Cytotoxicity of current adhesive systems: *in vitro* testing on cell culture of L929 and balb/c 3T3 fibroblasts

Citotoxicidade de sistemas adesivos atuais: teste *in vitro* em cultura de células fibroblásticas L929 e balbc 3T3

Abstract

Purpose: The aim of this study was to evaluate the cytotoxicity of three current adhesives: Prime&Bond NT (PBNT), Single Bond (SB) and XENO III (XENO).

Methods: After embedding and curing circles of filter paper with the tested adhesives, the filters were placed in contact with the solidified agar surface over L929 monolayer cells plated in 6-well cell culture plate and incubated for 24 h. The inhibition zone around the filter papers was measured in mm. MTT assay was performed using fibroblasts Balb/c 3T3 cell lines in multiwell culture plates. All assays were done in triplicate.

Results: All materials were cytotoxic (Kruskal-Wallis, $P<0.05$) in a similar level to latex ($P>0.05$). For intra-groups analysis, SB presented the lowest cytotoxicity ($P<0.01$), while there was no statistical difference between PBNT and XENO ($P>0.05$). MTT assay confirmed the cytotoxicity of the tested adhesives.

Conclusion: Considering the limits of this work, all adhesives tested were as cytotoxic as latex.

Key words: Cytotoxicity tests; adhesives; fibroblasts; cell culture; *in vitro*

Resumo

Objetivo: O objetivo deste estudo foi avaliar a citotoxicidade de três adesivos: Prime & Bond NT (PBNT), Single Bond (SB) e XENO III (XENO).

Metodologia: Após embebição e polimerização de filtros de papel com os referidos adesivos, estes foram colocados em contato com a superfície de agar solidificada sobre a monocamada de células L929 plaqueadas em cultura celular de 6-poços e incubadas por 24 h. A zona de inibição formada ao redor dos filtros de papel foi medida em milímetros. Outro teste realizado foi o do MTT, utilizando fibroblastos Balb / c 3T3 em placas de multi-poços, sendo os ensaios realizados em triplicatas.

Resultados: Todos os materiais testados foram citotóxicos (Kruskal-Wallis, $P<0,05$) e semelhantes ao látex ($P>0,05$). Para a análise intra-grupos, o SB apresentou a mais baixa citotoxicidade ($P<0,01$), enquanto não houve diferença estatística entre PBNT e XENO ($P>0,05$). O ensaio de MTT confirmou a citotoxicidade dos adesivos.

Conclusão: Considerando as limitações deste trabalho, todos os adesivos testados foram tão citotóxicos quanto o látex.

Palavras-chave: Testes de citotoxicidade; adesivos; fibroblastos; cultura de células; *in vitro*

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Introduction

A constant concern in Operative Dentistry is to prevent pulp injuries during restorative procedures. Some authors have demonstrated that most pulp alterations were related to bacterial contamination and their products arising from the microleakage, the major cause of failure in pulp capping (1). One of the important roles of the adhesive systems is to seal the tooth/restoration interface to prevent microleakage, decreasing postoperative sensitivity, marginal staining and, consequently, recurrent caries (2). Also, these materials could be used in direct and indirect pulp capping (3).

In spite of their wide and successful application in pulp capping, calcium hydroxide-based liners do not provide long-term protection against microleakage and the formed dentin bridge is not perfect. Because these materials may dissolve within 1-2 years (4) and do not bond to dental structure, researchers have investigated the use of dentin adhesives as pulp capping materials. Etch-and-rinse adhesives have not shown good results, as a moderate and persistent inflammatory response can occur, leading to the lack of dentin bridge formation (5). Their different compositions and the sensitive application technique led the authors not to recommend them for pulp capping.

Several self-etch adhesive systems have been introduced in the dental market. They do not require etching before its application, which simplifies the adhesive restorative procedures, and there is evidence that the clinical performance of a self-etch adhesive may remain excellent for years (6). An *in vitro* study (7) showed that an etch-and-rinse adhesive system was more cytotoxic than a self-etch adhesive. Results of *in vivo* studies (8,9) also indicated less inflammatory response in direct pulp capping with self-etching adhesives, with dentin bridge formation in some cases.

Cytotoxicity tests have been widely performed to assess the severe cytotoxic effect of different dentin adhesives (7,10-13). The International Standards Organization (ISO10993-5, 1992) classifies the cytotoxicity assay as the first step in the sequence of biocompatibility tests**.** The cell contact with the material can be direct (14) or indirect by means of diffusion in agar or through a Millipore filter (10). The agar acts as a cushion to protect cells from mechanical damage and allows the diffusion of leachable components of the polymeric specimens (15). Besides, another biocompatibility assay is the MTT, which includes the indirect extract of materials in cell culture medium.

It should be emphasized that comparative data on cytotoxicity of current self-etching and etch-and-rinse adhesives are limited (7,8,16). Consequently, the aim of the present study was to assess the cytotoxicity of a self-etch adhesive in comparison with two etch-and-rinse adhesives.

Methods

Test materials

The main characteristics of the test materials are listed in Table 1. PBNT and SB are etch-and-rinse adhesive systems with different solvents: acetone and ethanol/water, respectively. XENO is a self-etching adhesive with a modified smear layer primer.

Cells

L929 fibroblasts (ATCC cell line CCL 1, NCTC clone 929) were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin, 0.1 g/mL fungizone). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 for 24 h, until a monolayer, with greater than 80% confluence, was obtained. They were detached using a mixture of 0.125% trypsin and 0.025% ethylenediaminetetraacetic acid (EDTA), and transferred to a new culture flask until confluent monolayers were re-obtained. The cells were plated at $3x10^4$ cells/cm^2 in 6-well plates and maintained for 48 h in a humidified incubator with 5% CO_2 at 37 °C, to obtain a monolayer cell growth.

Balb/c 3T3 fibroblasts (clone A31, American Type Culture Collection) were cultured in DMEM and supplemented with 10% fetal calf serum (FCS) containing $NaHCO_3$ (1.2 g/L) and antibiotics (0.025 g/L ampicillin, 0.1 g/L streptomycin) at 37 °C in atmosphere of 5% CO_2. The assay was performed using 96-well plates, by plating Balb/c 3T3 cells at a concentration of $1.5x10^4$ cells/well.

Table 1. Product information.

Trade Name/Manufacturer	Etchant	Composition*	Lot #
Prime & Bond NT (PBNT) Dentisply De trey Konstanz, Germany	36% H_3PO_4	UDMA, PENTA, Resin RS-62 1 T-Resin, Polyacrylic acid, CQ, acetone, fluor	0504000268 OP. 494615
Single Bond (SB) 3M ESPE St Paul, MN, USA	35% H_3PO_4	Bis-GMA, HEMA, CQ, Polyalkenoic acid, Ethanol/water	SFG
XENO III (XENO) Dentisply De trey Konstanz, Germany		Bottle A: HEMA, purified water, ethanol, BHT, highly dispersed silicon dioxide Bottle B: Pyro-EMA, PEM-F, UDMA, BHT, CQ, ethyl-4-dimethylaminobenzoate	0412000452

* Composition description according to manufacturers. Abbreviations: UDMA urethane dimethacrylate, Bis-GMA bis-phenol A-glycidyl dimethacrylate, CQ canphorquinone, HEMA hydroxyethyl methacrylate, BHT butylated hydroxyl toluene, Pyro-Ema tetra-methacryl-ethyl-pyrophosphate, PEM-F penta-methacryl-oxy-ethyl-cyclo-phosphazenmonofluoride.

Agar diffusion test

After autoclave sterilization (121°C/30 min), circles of filter paper (nº 5, Whatman cellulose filters, England), 6-mm in diameter, were embedded with the adhesive systems and photocured according to the manufacturers' instructions. The light activator was the Curing Light XL 1000 (3M-ESPE, St Paul, MN, USA), with irradiance of 580 mW/cm^2, monitored with a radiometer (Demetron, Danbury, CT, USA). The embedded filters were placed in contact with the center of the solidified agar surface, over L929 monolayer cells, in 6-well cell culture plates, being three wells reserved for positive (latex), negative (PVC) and blank control. After incubation for 24 h at 37 °C, the inhibition zone around the filter papers was measured, as neutral red is free from dead cells (Fig. 1). The biological reactivity of the adhesive systems was scored from 0 to 4 in accordance with the criteria described in Table 2. Morphological analysis considered the cell integrity under and around the controls and the sample circles by inverted light microscopy. All assays were performed in triplicate for each adhesive system.

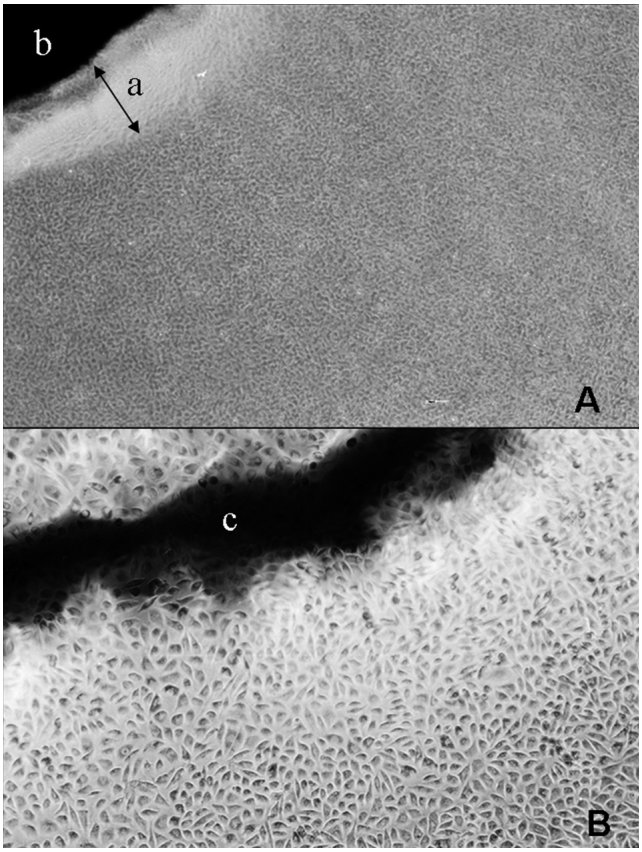

Fig.1. (A) Neutral Red uptake by filter paper embedded with SB + L929: cell appearance 24hs after adding an adhesive (light inverted microscopy, original magnification x4). Note the inhibition zone (a) around the filter paper (b); (B) Negative Control: PVC + L929: cell appearance 24hs after adding the PVC (light inverted microscopy, original magnification x10). Note the viable cells under and around the PVC (c).

Table 2. Biological reactivity for agar diffusion test.

Scores	Reactivity	Description of Reactivity Zone
0	none	No detectable zone around or under specimen
1	slight	Some malformed or degenerated cells under specimen
2	mild	Zone limited to area under specimen
3	moderate	Zone extends 5 to 10 mm beyond specimen
4	severe	Zone extends greater than 10 mm beyond specimen

MTT Assay

Cell viability was analyzed by the colorimetric assay described by Mosmann in which the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) is reduced to formazan blue only by viable mitochondria. For the extract preparation, 1 g of each adhesive, photocured for 20 s, and 10 mL of DMEM were incubated for 24 h without agitation at 37 °C in atmosphere of 5% CO_2. Then, the extracts were diluted in different concentrations (6.25 – 100%, ISO 10993-5, -12) and added to the 96-wells plates with DMEM and 10% FCS. After incubation for 24 h, the culture medium containing the extract was removed and the cells were rinsed with PBS-A (phosphate buffer saline) and treated with trypsin. Then, 0.5 mg of MTT/mL of DMEM was added to each well and the plates were incubated in a CO_2 incubator for 4 h. The MTT was aspirated and intracellular formazan crystals were solubilized with dimethyl sulfoxide and the absorbance of each 96-well plate was determined at 550 nm using a spectrophotometer. The mitochondrial function was then calculated, as a percentage of the control group (without any extract), considered as 100%. All assays were performed in triplicate (17).

Statistical Analysis

The statistical analysis of the agar diffusion test was performed by Kruskal-Wallis test to disclose differences among the groups. Next, each pair of the test materials was compared using a Mann-Whitney test. Statistical significance was determined at $P \leq 0.05$. MTT assay data was submitted to one-way analysis of variance (ANOVA) and Tukey's test if $P < 0.05$.

Results

All adhesive systems were severely cytotoxic, as the inhibition zone extended further than 10 mm from the specimen (Table 2), showing similar pattern as the positive control (latex, $P < 0.05$) (Fig. 2). SB was significantly less cytotoxic ($P < 0.01$) than PBNT and XENO adhesive systems, which were not statistically different.

MTT assay confirmed that all adhesive systems were highly cytotoxic ($P < 0.05$). XENO, PBNT and SB extracts (6.25%) promoted reduction of approximately 75% of the cell number (Fig. 3).

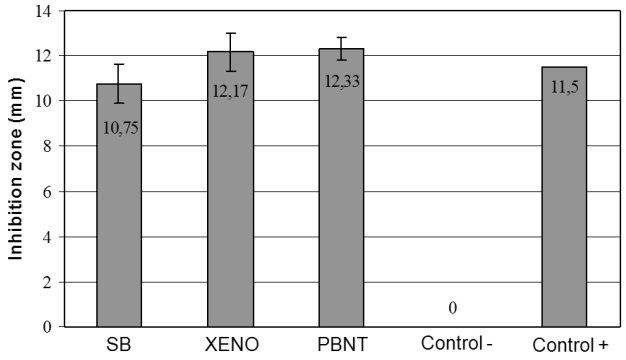

Fig. 2. Inhibition zone (mm) for all adhesive systems and controls

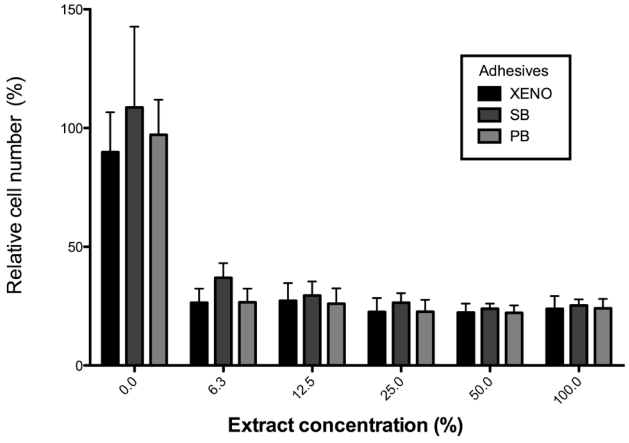

Fig. 3. Percentage of cell viability for three adhesive systems as a function of the extract concentration.

Discussion

Dentin adhesive systems often are used in deep cavities and their components, such as monomers, acids, or solvents, may pass trough the dentinal tubules before and after adhesive curing, particularly when the dentin permeability is high, causing pulpal irritation (10). The main goal of this study was to determine the immediate toxic action of current self-etch and etch-and-rinse adhesive systems applied on L929 and Balb/c cells. Previous works used these materials in diluted solutions (11) or just some components as HEMA (18). In the present study the materials were prepared following the manufacturers' instructions, similarly to a clinical situation, and extracts of the adhesives were obtained in different concentrations.

The L929 cell line is a established substrate and has been commonly used for cytotoxicity evaluation of biomaterials (2,7,14,19). In a previous study, toxic substances showed similar results on L929 fibroblasts and human gingival fibroblasts, indicating that L929 fibroblasts assays may represent sufficient screening models for *in vitro* evaluation of cytotoxicity (20). The technique using autoclaved and embedded filter papers with adhesive systems is convenient and reliable to determine the cytotoxicity potential and to investigate substances released from adhesives (12,21). Direct cell contact with the adhesive should be avoided as the heat coming from the exothermic polymerization reaction and from the light source may be harmful to the cells (21).

In the present investigation, all materials proved to be cytotoxic (score 4, Table 2) to cell culture, in agreement with previous *in vitro* and *in vivo* studies (2). Substances leached from these dentin adhesive materials may be responsible for the cytotoxic effects. The photoinitiator CQ (camphorquinone) is a cytotoxic (22) and mutagenic agent (19), and could explain the cytotoxic effects of the adhesive systems. Moreover, the degree of conversion of the composites as well as the adhesives systems is not complete, and monomers can be released from the incompletely polymerized material and the oxygen-inhibited surface, diffusing through the dentin to the pulp. Bond substances that are not polymerized seem to increase cytopathogenic effects (23), supporting the hypothesis that leached monomers are responsible for the adverse effects of adhesives on cell cultures. The light intensity was checked with a radiometer before the polymerization procedure, as the low intensity light may interfere with complete polymerization.

The present results showed that the cytotoxic effect of the tested adhesives was different. The SB adhesive was less cytotoxic to the L929 cells than PBNT and XENO, which were statistically similar. A recent study (24) also showed similar biological compatibility with human pulps for PBNT and XENO, when non-exposed pulp cavity restorations were performed. Other studies reported that the monomeric composition (Bis-GMA, UDMA, HEMA) of dentin adhesive agents may influence their cytotoxicity. In a recent study (18), GSH depletion was dependent on the number of methacrylate groups in resin monomers. GSH provides an antioxidative effect (25), and its depletion may impair protective capacity of the cell against toxic substances, leading to cell death (26). In that study, HEMA, with only one methacrylate group, caused a significantly lower depletion of GSH than TEGDMA and UDMA. Although SB contains Bis-GMA, with two methacrylate groups, it showed the lowest cytotoxic effect.

The cytotoxicity depends on the kind of the resin components and their molecular weights. HEMA, a lower molecular weight monomer, was shown to be less toxic than UDMA or TEGDMA and much less toxic than Bis-GMA (13). However, cytotoxicity is not influenced only by the individual components, but also by the synergistic or antagonistic interactions between them (13). A bonding agent that contained Bis-GMA was less cytotoxic than the other bonding agents that contained Bis-GMA+UDMA+HEMA and Bis-GMA+HEMA (11). In addition, it was demonstrated that a combination of Bis-GMA and HEMA was less cytotoxic than a combination of the three substances Bis-GMA, UDMA and HEMA (16).

Moreover, the dentin bond adhesives tested in the present study were either ethanol- or acetone-based. The ethanol-based material SB showed less cytotoxicity than the acetone-based material PBNT. However, the ethanol-based material XENO also showed poor results. Also, in a previous study no clear connection between solvent and cytotoxicity was found (16), as acetone-based materials PBNT and Syntac Sprint (Vivadent) showed the best and the poorest results, respectively.

The phosphoric acid-modified polymethacrylate resin did not have a negative effect on cytotoxicity, whereas the self-etching adhesive XENO did not show more cytotoxicity than PBNT. Szep et al. (16) also found that the pyrophosphate of the adhesive Etch&Prime 3.0 did not lead to more cytotoxicity. Another study (7) showed that total-etching bond systems were more cytotoxic to the cells underneath dentin discs than self-etching adhesive systems. This result may be explained by the fact that the above-mentioned study applied the adhesives on dentin discs and not directly on the cell culture. As these self-etching adhesives modify the smear layer, less toxic substances could penetrate through the dentin discs to the cells underneath. For the total etching bond system, the dentin etched with phosphoric acid has a higher permeability, allowing the toxic substances to penetrate (7).

As suggested previously (10), the cytotoxic effects caused by adhesives also depend on dentin permeability and residual dentin thickness. Thus, cytotoxic components of the adhesives and/or composites do not cause damage to all adhesive restorations and clinical cases. Recently, a long-term follow-up study (27) on composite restorations showed pulp complications in only 3.7% of the cases, which had indication for pulpectomy due to inflammation. It should be emphasized that these restorations were made in deep cavities with pulp protection. So, those authors concluded that the absence of pulp protection was not responsible for long-term pulp complications even in deep cavities with adhesives, specifically self-etching ones. Another study (28) also reported high success rates for adhesive restorations made in deep cavities without pulp protection. Dentin bridge formation was present in only few specimens, but could also be observed in direct pulp capping with adhesives (8,9). In spite of these considerations, direct pulp capping with adhesives may lead to pulp inflammation, infection, and necrosis (4,5,8), and a more conservative approach should avoid this procedure until definite biocompatibility evidences are available.

Conclusions

Within the limitations of this study, the results suggest that the tested adhesive systems had remarkable cytotoxic effects on cultured L929 and Balb/c cells similar to latex. Further *in vivo* and *in vitro* studies should be undertaken to clarify the cytotoxic effects of these materials in clinical procedures and to better address which is the toxic component in the materials formulation.

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