,1 a 313,0 µmols, com média de 143,82±111,03, enquanto para o Endofill estes valores foram significativamente menores (p=0.01), variando de 50,8 a 125,7, com média de 80.33±28,42 mols. Nos grupos controles positivo e negativo a média de liberação de óxido nítrico foi de 162,75µmols e 4,42µmols, respectivamente. Não houve diferença significativa entre o grupo controle positivo e o cimento Sealer 26 (p>0.05). Portanto, o cimento Sealer 26 causou, significativamente, maior toxicidade aos macrófagos, possivelmente devido aos seus componentes, a exemplo da resina epóxica e ao formaldeído, liberados durante a sua reação de polimerização.

Unitermos: Materiais obturadores do canal radicular; Óxido nítrico; Macrófagos.

INTRODUCTION

In periapical lesions of endodontic origin, macrophages represent the principal cells controlling tissue destruction (Soares and Queiroz[25], 2001). Originating from stem cells in bone marrow and circulating in the blood as monocytes, they are transformed into macrophages in the tissue (Metzger[15], 2000). The presence of microorganisms, endotoxins and subproducts of microbial metabolism in the root canal system, as well as substances used in endodontic treatment, such as cement sealers, activate these cells to produce innumerable substances with inflammatory activity, such as prostaglandins (PGE_2), cytokines, oxygenated intermediate products (peroxides and superoxides) and nitrogen oxides, such as nitric oxide (Perassi, et al.[18] 2004). The continuous secretion of these biomolecules maintain the inflammatory reaction with destructive periapical consequences (Blix and Helgeland[4], 1998; Perassi, et al.[18] 2004).

Due to the complexity of responses of the various cells in the periapical region to endodontic cements, a cell culture represents a simplified method of evaluating the behavior of a specific cellular group in the context of a tissue, with the advantage that all factors and variables may be controlled. Among the myriads of responses that the cells activate when challenged by an aggression or condition of stress, the production of nitric oxide has been most commonly evaluated (Azar, et al.[2] 2000; Blix and Helgeland[4], 1998; Flora Filho and Zilberstein[7], 2000; Shimauchi, et al.[23] 2001; Takeichi, et al.[27] 1998).

This substance is derived from the amino acid L-arginine through the enzyme activity of three deoxygenases, denominated nitric oxide synthases (NOS). Two isomers of this enzyme are expressed in the constitutive form (cNOS) in endothelial cells, neurons and skeletal muscle. The third induced isomer (iNOS) is identified in cells of the immune system, as in the example of macrophages (Flora Filho and Zilberstein[7], 2000). This free radical gas participates in various physiological and pathological processes, for example: 1) regulating blood pressure, 2) suppressing the activity of macrophages, 3) inhibiting the adhesion of leucocytes to the vascular endothelium and 4) promoting apoptosis (Moncada, et al.[16] 1991). According to Blix and Helgeland[4], (1998), prostaglandin E_2 and endotoxins of *Actinobacillus Actinomycetemcomitans* and *Escherichia coli* induce the production of nitric oxide in cultures of macrophages. Takeichi, et al.[27] (1998) verified that a variety of cells present in apical cysts produce nitric oxide, with examples of epithelial and endothelial cells, macrophages, neutrophils, fibroblasts and lymphocytes principally those situated in the proximity of blood vessels. Therefore, it can be inferred that nitric oxide participates in maintenance mechanisms of the inflammatory periapical response, which is in agreement with Shimauchi, et al.[23] (2001) who identified elevated concentrations of nitric oxide in the exudates from periapical lesions. Therefore, nitric oxide has a significant association with the tissue destruction mediated through the chronic inflammatory process.

One of the objectives of sealing root canals is the recuperation of normal clinical, radiographic and histological aspects of the apical and periapical region. Therefore, the function of sealants and gutta-percha for endodontic treatment must be analyzed in terms of their physicochemical and biological properties. In all techniques for obturation of the root canal system gutta-percha is the principal component, which has been observed to behave biologically as an inert material (Rasquin[19], 1997). Effective obturation depends on the endodontic cements which are fundamental in their ability to fill the irregularities between the gutta-percha and the walls of the root canals, reducing marginal microleakage. Due to their flow properties, cements integrate directly with the tissues of the root canal system and apical region (Cohen, et al.[6] 2000; Leonardo, et al.[12] 2000).

Faced with a physical microbial or chemical challenge, an acute inflammatory reaction is initiated, with vascular alterations, plasma and cellular exudates, principally of polymorphonuclear cells such as neutrophils. Subsequently, a chronic inflammatory infiltrate predominates with the presence of mononuclear cells, such as macrophages and lymphocytes (Catanzaro and Percinoto[5], 1984). The macrophages participate in defense mechanisms mediated by the presentation of antigens to T lymphocytes, phagocytosing foreign bodies and liberating various cytokines such as interleukins–1, alpha and beta (IL-1α and IL-1β), tumor necrosis factor (TNF), prostaglandins, free intermediaries derivatives of oxygen or nitrogen, for example hydrogen peroxide and nitric oxide (Ialent, et al.[9] 1992; Soares and Queiroz[25], 2001). It is important to emphasize that once the cytotoxic stimulus ceases, these cells start to participate in the process of repair mediating in the stimulation or production of growth factors. In this way, persistence of macrophage stimulation with the release of pro-inflammatory substances impedes complete periapical repair, as observed histologically. As a result, after debridement, shaping and intra-canal infection control, sealing must be undertaken with substances that minimize aggression or cellular stress. Therefore, since macrophages influence the processes of defense and repair, this study has the objective of evaluating the cytotoxicity of two endodontic cements, one based on calcium hydroxide and epoxy resin, and the other based on zinc oxide eugenol, with respect to the release of nitric oxide in cultures of peritoneal macrophages.

MATERIALS AND METHODS

This research study was approved by the Institutional Review Board of the Dental School of São Paulo State University at Araraquara (UNESP). Two cements were used in this study: Sealer 26 and Endofill. The Sealer 26 (Dentsply Industria e Comércio Ltda, Petrópolis-RJ-Brazil)) is based on epoxyamine. The powder component contains calcium hydroxide, bismuth oxide, tetramine hexamethylene and titanium dioxide. The other component contains epoxide resin (diglyceride biphenol ether A). The cement Endofill (Dentsply Industria e Comércio Ltda, Petrópolis-RJ, Brazil)

is composed of a powder containing zinc oxide, hydrogenated resin, bismuth subcarbonate, barium sulfate and sodium borate, and the liquid component containing eugenol and sweet almond oil. The cements were prepared according to the directions of the manufacturer and after hardening were processed by breaking and then sonically pulverizing (Vibra Cell,Sonics & Materials, Inc. Danbury, CT. USA). They were weighed on a precision balance (Mettler AJ150, Mettler-Toledo AG, Greifensec, Switzerland) and diluted in polyethylene glycol 400 resulting in a concentration of 18mg/mL and kept in 15-mL centrifuge tubes (Corning Inc.) for autoclaving. To obtain macrophages, 3.0mL of a solution of sodium thioglycolate (Difco, São Paulo, Brazil) was injected into the abdominal cavity of six Swiss male mice of 6 to 8 weeks age, weighing between 25 and 30 grams. The subsequent procedures were undertaken in a laminar flow chamber. Ninety-six hours after the stimulation/activation, the animals were sacrificed by asphyxiation, and after antisepsis of the abdomen with 0.3% iodated alcohol, the peritoneum was revealed. Then 5.0ml phosphate buffered saline (PBS), pH 7.2 (Difco), were introduced into the abdominal cavity using a sterilized disposable syringe and needle. After massaging the abdominal cavity, the contents were recovered with a syringe and needle, and transferred to a sterilized centrifuge tube, with a capacity of 15mL. The peritoneal washing of each animal was centrifuged (Hermle ZK380, B. Hermle AG, Hettich, Tuttlingeen, Germany) at 1500rpm for 5 minutes. After discarding the supernatant, 1.0ml of PBS was added to the cells, which was homogenized and re-centrifuged, a process that was repeated twice. The supernatant was discarded and the cells re-suspended in 1.0mL of RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO), containing 100U/mL of penicillin (Sigma), 100 µg/mL of streptomycin (Sigma), 2µg of L-glutamine (Sigma) and 5.0% fetal bovine serum (Cutilab, Campinas/SP, Brazil). Cell counts were undertaken in a Neubauer counting chamber, to obtain a final concentration of 5.0×10^6 cells/mL of RPMI-1640. Aliquots of 100µL of the suspension containing the cells were placed in sterile 96-well tissue culture plates (Corning, New York 14831, USA). After the addition of 100µL of each solution of cement to the tissue culture plates containing the macrophages, they were incubated for 48 hours, at 37^0C, in an atmosphere of 5.0% CO_2. In the final phase, 50µL of the supernatant of culture cells were removed and transferred to another plate. Then, 50µL of Griess reagent were as added and allowed to rest for ten minutes at room temperature, and then read using automatic ELISA (Organon, Reader 2001, USA) with a 540 nanometer filter. Each cement was experimented in quadruplicate as a function of each animal, and the mean release of nitric oxide obtained in micromoles. In the positive control group, endodontic cements were not used. This group containing the cellular suspension received addition of 100µL of a solution containing 10mg/mL of lipopolysaccharide (LPS) of *Escherichia coli* 026:B6 (Difco). The negative control group received only the cellular suspension. The results were expressed in micromoles (mmols) from a standard curve established in each test, made up of known micromolar concentrations of nitric oxide. The results were statistically analyzed using the analysis of variance and Tukey test with a level of significance established at $p < 0.05$.

RESULTS

The release of nitric oxide in micromoles (µmols) in the presence of Sealer 26 and Endofill are expressed in Tables 1 and 2 and in Figure 1. It can be seen that the production of nitric oxide by Sealer 26 varied from 36.1 to 313.0µmols, with a mean of 143.82µmols. For Endofill this value oscillated between 50.8 and 125.7µmols, with a mean of 80.33 µmols. Most nitric oxide was liberated in the presence of Sealer 26, which was statistically significant ($p=0.01$). In the positive control group, the release of nitric oxide varied from 88.46 to 386.22µmols, with a mean of 162.75µmols, and for the negative control this value varied from 0 to 10.45µmols, with a mean of 4.42µmols. There was no significant difference between the positive control group and cement Sealer 26 ($p>0.05$).

DISCUSSION

The response of apical and periapical tissue to sealant materials is the sum of individual reactions of each cellular group as well as reactions of the extracellular matrix. Based on the type of response in the short, medium and long-term, predictions can be made about the biocompatibility or tolerance of materials used. It is important to emphasize that among the different systems of evaluation of dental materials,

TABLE 1- Mean nitric oxide release in µmols of Sealer 26 cement in peritoneal macrophage culture obtained from six Swiss mice

Animal	1st reading	2nd reading	3rd reading	4th reading	Mean
1	43.5	38.2	30.3	32.4	36.1
2	31.3	29.7	31.3	191.5	71.0
3	429.0	304.3	262.5	256.2	313.0
4	58.9	47.1	41.6	49.2	49.2
5	246.7	155.2	144.2	130.2	169.0
6	278.3	197.8	197.8	224.6	224.6
				Mean	143.82

cell culture represents an initial stage of investigation, since the response of isolated cells is of considerable value in understanding the complex tissue response. For Leonardo, et al.[12] (2000), it should be treated as a relatively simple, low cost, relevant, reproducible and adequate methodology to evaluate the basic properties of dental materials. However, Azar, et al.[2] (2000) emphasize that this methodology has a number of limitations, for example: 1) in the majority of studies, the materials are evaluated after the hardening period during which there is a significant reduction of solubility and release of its components, while clinically, the recently mixed material is in direct contact with the apical and periapical tissues. 2) the dynamics of periapical tissue is not reproduced in tissue culture, for example the buffering capacity of plasma and the interaction of other cells with the material, as well as cell to cell interactions, 3) the progressive dilution of substances due to renewal of culture medium and 4) cell cultures are exposed to only short periods of evaluation, often 72 hours, after which the cells deteriorate.

Endodontic cements based on zinc oxide and eugenol have been most commonly used in clinical endodontics. However, with the objectives of improving physicochemical properties, developing adhesion to the dental structure, improving working time, mixing time and apical sealing, cements based on epoxy resin have been developed since the 1950's such as AH26 introduced in the United States in 1957, by Schroeder (Cohen, et al.[6] 2000). Since then, with a view to improve the biocompatibility of cements based on zinc oxide eugenol or resin, a new generation of hybrid cements have appeared resulting from addition of other components. As a result, Sealer 26 is a derivative of AH26, in which Berbet[3] (1978) added 20% calcium hydroxide PA, which is a formulation offering better biocompatibility, compared to AH26, and is notable because of its compatibility with the growth of new periapical cementum has been observed in dogs.

A pilot study was used to determine the concentration of endodontic cement necessary. Thus it was shown that in reduced concentrations, for example, 0.1mg/mL, posed insufficient stimulation to release nitric oxide; however, in elevated concentrations such as 180mg/mL, was lethal to all cells. Therefore a concentration was used that represented an intermediate value. In the present study, macrophages from the peritoneum of mice, in contact with similar concentrations of cements in suspension, produced larger quantities of nitric oxide in contact with Sealer 26, compared to Endofill. Similarly, many other *in vitro* studies have shown an increased cytotoxicity of resin based cements, principally AH26. For example Gerosa, et al.[8] (1995), reported that AH26 caused a greater degradation of gingival fibroblasts, while Pulp Canal Sealer caused less cellular death. Evaluating the cytotoxicity by measuring the release of chrome, Safavi, et al.[21] (1989) demonstrated greater cellular damage for AH26 than for Tubli-seal, which released a lower quantity of radioactive chrome. After mixing, Matsumoto, et al.[14] (1989) inserted a number of endodontic cements in polyethylene tubes, with an aperture equivalent to a #40 file, and placed them in cell cultures of rat pulp, containing radioactive thymine. They showed that AH26 completely inhibited the synthesis of DNA, while cements based on zinc oxide and eugenol had a moderate effect. The sealant Sealapex was shown to have the best cellular compatibility. In a study by Nakamura, et al.[17] (1986), suspensions of up to 20% of AH26 showed increasing epithelial cell death, as a function of the period of incubation, with percentages varying from 85.6% to 99.7%, in a period of 1 to 3 days, respectively. In contrast the cement Neodyne, which contains zinc oxide, calcium hydroxide and eugenol, revealed a reduced cytotoxicity, behaving in a similar way to the cement Canals, based on zinc oxide and eugenol. However, a cement containing zinc oxide, calcium hydroxide and formaldehyde showed behavior similar to AH26.

The cytotoxicity of AH26 has been attributed to the amine and to the epoxy resin bisphenol (tetramine hexamethylene).

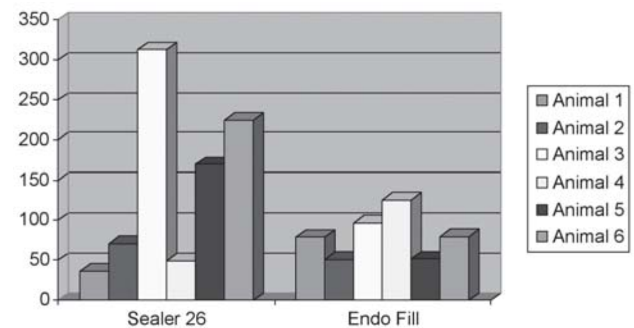

FIGURE 1- Nitric oxide release in micromoles, by Cement Sealer 26 and Endofill, in a peritoneal macrophage culture from Swiss mice

TABLE 2- Mean nitric oxide release in μmols of Endofill cement in peritoneal macrophage culture obtained from six Swiss mice

Animal	1st reading	2nd reading	3rd reading	4th reading	Mean
1	103.1	72.6	65.2	72.5	78.3
2	51.8	47.9	39.2	64.5	50.8
3	86.5	96.8	114.2	88.9	96.6
4	129.2	104.7	143.4	125.7	125.7
5	77.1	42.4	41.6	44.7	51.4
6	62.4	86.5	88.9	79.2	79.2
				Mean	80.33

The former undergoes decomposition during hardening, releasing ammonia and formaldehyde (Koulaouzidou, et al.[10] 1998; Leonardo, et al.[11] 1999; Spangberg, et al.[26] 1993). Schweikl, et al.[22] (1995) have shown, for AH26 cement, an elevated cytotoxic and mutagenic effect, which was greater for recently mixed material, and reduced significantly after a period of 1 to 7 days. Similar results were observed for the AH26 liquid, or in other words epoxy resin. However, it should be emphasized that the toxicity of AH26 is not only due to the release of formaldehyde during the hardening process.

After curing, cements based on zinc oxide eugenol release free eugenol, zinc oxide (ZnO) and zinc ions (Zn^{2+}), which are the principal causes of cytotoxicity (Azar, et al.[2] 2000; Arenholt-Bindslev and Horsted-Bindlev,[1] 1989), but this is less cytotoxic than tetramine hexamethylene, guaiacol or formaldehyde (Nakamura, et al.[17] 1986). Moreover, no positive correlation was found between the liberation of eugenol and the cytotoxicity of these cements (Safavi, et al.[21] 1989; Maseki, et al.[13]1991).

In our *in vivo* experiments, Sealer 26 has shown unfavorable effects on periapical repair. Thus, after vital pulp therapy in dogs, over a period of 180 days, Silva, et al.[24] (1997) showed absence of apical biological seal in 100% of the root canals sealed with this cement. In cases of pulp necrosis associated with periapical lesions, 270 days after sealing the root canals, Rasquin[19] (1997) showed a high failure level of biological apical sealing. Silva, et al.[24] (1997) emphasized that the occurrence of tissue necrosis with Sealer 26, was independent of the apical level of sealing. Similar tissue aggression was also shown in the subcutaneous connective tissue of isogenic BALB/c mice. Recently, Sacomani, et al.[20] (2001) made alterations to Sealer 26, removing bismuth trioxide, titanium dioxide and increasing calcium tungstate, salol and butyl phthalate, creating Modified Sealer 26. However, 180 days after vital pulp therapy in dogs, the modifications did not show histological improvements in their biological performance. The present study has shown, through the measurement of released nitric oxide, that cement Sealer 26 has a greater toxicity to peritoneal macrophages compared to Endofill. These results corroborate various *in vitro* studies that used other types of cells and methodologies to evaluate cellular response, as well as various *in vivo* studies demonstrating unfavorable periapical repair with the cement Sealer 26.

CONCLUSIONS

1- Using the technique of cell culture, peritoneal macrophages of mice produced variable quantities of nitric oxide in contact with endodontic cement Sealer 26 and Endofill.

2- Considering that cells in conditions of aggression or stress produce greater quantities of nitric oxide, the cement Sealer 26 showed greater toxicity than Endofill.

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