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ORIGINAL ARTICLE

Cytotoxicity of a calcium aluminate cement in comparison with other dental cements and resin-based materials

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Abstract

The objective of this study was to compare the cytotoxic effects of a calcium aluminate cement with several currently used direct restorative materials. Specimens of three composites (QuiXfil, Tetric Ceram, Filtek Supreme), one zinc phosphate cement (Harvard Cement), one glass ionomer cement (Ketac Molar), and one calcium aluminate cement (DoxaDent), were used fresh or after 7-days' preincubation in cell culture medium at 37°C, pH 7.2. PVC strips for ISO 10993-5 cytotoxicity test were used as positive control and glass specimens as negative control. L-929 fibroblasts (5-ml aliquots, containing 3×10^4 cells/ml), cultivated in DMEM with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin at 37°C/5% CO_2 and trypsinized, were exposed to the specimens for 72 h. The cells were harvested, centrifuged, and resuspended in 500 µl DMEM and then counted in 500 µl DMEM for 30 s with a flow cytometer at 488 nm. The analysis of variance comparing the six materials showed different influences on L-929 fibroblast cytotoxicity ($p < 0.0001$). The cytotoxicity of all specimens diminished with increasing preincubation time ($p < 0.0001$). Fresh DoxaDent exhibited the lowest cytotoxicity, followed by QuiXfil. Ketac Molar showed the highest cytotoxicity. After 7 days of preincubation, Harvard Cement and Filtek Supreme demonstrated more cytotoxicity than the other materials ($p < 0.005$).

Key Words: *Biocompatibility, cell culture techniques, ceramics, dental materials*

Introduction

Restorative materials interact with the biological environment they are placed in [1]. Contributing factors for this interaction are for example the release of substances from the material before and/or after setting [2] or the surface characteristics of the material [3]. Adverse reactions may be clinical or subclinical and they can be toxic and/or allergic [4,5]. Tests developed for dental materials that are considered as medical devices are described in ISO 10993-5, ISO 7405, and the ADA guidelines for posterior restorations [6–8]. The first step when dealing with dental material toxicity is to analyze the toxic potential in cell culture systems; this provides a controllable and reproducible test by which to initially assess the biological response [4,9–11].

The popularity of posterior tooth-colored restorations has increased during recent years because of a growing demand for esthetics and concern about the biocompatibility of amalgam. This has been paralleled by an increasing number of reports describing occupational skin reactions among dental personnel caused by the uncured acrylic resins of the dental resinous materials [12–15]. The range of dental materials has been much diversified with the introduction of different intermediary materials between resin composite and glass ionomer cements. However, the use of non-resinous alternative materials such as the glass ionomer is limited to non-stress-bearing areas because of their relatively poor mechanical properties.

Recently, a calcium aluminate cement has been developed in Scandinavia intended for use in Class I, II, and V direct restorations [16]. The manufacturer claims that the material is a "bioceramic" alternative to amalgam and resin composite. The material is inorganic and non-metallic and thus meets the

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Table I. Investigated materials and manufacturers

Material	Manufacturer (lot no.)	Restorative	
DoxaDent	Doxa Certex AB, Uppsala, Sweden (SC017)	Calcium aluminate cement	Katoite [$(CaO)_3(Al_2O_3)(H_2O)_6$]
Harvard Cement-Powder, Liquid	Richter and Hoffmann Harvard Dental GmbH, Berlin, Germany (2122302005) (2121002001)	Zinc phosphate cement	A 100 g quantity of powder contains: 90 g of zinc oxide, 9 g of magnesium oxide. 100 g of liquid contains 53 g of phosphoric acid.
Ketac-Molar	3M ESPE Dental Products St. Paul, Minn., USA (117586)	Highly filled glass ionomer cement	Powder: calcium aluminum lanthan fluorosilicate glass, acrylic acid/maleic acid copolymer, pigments. Liquid: acrylic acid/maleic acid copolymer, tartaric acid.
Filtek Supreme	3M ESPE Dental Products St. Paul, Minn., USA (20030422)	Resin composite	Bisphenol A polyethylene glycol diether dimethacrylat (5–15w%), Diurethan dimethacrylat (5–15%), Bisphenol A diglicidyl methacrylat (1–10%), Triethylene glycol dimethacrylate (<5%), silica zirconia nanoclusters.
Tetric Ceram	Ivoclar, Vivadent AG, Schaan, Liechtenstein (E40866)	Resin composite	Bis-GMA 8.5w%, Urethane dimethacrylate 7.8w%, TEGDMA 4.4w%, Bariumglass silanized 49.5 w%, ytterbiumtrifluoride 17w%, mixed oxide, silanized 5w%, Ba-Al-Fluorosilicate glass, silanized 5%, high dispersed silica, silanized 1w%, additives 1.4w%, catalysts and stabilizers 0.3w%, pigments <0.1w%.
QuiXfil	Dentsply DeTrey GmbH Konstanz, Germany (0310000267)	Resin composite	BisEMA, UDMA, TEGDMA, TMPTMA, TCB, new patented filler 66vol% (86w%) camphorquinone, dimethylaminobenzoic acid ethyl ester.
Positive Control PVC Strips	Portex Ltd. Hythe, Kent, UK (30375)	–	

Bis-EMA, ethoxylated bisphenol-A-dimethacrylate.
Bis-GMA, bisphenol A -glycidyl methacrylate.
TCB, butane-1,2,3,4-tetracarboxylic acid, bis-2-hydroxyethyl methacrylate.
TEGDMA, triethylene glycol dimethacrylate.
TMPTMA, trimethylolpropane trimethacrylate.
UDMA, urethane dimethacrylate.

definition of a ceramic material. It is based on two essential constituents, alumina (Al_2O_3) and calcium oxide (CaO), and small amounts of ZrO_2, TiO_2, Fe_2O_3, and SiO_2. Fused together at high temperature, small particles of calcium-aluminates are formed that have a cement-forming potential. To start the hardening reaction the calcium aluminate tablets are brought into contact with the supplied liquid, which contains water and small amounts of Li^{2+} as accelerator. An acid-base reaction is initiated. Water acts as a weak acid and calcium aluminate dissolves to form Ca^{2+}, $Al(OH)_4^-$ and OH^-. The solutes precipitate to form a gel. Gradually, the amorphous gel changes into a crystalline phase of mainly katoite [$(CaO)_3(Al_2O_3)(H_2O)_6$]. The post-set hardening takes several days.

Hardness, wear, and surface characteristics of the calcium aluminate cement have been studied *in vitro*, showing that the material is hard and has a relatively low resistance to wear [17]. The cytotoxic effect of the material is not known.

It is hypothesized that the cytotoxic effect of the calcium aluminate cement is lower than the cytotoxic effects of the commonly used resin-based restoratives and dental cements. The aim of this study was to compare the cytotoxicity of the calcium aluminate cement with several currently used direct restorative materials.

Material and methods

The restorative materials studied, a calcium aluminate cement (DoxaDent), a zinc phosphate cement (Harvard Cement), a highly filled glass ionomer cement (Ketac Molar), and three resin composite materials (QuiXfil, universal shade; Filtek Supreme, A3; Tetric Ceram, A3) are described in Table I.

Manufacture of specimens

The materials were prepared in accordance with the manufacturer's recommendations. All except DoxaDent were filled into polyethylene blocks containing 5-mm diameter cylindrical holes standing on a glass plate. Cylinder height was 2 mm. The composite specimens were covered with Mylar and light-cured from one end (QuiXfil 20 s, Filtek Supreme 20 s, Tetric Ceram 40 s). Light curing was done with a Demetron Optilux curing light (Kerr Co, Orange, Calif., USA; light intensity 550 mW/cm^2).

A high frequency of fractures of the DoxaDent specimens was observed during removal from the 2-mm height molds, so polyethylene blocks containing 4-mm diameter cylindrical holes, standing on a glass plate, with a cylinder height of 4 mm, were used. In another series of experiments, it has been shown that specimens of the same materials with sizes different from those used in this study did not show statistically different cytotoxicity levels (unpublished data). A DoxaDent tablet was partially immersed in the supplied liquid and allowed to absorb the liquid for 5 s. Subsequently, the tablet was totally immersed in the liquid for another 5 s and then blot-dried on a piece of absorbant tissue before insertion in the mold. The material was packed with a condensing instrument (DoxaDent) under maximum hand pressure. The procedure was repeated with new tablets until the mold was full. The DoxaDent specimens were then covered with a wet paper towel to minimize desiccation.

The resin composite specimens were removed immediately after curing, while the cement specimens were removed 1 h after preparation. All specimens were sterilized with UV radiation 1 h after preparation for 40 min at each side [9].

Glass specimens of 5-mm diameter and 2-mm height were used as negative controls. PVC strips (Portex Ltd. Hythe, Kent, UK) for ISO 10993-5 cytotoxicity test [1] were used as positive controls. Fresh and 7 days' preincubated specimens of each material were prepared in triplicate. Experiments were repeated 6 times, resulting in 36 observations per material (18 for fresh and 18 for 7-day-old specimens).

Preincubation of specimens

Half of the specimens were added to the cultures immediately after preparation and sterilization. The other half were preincubated at 37°C, pH 7.2 for 7 days in cell culture medium (one specimen in 10 ml of Dulbecco's Modified Eagle Medium [DMEM; Sigma, Germany]) without agitation. The culture medium was then removed and the specimens were used for the experiments.

Culture of L-929 fibroblasts

The murine fibroblast cell line L-929 was obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA). L-929 cells were cultivated in Costar 162 cm^2 flasks (Cambridge, Mass., USA) in DMEM supplemented with 10% FCS (fetal calf serum; Seromed, Linz, Austria), 1% glutamine, and 1% penicillin/streptomycin at 37°C in a fully humidified air atmosphere containing 5% CO_2 and were passaged by trypsinization. Fibroblasts (5-ml aliquots, containing 3×10^4 cells/ml) were exposed to freshly prepared or preincubated specimens in polystyrene 6-well tissue culture plates (Costar) for 72 h at 37°C/5% CO_2. Cells were then harvested with trypsin (2.5% in Ca^{2+} and Mg^{2+} free Hanks balanced salt solution; JRH Biosciences, Ks., USA), centrifuged, and resuspended in 500 µl DMEM [9,10].

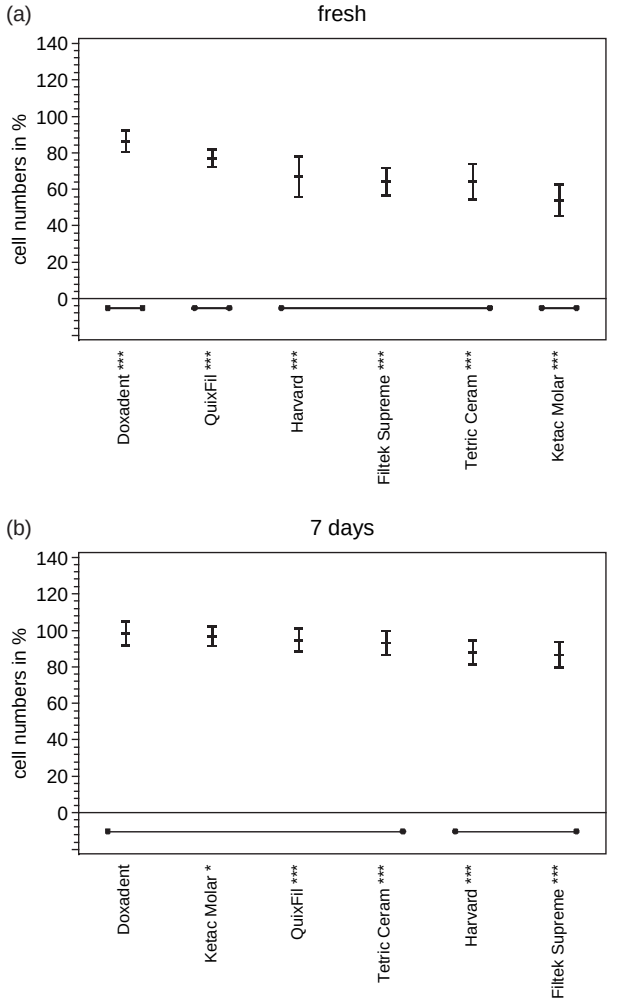

Figure 1. Grouping of materials with no different effects: (a) freshly prepared, (b) 7-day preincubated. Specimens were incubated with L-929 fibroblasts for 72 h and cell numbers determined by flow cytometry. Cell numbers were expressed as a percentage of controls (cultures with glass specimens). Vertical bars show ls-means $\pm$ standard deviations of 18 observations ($=6$ independent experiments with triplicates); materials covered with the same horizontal bar are not significantly different from each other. Stars indicate statistical significant difference to control (100%): $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. (Because of multiple testing, a p-value < 0.005 was considered to indicate statistical significance for this special question).

Flow cytometry

Cells were counted in a volume of 500 µl DMEM over a fixed time of 30 s with a flow cytometer (FACS Calibur; Becton Dickinson, San José, Calif., USA) equipped with an argon laser tuned at 488 nm. Cell numbers after exposure to test specimens were compared to controls (cultures with glass specimens). Cell density has been checked visually with an inverted microscope (Diaphot 300; Nikon Instruments Europe B.V., Badhoevedorp, The Netherlands).

Statistics

An analysis of variance was evaluated to explain the effects of materials and aging on cell numbers. In this model, materials and aging time (fresh and 7 days) were included as fixed factors. The interaction material with aging was also considered in the

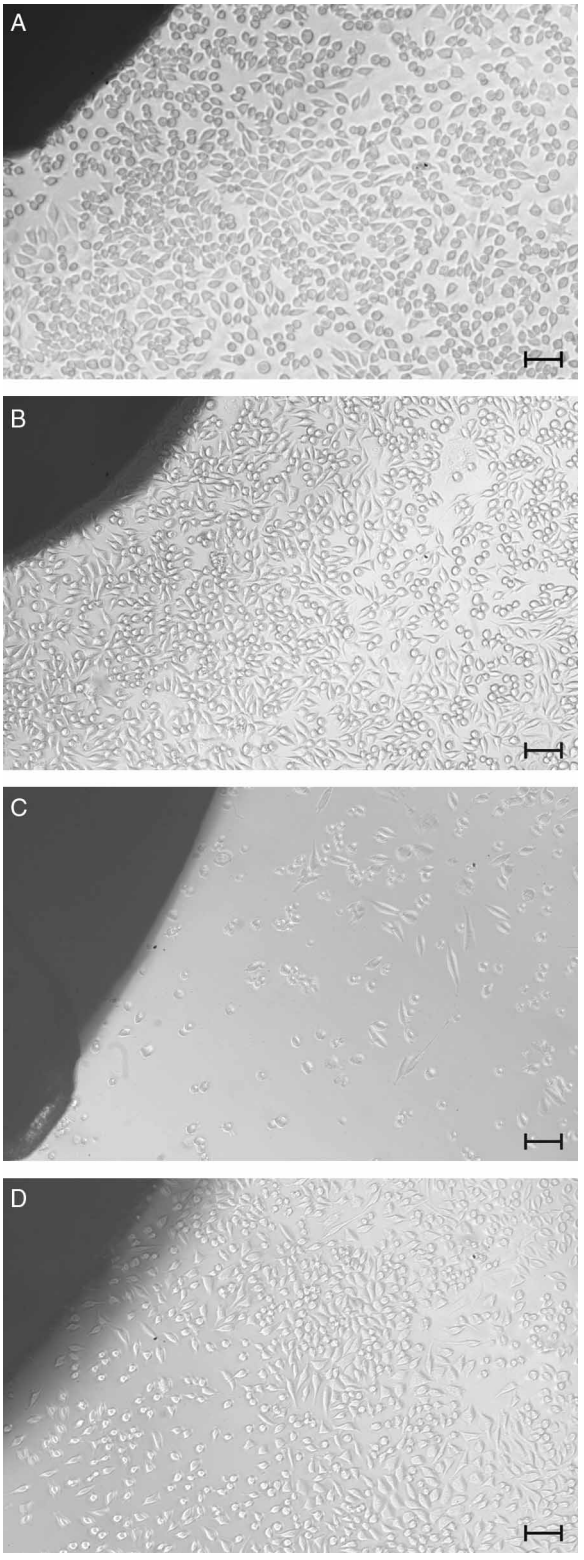

Figure 2. Effects of DoxaDent and Filtek Supreme on L-929 fibroblasts in culture. (A) DoxaDent, fresh, (B) DoxaDent, 7-days preincubated specimen, (C) Filtek Supreme, fresh, (D) Filtek Supreme, 7-days preincubated specimen. Cells were incubated with specimens as described in the Materials and Methods section (bar $=50$ µm).

model. For pooling the six materials tested, the approach developed by Ryan [18,19], Einot & Gabriel [20], and Welsch [21] was used both over aging and at each point of aging. The dependent variable was cell numbers in percent (=cell numbers/cell numbers of control [cultures with glass specimens]). The data of the positive control were not used in statistical analyses. A two-sided p-value <0.05 was considered to indicate statistical significance.

At each point of aging (0d, 7d), means were tested if they were significantly different from control (100%). For this special question, a p-value <0.005 was considered to account for multiple testing and control the probability to perform at least one type I error. All calculations were performed with SAS © Release 8.2.

Results

The effects of the materials on L-929 fibroblasts varied significantly ($p<0.0001$). Cytotoxicity of all materials diminished after 7 days of preincubation ($p<0.0001$). Materials showed different reductions of cytotoxicity after 7 days of preincubation (interaction material with time: $p<0.0001$). The validity of the trial was checked by a positive control which reduced cell numbers to 18.7 ($\pm$9.8%) of the negative control (glass specimens), as expected from previous experiments.

The results in Figure 1 demonstrate that all freshly prepared materials show reduced cell numbers compared to the negative control. A rank order of significantly different cytotoxic effects was established for fresh and 7 days' preincubated specimens (Figure 1a, b). Fresh specimens of DoxaDent exhibited least cytotoxicity, followed by QuiXfil and a group of three materials (Tetric Ceram, Filtek Supreme, Harvard Cement). Ketac Molar showed the highest cytotoxicity. After 7 days of preincubation, Harvard Cement and Filtek Supreme showed significantly more cytotoxicity than the other materials tested.

In cultures containing Filtek Supreme, an area of reduced cell density is seen in the near vicinity of specimens. This is shown for fresh and 7 days' preincubated specimens in Figure 2A, B, whereas this effect is more pronounced for fresh specimens. Fresh and 7-days' preincubated specimens of DoxaDent demonstrate full cell densities around the materials (Figure 2C, D).

Discussion

The biocompatibility of restorative materials has been assessed by *in vitro* and *in vivo* studies [9,22–27]. Cell culture systems provide a controllable and reproducible method to initially assess the cytotoxicity of a restorative [9,28]. These *in vitro* studies are relatively easy to implement and provide information about the mechanism of cellular toxicity, but the *in vivo* importance is difficult to predict [11]. In the current study, L-929 fibroblasts were used with a standardized test system. Many *in vitro* studies assessing the cytotoxicity of dental materials have utilized fibroblast cell lines, because these represent a common cell type in pulp and gingival tissues, and also because of their reproducible growth rates [6,9–11,28–30]. Owing to specimen size and corresponding culture volumes (6-well plates), cell counting is more accurate than other proliferation assays (e.g. ^{3}H-thymidine incorporation designed for 96-well plates) and flow cytometric analysis is quicker and more precise than counting in a hemocytometer [31]. Although it has been shown that different cytotoxicity test methods can produce different results for the same materials [9,31], the method applied in this study (cell counting with a flow cytometer) is well established for cytotoxicity testing of dental materials [9,10].

It has been shown that low cell numbers correspond to reduced cell densities in the vicinity of specimens [9]. This was confirmed in the present study (Figure 2).

In vitro studies have shown that the cellular toxicity of dental materials was related to leachables containing residual monomers and components such as degradation products or not reacted initiators, activators, and/or stabilizers [22,23,30].

We have no information about the biological risks of the calcium aluminate cement. The cytotoxic response may not be congruous with those of traditional posterior dental restoratives like resin composites and glass ionomer cements. These materials contain initially unbound components. In a clinical situation, these may be released through the dental tubules into the pulp or through the surface of the restoration into the oral cavity. The calcium aluminate cement consists of a high content of aluminate hydrates with unknown stability and cell response in contrast to the monomers, initiators, and stabilizers of resin composites. The fresh specimens of DoxaDent showed less cytotoxicity than specimens of the other restorative materials, but compared to the glass control there is still a reduction of cell growth. This might be due to a leakage of substances [32]. Although DoxaDent is slightly cytotoxic, it may be biocompatible in the dentin-pulp system, since substances released from the cement, i.e. calcium oxide and alumina together with ZrO_2, TiO_2, SiO_2, etc., are probably precipitated in the dentinal tubules with dentin fluid and may act as a "wound bandage" for pulp tissues. The DoxaDent specimens were made in different molds because of the difficulty obtaining acceptable specimens in the ordinary molds used for the other materials. In a recent study, we observed that the cytotoxicity of specimens with shapes as used in the

present study (5×2 vs 4×4 mm) were similar (unpublished observations).

The other two dental cements investigated, glass ionomer cement and zinc phosphate cement, showed significantly higher toxicity than DoxaDent. Several studies have demonstrated the cytotoxicity of glass ionomer cements [33,34]. Doherty [35] found that the toxic agent was possibly fluoride, which was effectively removed by an extraction method, and thereafter the glass ionomer cement was cytocompatible. Hanks et al. [28] showed that immediate-set glass ionomer cements are cytotoxic, whereas long-set (7 days) glass ionomers are not, which concurs with our results. Similar results have been obtained by others [33,34,36]. Extracts from the powder components of chemically cured glass ionomer cements were severely cytotoxic using MTT and NR assay. Fuji II powder contains polyacrylic acid, which has been shown to be seriously cytotoxic [37].

Zinc phosphate cement was included in this study because it is one of the most used dental cements, with a similar flexural strength as calcium aluminate cement [38]. It is traditionally applied as luting material or temporary restorative. It has been shown that the cytotoxicity of phosphate cements is attributed to the release of zinc ions, the acidity of the materials and the release of other chemical substances [28,39–41].

The hardening reaction of zinc phosphate cements and glass ionomer cements is like that of the calcium aluminate cement, based on an acid-base reaction; the cements therefore bear more similarities with each other than with resin composite materials. De Souza Costa et al. [23] suggested that the early cytotoxic effects of dental materials could be provided by the acidity of the materials. The calcium aluminate cement shows a high pH during setting, while the other cements are acidic immediately after mixing, approaching neutral pH after 24 h [42,43]. We therefore measured the pH changes in DMEM with freshly prepared specimens of DoxaDent, QuiXfil, Ketac Molar and Harvard Cement. Independently of the dental material chosen, the pH was stable with only minor variation (unpublished observations). The buffering system in DMEM seems to be efficient and therefore the effect of the pH changes on the fibroblasts in the present study is limited.

The cytotoxic potential of various other dental resin composites and compomers was recently demonstrated with the same standardized cell culture system. The results of these studies are in line with the initial cytotoxicity found in the present study [9,10,30]. In this study, the composites were more cytotoxic than DoxaDent (QuiXfil 77% [mean of cell numbers as percentage of control], Tetric Ceram 64%, Filtek Supreme 65%, DoxaDent 87%). In previous similar investigations, resin composites with less toxicity have also been found [10]. Tetric Ceram was chosen as an established composite ("gold standard") along with two relatively new resin composites. QuiXfil is a material which can be cured more quickly because of its high translucency, and the low cytotoxicity can be due to a higher conversion rate. Filtek Supreme is a composite based on nanofiller technology containing the same monomers as most of the commonly used resin composites.

The initial cytotoxic effects of all materials investigated diminished after 7 days of preincubation. Filtek Supreme and Harvard Cement were clearly more cytotoxic than the other materials.

The high initial effect could be due to the fact that the leakage of substances from the materials occurs mainly during the first days [44,45]. The trend toward decreasing cytotoxicity by time of resin composites, as found in the present study, is confirmed in earlier investigations [9,10,30]. In contrast to the cell culture tests, where non-aged materials were studied, no differences in gingival crevicular fluid cytokine levels of IL-1 were observed in contiguous aged Class V restorations of calcium aluminate cement, and resin composite and enamel. In general, the concentrations of cytokines were low [46].

The issue of biocompatibility of a dental restorative has to be considered on different levels. The guidelines for posterior restoratives of the American Dental Association and American National Standard Institute (ADA/ANSI) recommend biocompatibility testing of dental materials in order to obtain full clinical acceptance [47]. "Initial", "secondary", and "usage" tests are included. As initial tests, cytotoxicity tests at the cellular level are mostly employed, indicating the early cytotoxic effects of the materials. In the usage tests, the restoratives can be evaluated in longitudinal follow-ups in order to observe their durability. A "biomaterial" was recently defined in a working consensus conference as a non-viable material used in a medical device, intended to interact with biological systems. "Biocompatibility" of the material is its ability to perform with an appropriate host response in a specific situation [1]. For a dental material this means that besides acceptable low initial reactions, the restorative should also show acceptable durability. The poor mechanical properties of the calcium aluminate cement material, expressed by a low flexural strength, resulted in an unacceptably high fracture rate of Class II restorations in a 2-year follow up [16]. Despite the favorable results of DoxaDent in cell culture and its possible ability to act as a "wound bandage" for pulp tissues, the overall biocompatibility of DoxaDent is not acceptable due to its low durability. Conversely, the positive biological properties of DoxaDent suggest further developing this material to overcome its low mechanical properties.

In conclusion, the fresh DoxaDent specimens showed the lowest toxicity in the cell culture medium, directly followed by QuiXfil, whereas Ketac Molar exhibited the most pronounced cytotoxicity. All materials showed diminishing toxicity after the 7-day period.

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