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Cytotoxicity of Root Canal Sealers on Endothelial Cell Cultures

Vagner José Medeiros Martins[1], Renata Ximenes Lins[2], Teresa Cristina Ávila Berlinck[2], Rivail Antônio Sérgio Fidel[2]

[1]UFJF - Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil
[2]UERJ - State University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Correspondence: Vagner José Medeiros Martins, Rua José Lourenço Kelmer, S/N, 36036-900 Juiz de Fora, MG, Brasil. Tel: +55-32-2102-3857. e-mail: vagner.jose@ufjf.edu.br

This study evaluated, *in vitro*, the cytotoxicity of six root canal sealers after 12, 24 and 72 h of contact time, using an endothelial ECV-304 cell line. The MTT assay was used for analysis of cell viability. Twelve specimens of each sealer were prepared and randomly assigned to 6 groups according to the commercial brands (n=4/time). A control group was also formed, which was not subjected to the contact with sealers. To assess the effects of sealers on endothelial cells, the specimens were placed in culture plate wells and incubated at 37°C with 5% CO_2 and 100% humidity. MTT assays were performed in quadruplicate after 12, 24 and 72 h of contact of the sealer specimens with monolayers. Statistical analysis was performed by two-way ANOVA with Bonferroni post-hoc test at a significance level of 5%. Analysis of absorbance in the experimental groups showed that GuttaFlow presented the lowest cytotoxicity, with a mean absorbance of 0.048, followed by Pulp Canal Sealer (0.038), Sealer 26 (0.038), Endo Densell (0.036) and Pulp Fill (0.035). The control group had a mean absorbance of 0.098. Based on the results, Endofill and GuttaFlow were the most and the least cytotoxic sealers, respectively.

Key Words: cell cytotoxicity, root canal sealers, cell culture, culture media, endothelial cells.

Introdution

Cytotoxicity is a complex *in vivo* phenomenon that can trigger a broad spectrum of effects from a simple cell death to metabolic aberrations with functional or route-specific changes (1,2).

Among the various recommended methodologies for assessing biocompatibility and cytotoxicity of dental materials at different levels of research, *in vitro* cell culture tests are part of the initial protocols most widely used by some researchers (3-7).

The first *in vitro* studies using cell cultures to assess the cytotoxicity of dental materials were carried out in the late 1960's (8). Cell culture refers to culture derived from scattered cells removed from the original tissue, a primary culture, or a cell line that had already been established in culture by enzymatic, mechanical or chemical disruption (9).

Permanent cell lines or primary cultures (e.g., gingiva, mucosa and pulp fibroblasts) can be used, but primary cultures are known to reflect more accurately *in vivo* situations despite being difficult to cultivate (10). ECV304 endothelial cells are obtained from the human umbilical cord vein. This line is characterized by a monolayered growth pattern, high proliferative potential, with no growth factor-specific requirement (11).

The MTT solution is a tetrazolium salt reduced to formazan by mitochondrial enzymes of viable cells proportionally to the dehydrogenase activity, and is defined as a 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide colorimetric assay. This method is applied to assess cell survival and proliferation and detects the signal produced by the activation of living cells. This is a low-cost procedure that yields results within 48 h (12).

Root canal sealers have a major role in avoiding apical percolation by sealing branches and providing improved filling adjustment to the irregularities found on the dentin/filling material interface. These materials must fill the dentinal tubules, closely adhere to the organic and inorganic phases of the dentin, destroy or neutralize microorganisms and their byproducts, induce new cementum formation and strengthen the root canal system. From this perspective, all sealers in use can be considered inappropriate (13).

In this study, the MTT assay was used to assess the *in vitro* cytotoxicity of Densell Endo, Pulp-Fill, Endofill, Sealer 26, Pulp Canal Sealer and GuttaFlow root canal sealers after 12, 24, and 72 h of contact with an endothelial ECV-304 cell line from the human umbilical cord veins.

Material and Methods

ECV-304 endothelial cells were obtained from the Microbiology and Immunology (DIMI) courses from the Medical Sciences School at the Biomedical Center of the State University of Rio de Janeiro, RJ, Brazil.

In compliance with ISO 10993-5:2009 standard "Biological evaluation of medical devices. Part 5. Test for in vitro cytotoxicity", cells were kept frozen at -70°C in a F12 medium supplemented with 5% DMSO and 95% fetal bovine serum (Gibco, Grand Island, NY, USA). For defrosting, cells were rapidly shaken in hot water bath at 37°C, while still in the stock medium (5% DMSO and 95% fetal bovine serum). The tubes were then washed with 70% ethanol and

placed in a laminar flow to ensure complete drying before being effectively opened. For cell culturing, the contents in the cryogenic tube were transferred to 25-cm^3 sterile bottles. Cell suspension was kept in an incubator (Shel Lab Laboratory; Sheldon Manufacturing, Cornelius, OR, USA) with temperature and pressure control in a humid setting at 37°C and 5% CO_2.

The first medium replacement was established at 24 h in order to remove the remaining DMSO. Cell passaging was performed after 48-h periods until confluent cultures could be obtained. The distribution of 100 μL of the medium containing cells in a concentration of 1.5 x 10^6 cells/mL *per* micro-plate well was done in order to culture cells from 96 wells, incubated at 37°C in the presence of 5% CO_2. After distribution, cells were kept at 37°C with 5% CO_2 for 48 h for experimentation.

In order to assess cell viability, an MTT cytotoxicity assay consisting of tetrazolium salt reduced to formazan by mitochondrial enzymes of viable cells was used proportionally to its dehydrogenase activity and defined as a 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide colorimetric assay (11).

Twelve test specimens were prepared for each sealer (3 mm diameter and 1 mm high) using six stainless steel devices (mold + extractor) specifically designed for this study. The devices were properly sterilized and filled with freshly mixed sealer according to each manufacturer's instructions.

After the setting time required for each sealer, the specimens were allocated to 6 experimental groups according to the commercial brands, and one control group that was not subjected to the contact with sealers. Immediately after fabrication, the test specimens were allocated onto Petri dishes, which were placed in a microwave oven for two 5-min cycles at maximum output for the sterilization process. Following this, the test specimens were exposed to ultraviolet light for a period of approximately 30 min for surface disinfection.

In order to assess the effect of sealers on the ECV-304 endothelial cells, test specimens were placed in culture plate wells containing 25 mg/mL suspension in a culture medium and incubated at 37°C with 5% CO_2 and 100% humidity. MTT reduction tests were done in quadruplicate at 12, 24, and 72 h using 96-well cell culture microplates. Samples were sequentially incubated 72, 24, and 12 h before analysis. This procedure was chosen so that all the samples could be analyzed at the same time.

For the cell viability test, the specimens were removed after an incubation period of 12, 24, and 72 h, and the cultures were washed with phosphate buffer saline (PBS) and incubated in a 1 mg/mL MTT solution in a DMEM medium for 1 h at 37°C with 5% CO_2. In each well, 200 μL isopropyl alcohol (Merck & Co. Inc, Whitehouse Station, NJ, USA) was added for formazan solubilization, and two aliquots of 100 μL alcohol containing solubilized formazan were removed from each well and transferred onto another microplate also containing 96 wells, and thus the number of samples doubled. The microplate was transferred to an ELISA spectrophotometer (Biorad Laboratories, Richmond, CA, USA) where the absorbance values were determined at 570 nm (A_{570}).

Two-way ANOVA test was used in combination with Bonferroni's post-hoc test to identify differences among groups and times, with a significance level of 5%.

Results

At the 12-h analysis the GuttaFlow had the least cytotoxicity with a mean absorbance of 0.055, followed by Sealer 26 (0.038). Pulp Canal Sealer and Densell Endo showed the same mean absorbance (0.031). Pulp Fill and Endofill showed the greatest cytotoxicity (mean absorbance 0.024 and 0.021, respectively) among all sealers. The control group had a mean absorbance of 0.158 (Fig. 1A).

At the 24-h analysis, GuttaFlow and Sealer 26 once again had the greatest mean absorbance (0.041 and 0.037, respectively) proving to be the least cytotoxic, followed by the Pulp Canal Sealer which had a mean absorbance of 0.035. Densell Endo and Pulp Fill, however, had similar cytotoxicity with mean absorbance of 0.033 and 0.032, respectively. The Endofill once again proved to be the most cytotoxic (0.026). The control group had a mean absorbance of 0.086 (Fig. 1B).

At 72 h (Fig. C), Pulp Canal Sealer had the least cytotoxicity with a mean absorbance of 0.049, followed by the GuttaFlow and Pulp Fill, both with a mean absorbance of 0.048. Densell Endo, Sealer 26, and Endofill had mean absorbance of 0.044, 0.040 and 0.036, respectively. Most important, the control group's mean absorbance (0.050) was close to those exhibited by the experimental groups.

As shown in Figure 2, the control group differed significantly from all groups at all times. The results showed the greatest cytotoxicity for Endofill while GuttaFlow had the lowest absorbance values. When compared over time, the experimental groups were found to behave differently in addition to having different absorbance values. It is worth noting that all groups tended to reach the same levels within 72 h due to the fact that they were near confluence.

When overall mean absorbance values for all groups were analyzed (Fig. 3), GuttaFlow was found to have the lowest cytotoxicity level, with a mean absorbance of 0.048. Pulp Canal Sealer and Sealer 26 were next with equal mean absorbance values of 0.038, followed by Densell Endo and Pulp Fill, with 0.036 and 0.035, respectively. The sealer with the lowest mean absorbance, and therefore the most

cytotoxic in the study, was Endofill (0.027). The control group had a mean absorbance of 0.098.

Discussion

Assessing cytotoxicity is important because it allows understanding the biological mechanism that produces the cytotoxic effect and the mechanism of action of different materials during material/tissue interaction. However, it is recognized that the test has its limitations. The use of cell cultures in monolayers is not physiological, and does not reproduce the *in vivo* tissue architecture in which underlying cells could repair surface aggression. The presence of an *in vitro* cytotoxic effect does not guarantee that the material is toxic when applied *in vivo*. On the other hand, absence of a cytotoxic effect ensures good clinical response.

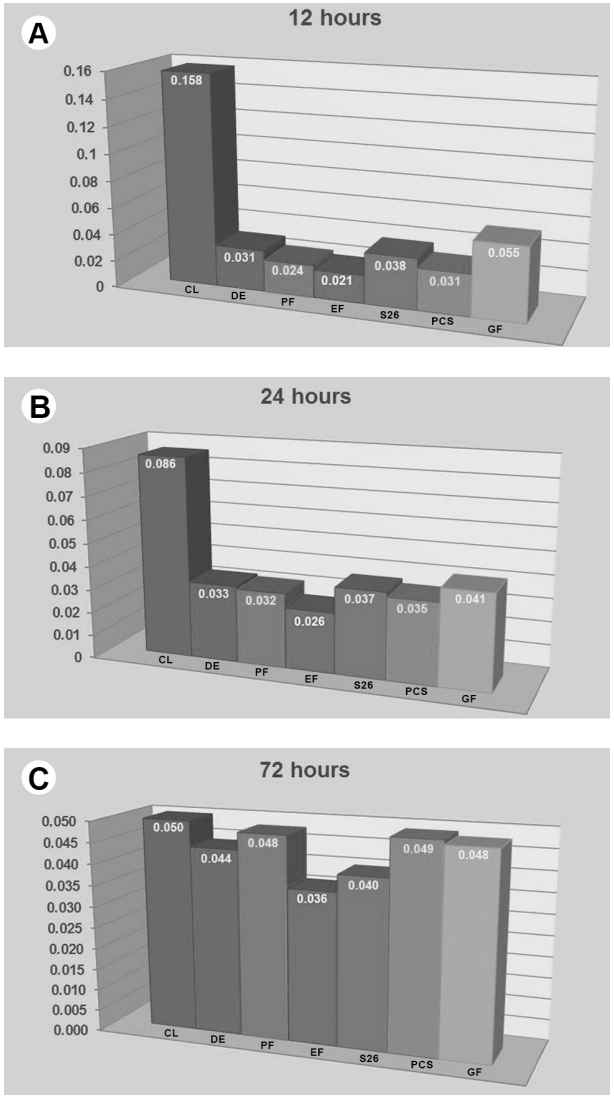

Figure 1. Mean absorbance values at 12 h (A), 24 h (B) and 72 h (C). CL: Control. DE: Densell. PF: Pulp Fill Endo. EF: Endofill. S26: Sealer 26. PCS: Pulp Canal Sealer. GF: GuttaFlow.

It should be noted that NCTC clone 929 is still used in cytotoxicity studies. Some authors (9) have described cell culture as a culture derived from scattered cells removed from the original tissue, a primary culture, or a cell line that had already been established in culture by enzymatic, mechanical or chemical disruption.

ECs are also an important source of pro-inflammatory mediators and vascular tone modulators, besides expressing adherence molecules and secreting/activating chemoattraction factors that control leukocyte draft. EC culture can demonstrate its specific capacity to produce endothelin and NO (14).

ECV-304 endothelial cells are obtained from the human umbilical cord vein, spontaneously immortalized, and are characterized by a monolayered growth pattern, high proliferative potential, with no growth factor-specific requirement (11). For this study, ECV-304 cells were used and cultured until confluent cultures at 37°C could be obtained.

Subculturing also involves removing the culture medium and dissociating monolayer cells with trypsin and/

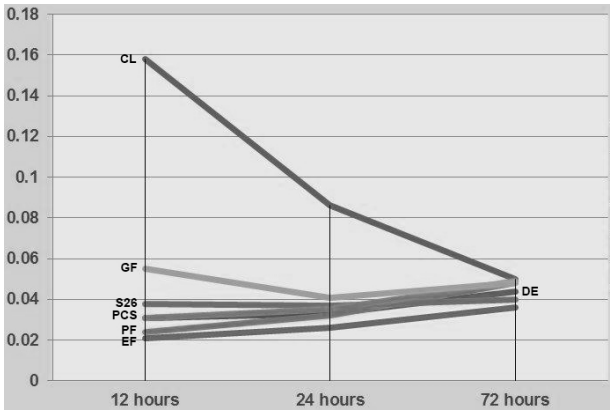

Figure 2. Mean absorbance values of the cements at the different time points. CL: Control. DE: Densell. PF: Pulp Fill Endo. EF: Endofill. S26: Sealer 26. PCS: Pulp Canal Sealer. GF: GuttaFlow.

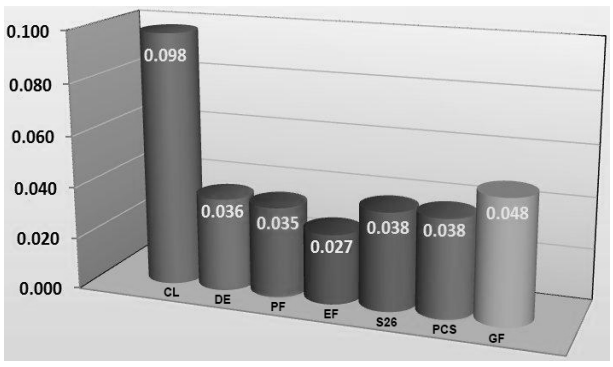

Figure 3. Overall mean absorbance values of the cements regardless of the evaluation time. CL: Control. DE: Densell. PF: Pulp Fill Endo. EF: Endofill. S26: Sealer 26. PCS: Pulp Canal Sealer. GF: GuttaFlow.

or EDTA. These substances were also used in this study (10 mL solution containing 0.1% trypsin and 0.01% EDTA at 37°C) for treating cells removed from cryogenic tubes and transferred to 25 cm^3 sterile bottles (13).

As reported by some authors (15), changes in culture and subculture media have varied intervals according to the used cell line. Therefore, the line should be properly considered when selecting a subculturing protocol, and choose the protocol that causes the least damage to the cells, as advocated by other authors. For this study, subsequent media changes were done after 48 h until a confluent culture could be obtained.

Cell culture techniques are widely used because they are cost-effective, relatively easy to maintain and require little physical space. Additionally, they can be used to prepare antigens, monoclonal antibodies and vaccines, as well as to isolate microorganisms, especially viruses, and to assess cytotoxicity in a wide range of products.

Cytotoxicity is a complex phenomenon that can result in a broad spectrum of effects from a simple cell death to metabolic aberrations with functional or route-specific changes (2,9). The definition of cytotoxicity in a trial may vary depending on the nature of the trial. Some experiments can determine a material as non-cytotoxic because it does not change cell proliferation, mitochondrial activity, or the DNA synthesis. However, the tested material may change some metabolic pathway, which the trial is unable to detect (1). The regulation document that standardizes *in vitro* cytotoxicity tests and selects the most suitable method is the ISO 10993-5 standard, as established in 1992 (9).

Benefits from *in vitro* toxicity tests when compared with animal experiments and clinical studies in humans include control of the experimental conditions, low cost, fast performance and absence of ethical issues (9,11).

In vitro tests can be done by using permanent cell lines or primary cultures (e.g., gingiva, mucosa, and pulp fibroblasts); however, primary cultures are known to reflect *in vivo* situations more precisely despite being difficult to culture (10). The first *in vitro* study using cell cultures to assess dental material cytotoxicity were carried out by Kawahara (8) and, according to most authors (1,4-7,9,10), *in vitro* cell culture tests are part of the most commonly used protocols within the recommended methodologies for assessing dental material biocompatibility and cytotoxicity at different levels of research.

This type of test is beneficial because it can be experimentally controlled and allows for methodological standardization, in addition to being fast, relatively inexpensive, simple and able to replace experiments on animals and human patients. Because they are carried out away from any contact with the organism, many complex interactions that mask biological response in the body are not present (1,9). In view of that, *in vitro* experiments were chosen for this study because, as pointed out by most authors, they are important in producing knowledge as well as enabling modifications in the substance structure before being used on animal and/or human models.

The latter categorization was chosen for this experiment where the test specimen was placed over confluent cells, as determined by some authors (16), with special emphasis on the changes that the material can cause to cells during the experiment. Other authors (17) have pointed out that direct contact of test specimens obtained from different materials may inhibit cell growth due to physical contact, not from the toxic substances that are released. For some authors (8), there is no standard time for cytotoxicity tests, since each material is cytotoxic at different levels. For this investigation, analyses were done by direct contact with non-water-soluble materials at 12, 24, and 72 h.

In vitro tests assess material properties directly in cell cultures that react to the effects of the products being analyzed. An example is the MTT assay. In this assay, the yellow MTT salt, which has a ring-shaped molecular structure, is absorbed by cells and cleaved by an enzyme inside the mitochondria, giving origin to a product named formazan in the form of purple-colored non-soluble crystals (12,18,19). To achieve this result, the identification of the color intensity of the solution is done by an ELISA spectrophotometer, with a high accurate reading in cytotoxicity studies (18). As previously outlined (12), this method assesses cell survival and proliferation, and detects the signal produced by the activation of living cells. The product that builds up inside the cell is extracted by adding an appropriate solvent and observing it with a digital spectrophotometer.

Placing material to be tested either directly or indirectly in contact with a cell culture is defined as a cytotoxicity test. Additionally, cell changes brought on by the material must be observed by taking into account the appropriate time. It is the understanding of the authors (16) that there are several methods for observing these changes like, for example, the incorporation of vital dyes or the inhibition of cell colonies.

An advantage of the MTT assay is that it can identify changes in metabolism and cell function from contact within cells and materials, even in the absence of dead cells (20). Additionally, it is a low-cost procedure that yields results within 48 h (12,13,18,19), and is therefore the most commonly used procedure for determining the cytotoxicity of different types of materials (20). Due to these characteristics and the endorsement by some authors (13,15,21-25), this assay was used in this study for assessing the cytotoxicity of all the six endodontic sealers.

For this study, test specimens were prepared after

handling sealers according to each manufacturer's instructions (11,14,22) and using devices (mold + extractor) specifically designed for the study and properly sterilized before use. For correct sterilization, test specimens were placed on a Petri plate and put in a microwave oven for two 5-min cycles at maximum output. For surface disinfection, test specimens were exposed to ultraviolet light (14,25), for approximately 30 h. However, some authors (14) used a 2-h period whereas others (25) used a 24-h period.

This type of incubation used in the present study to assess the effect of the sealers on ECV-304 cells has been used elsewhere (13,15,23-25). A previous study (13) also used ECV-304 cells that were incubated at 37°C in 5% CO_2 and 100% humidity for 7 days and cultured in an F12 medium supplemented with 10% fetal bovine serum.

In a previous study (23), confluent cells were enhanced with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. After a 24-h incubation period, cytotoxicity was assessed by an MTT assay. In a research by other authors (15), a subculture was conducted with confluent culture cells treated with 0.5 g/L/0.2 g/L EDTA in phosphate buffered saline (PBS). In this research, for ECV-304 cell culturing (ISO 10.993-5), the contents in the cryogenic tube was transferred to 25-cm^3 sterile bottles, washed with PBS, in phosphate buffer 0.1M pH 7.2, and treated with 10 mL solution containing 0.1% trypsin and 0.01% EDTA at 37°C.

The MTT assay was used to assess cell viability (12,13,15,18,20,22-25). The MTT reduction tests were performed in quadruplicate at 12, 24, and 72 h using 96-well cell culture microplates. Ninety-six wells were filled in order to have a safety margin, since only 84 wells would be analyzed. This procedure was chosen so that all the samples could be analyzed at the same time. The microplate was transferred to an ELISA spectrophotometer where absorbance values were determined at 570 nm (A_{570}).

The biological tests are important considering that materials used in the oral cavity should be nontoxic, non-absorbable by the circulatory system, and should not cause injuries to the oral tissues. Non-biocompatible materials may prove mutagenic or influence inflammation mediators causing systemic responses, including cytotoxic, teratogenic or carcinogenic effects. These materials must be free of agents that may cause allergic responses to individuals who are sensitive to these substances.

According to the categories in which dental materials are classified, different research protocols should be developed in order to evaluate and determine the biological behavior and possible indications for clinical use. For a dental sealer to be clinically successful, the whole system must be biocompatible. Therefore, tests are recommended to evaluate biocompatibility. They are a preliminary approach to assess a particular dental material and characterize its cytotoxicity profile in a specific biological system.

At the 12-h analysis, Pulp Fill and Endofill showed the highest cytotoxicity. GuttaFlow showed the lowest cytotoxicity, followed by Sealer 26, Pulp Canal Sealer, and Densell Endo. For the 24-h period, GuttaFlow and Sealer 26 continued to show lower cytotoxicity, followed by the Pulp Canal Sealer. However, Densell Endo and Pulp Fill had similar cytotoxicity. Endofill once again was found to be more cytotoxic then the other sealers. After 72 h, Pulp Canal Sealer showed the lowest cytotoxicity, followed by GuttaFlow, Pulp Fill, Densell Endo, and Sealer 26. However, Endofill remained the most cytotoxic.

Considering the mean absorbance values obtained at three time points, Endofill showed the highest and GuttaFlow the lowest cytotoxicity, respectively.

Resumo

Este estudo avaliou, *in vitro*, a citotoxicidade de 6 cimentos endodônticos após 12, 24 e 72 h de tempo de contato, utilizando-se uma linhagem de células endoteliais ECV-304. Para a avaliação da viabilidade celular, utilizou-se o teste de citotoxicidade MTT. Para cada cimento foram preparados 12 corpos de prova que foram distribuídos em 6 grupos experimentais de acordo com as marcas comerciais, sendo 4 para cada tempo. Foi criado um grupo controle que não foi submetido à ação de cimento. Para avaliação do efeito dos cimentos sobre as células endoteliais, os corpos de prova foram inseridos nos poços da placa cultura, incubados a 37°C em presença de 5% de CO_2 e 100% de umidade. Os testes MTT foram realizados em quadruplicata, após 12, 24 e 72 h de contato das amostras com o tapete celular. Foi utilizada a prova two-way Anova com o teste *post hoc* de Bonferroni com nível de significância de 5%. Quando analisadas as médias gerais de absorbância dos grupos analisados observou-se que o cimento GuttaFlow se apresentou como o cimento com menor índice de citotoxicidade, apresentando média de absorbência de 0,048. Logo após, apresentando médias de absorbância iguais (0,038) encontraram-se os cimentos Pulp Canal Sealer e Sealer 26; seguidos do Densell Endo e do Pulp Fill, com 0,036 e 0,035, respectivamente. O grupo controle apresentou média de absorbância de 0,098. Portanto, tendo como base os resultados obtidos, pôde-se concluir que o cimento Endofill foi o que apresentou maior citotoxicidade e o cimento GuttaFlow, o menos citotóxico.

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