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Transdental cytotoxicity of experimental adhesive systems of different hydrophilicity applied to ethanol-saturated dentin

Luciana Bianchi,

UNESP – Univ. Estadual Paulista, Araraquara School of Dentistry, Department of Pediatric Dentistry and Orthodontics, Araraquara, SP, Brazil

Ana Paula Dias Ribeiro,

UnB – University of Brasília, Department of Dentistry, Brasília, DF, Brazil

Marcela Rocha de Oliveira Carrilho,

UNIBAN - Universidade Bandeirante de São Paulo, School of Dentistry, Department of Biomaterials, São Paulo, SP, Brazil.

David H. Pashley,

GHSU – Georgia Health Sciences University, College of Dental Medicine, Department of Oral Biology, Augusta, Georgia, USA.

Carlos Alberto de Souza Costa, and

UNESP – Univ. Estadual Paulista, Araraquara School of Dentistry, Department of Physiology and Pathology, Araraquara, SP, Brazil

Josimeri Hebling

UNESP – Univ. Estadual Paulista, Araraquara School of Dentistry, Department of Pediatric Dentistry and Orthodontics, Araraquara, SP, Brazil

Abstract

The aim of this study was to evaluate the transdental cytotoxicity of experimental adhesive systems (EASs) with different hydrophilicity and dentin saturation solutions (ethanol and water) on odontoblast-like cells. One hundred 0.4-mm-thick dentin discs were mounted in in vitro pulp chambers and assigned to 10 groups. Odontoblast-like cells MDPC-23 were seeded onto the pulpal side of the discs, incubated for 48h. The EASs with increasing hydrophilicity (R2, R3, R4 and R5) were applied to the occlusal side of the discs after acid etching and saturation of demineralized dentin with water or ethanol. R0 (water and ethanol- no adhesive) served as controls. After 24h, cell metabolism was evaluated by SDH enzyme production (MTT assay; n=8 discs) and cell morphology was examined by SEM (n=2 discs). The type of cell death was identified by flow cytometry and the degree of monomer conversion (%DC) was determined by infrared spectroscopy (FTIR) after two photoactivation times (10 s or 20 s). Data were analyzed statistically by the Kruskal-Wallis and Mann-Whitney tests ($\alpha=0.05$). Dentin saturation with ethanol resulted in higher necrotic cell death ratios for R3, R4 and R5 compared with water saturation, although R3 and R4 induced higher SDH production. Photoactivation for 20 s significantly improved the %DC of all EASs compared with 10 s. A significant positive correlation was observed between the degree of hydrophilicity and %DC, for both photoactivation times. In conclusion, except for R2, dentin saturation with ethanol increased the cytotoxicity of EASs, as expressed by the induction of necrotic cell death.

Keywords

adhesive systems; cytotoxicity; ethanol; dentin; odontoblast-like MDPC-23 cells

INTRODUCTION

Several factors can influence the adhesion of polymeric materials to dentin substrate, such as the type, composition and application mode of adhesive systems, and the intrinsic characteristics of this tissue, which determine its regional heterogeneity. For this reason, resin bonding to dentin is not considered as safe and predictable as to enamel [1] and therefore, producing a resin-dentin bond strong enough to resist oral conditions remains a challenge.

Collagen fibrils may remain exposed after formation of resin-dentin bonds due to incomplete impregnation of demineralized dentin by resin monomers [2,3]. This occurs as a result of a series of factors namely gradual reduction of the interfibrillar spaces towards the mineralized dentin, the high molecular weight and hydrophilic nature of some resin monomers present in the adhesive systems [4,5] and moisture gradient of dentin [6], which permit the occurrence of the phase separation phenomenon [7]. In addition to these immediate factors, collagen fibrils may also be exposed due to long-term hydrolytic degradation of polymer [8] and leaching of residual monomers and low-weight polymers. Once exposed, these fibrils become susceptible to hydrolytic and enzymatic degradation, which is mediated by dentin proteases (matrix metalloproteinases - MMPs and cathepsins). Although the effects of enzymatic degradation can be minimized by the simple incorporation of chlorhexidine to dentin bonding protocols [9,10], hydrolytic degradation also exerts a significant, and perhaps predominant, effect on resin-dentin bond deterioration, affecting not only the organic, but also the polymeric content.

The concept of dentin saturation with ethanol to improve dentin infiltration by hydrophobic monomers and subsequently reduce the susceptibility of the resin-dentin interface to hydrolytic degradation was introduced in 2007 [11,12]. According to Tay *et al.* [13], the possibility of dentin infiltration by hydrophobic monomers, such as BisGMA, is based on changing the intrinsically hydrophilic nature of etched dentin to a more hydrophobic characteristic by replacing water by ethanol to maintain dehydrated collagen matrices in an extended state to facilitate resin infiltration, in a technique known as ethanol-wet bonding. A significant increase in the bond strength and durability of adhesive interfaces is observed when this technique is used. This result can be justified by a better resin-dentin infiltration [14] and sealing of the collagen matrix, and minimizing the endogenous collagenolytic action of dentin MMPs [15]. Additionally, a significant decrease of dentin permeability has been demonstrated after application of experimental adhesive systems (EASs) with known solubility parameters on demineralized dentin saturated with ethanol [16].

Although dentin saturation with an alcoholic solution has been shown to enhance monomer infiltration quantitatively and qualitatively and improve the long-term stability of resin-dentin bonds, it remains unclear whether this technique enhances the cytotoxicity of adhesive systems to pulp cells. It has been demonstrated that resin monomers can diffuse through dentin and reach the subjacent pulp tissue at concentrations high enough to elicit adverse reactions [17,18] in a dose- and time-dependent manner. Resin monomers can induce apoptosis *in vitro* [19,20] and cause untoward cellular and biological events such as changes in ATP synthesis and dentin matrix mineralization, immunological reactions and genetic alterations [21,22].

As ethanol-wet bonding facilitates resin infiltration, especially hydrophobic monomers, this study investigated whether this technique increases the cytotoxicity of EASs to cultured odontoblast-like cells. The null hypothesis is that EASs bonded to ethanol-saturated acid-etched dentin are no more cytotoxic than the same EASs bonded to water-saturated acid-etched dentin.

MATERIAL and METHODS

Preparation of dentin discs

One hundred noncarious third molar human teeth free of cracks, defects or morphological alterations were obtained upon approval by the Ethics Committee of the School of Dentistry at Araraquara - UNESP (Protocol #25/09). After removal of surface-adhered debris, the teeth were stored in 0.12% thymol solution at 4°C and used within 3 months after extraction.

On the day of the experiment roots were removed 2 mm above the cemento-enamel junction by a precision cutting machine equipped with a water-cooled diamond saw (Isomet 1000, Buehler Ltda., Lake Bluff, IL, USA). Sequential transversal cuts were made until reaching a flat dentin surface immediately above the pulp horn region. For each tooth, one 0.5-mm-thick dentin disc was cut from the mid-crown dentin as close as possible to the pulp region but without the presence of pulp horn projections. The teeth were carefully examined with a stereoscopic microscope (SZX7, Olympus, São Paulo, SP, Brazil) to confirm the absence of enamel islets on the occlusal side and defects resulting from pulp horn projections on the pulpal side. Next, the occlusal side of the discs was manually polished with wet 320-grit silicon carbide paper to reach a final thickness of 0.4 mm, as measured with a digital caliper accurate to the nearest 0.01 mm (Mitutoyo South Americana Ltd, Suzano, SP, Brazil). The dentin discs (n=100) were stored in phosphate buffer saline (PBS, pH 7.2) at 4°C, until the measurement of dentin permeability.

Measurement of Dentin Permeability (Hydraulic Conductance)

Dentin permeability was measured to permit a homogeneous distribution of the dentin discs into the groups, eliminating the influence of this variable on the analysis of transdental cytotoxicity of the EASs applied to the discs. The simplest form to measure dentin permeability is calculating its hydraulic conductance (L_p) by filtration. Permeability is calculated from the hydraulic conductance or ease of fluid flow through a surface with known area (e.g.: dentin disc) and under a constant hydrostatic pressure within certain amount of time, using the following equation:

$$L_p = \frac{J_v}{A \cdot \Delta P \cdot t}$$

where, L_p is a hydraulic conductance ($\mu\text{L} \cdot \text{cm}^{-2} \cdot \text{min}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$); J_v is the volume of fluid in μL or distance traveled by a microbubble in mm; A is the dentin surface area in cm^2 ; ΔP is the pressure gradient in $\text{cm H}_2\text{O}$; and t is the time in minutes.

For determination of the hydraulic conductance, the discs were individually placed in *in vitro* pulp chambers (IVPCs) developed originally by Hanks et al. [23]. In all dentin discs, the area for measurement of hydraulic conductance was standardized as 0.28 cm^2 . Both sides of the discs were etched with 0.5 M EDTA (acid ethylenediaminetetraacetic), pH 7.2, for 60 s to remove the smear layer created during disc polishing, followed by copious rinsing with distilled water. A metallic cannula attached to the middle compartment of the IVPC was connected to a 180 cm column of water through a 0.5-mm-diameter polyethylene tubing. The disc remained under this pressure for 5 min, after which time the movement of a

microbubble introduced through the cannula was recorded during 1 min and the obtained values (Jv) transformed into conductance values using the equation presented above.

After measurement of hydraulic conductance, the dentin discs were allocated to 10 groups of 10 discs each, in such a way that the statistical analysis of the hydraulic conductance data showed similar dentin permeability among the groups (ANOVA, $p > 0.05$). The discs were frozen individually in PBS (pH 7.2) until when they were used for analysis of cytotoxicity.

MDPC-23 Cell Culture on Dentin Discs

Odontoblast-like MDPC-23 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) and containing 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine (Gibco, Grand Island, NY, USA) in an humidified incubator with 5% CO₂ and 95% air at 37°C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The MDPC-23 cells were sub-cultured every 3 days until an adequate number of cells were obtained for the study. All cell culture procedures were carried out in a vertical laminar flow chamber (Veco do Brasil Ind. Com. de Equip. Ltda, Campinas, SP, Brazil). In preparation for the cytotoxicity assays, the occlusal side of the discs was hand polished with 600-grit silicon carbide paper for 10 s to produce a fresh smear layer and simulate clinical condition. Then, the discs were mounted in modified IVPCs and both sterilized in ethylene oxide.

The odontoblast-like MDPC-23 cells (3×10^4) cultivated in plain DMEM medium were seeded onto the pulpal side of the dentin discs (0.28 cm²). The IVPCs were placed in 24-well plates (COSTAR 3595 - Corning Incorporated, Corning, NY, USA) in an inverted position and maintained in an incubator with 5% CO₂ and 95% air at 37°C for 48 h for adherence of cells on the pulpal dentin surface. After this time, the IVPCs were placed with the occlusal side of the discs turned upwards to receive the adhesive procedures.

Dentin saturation solutions and EASs

In all groups, the smear-covered dentin was etched with 35% phosphoric acid (Scotchbond Etchant, 3M ESPE, St. Paul, MN, USA) for 15 s, copiously rinsed with sterile distilled water for the same amount of time, and blotted dry with sterile absorbant paper to maintain a moist dentin surface. Then, 20 µL of distilled water or 100% ethanol were passively applied to dentin surface for 60 s and excess was removed with a sterile absorbant paper to keep dentin moist.

Four EAS (R2, R3, R4 and R5) with known increasing degrees of hydrophilicity were evaluated. These experimental adhesive systems have been used in many studies, which focused mainly on mechanical tests [16,24,25]. The composition and Hoy's solubility parameters of the EASs are presented in Table 1. R2 is similar to the non-solvated hydrophobic component used in 3-step etch-and-rinse adhesive systems or 2-step self-etch systems. R3 represents the basic composition of a simplified etch-and-rinse adhesive system, and R4 and R5 contain carboxylic and phosphoric acid derivatives, respectively, representing simplified self-etch adhesive systems. R0 was the control group (no adhesive).

Two layers of each EAS were applied to the discs saturated with distilled water or ethanol. Five microliters of each EAS were first applied to dentin, gently scrubbed with a microbrush for 10 s, and other 5 µL were applied. All EASs were photoactivated for 20 s with a halogen light-curing unit (Optilux 500, Kerr Company, Orange, CA, USA) with 510 ± 10 mW/cm² light intensity, as measured with a curing radiometer.

All adhesive procedures were performed in a vertical laminar flow chamber to avoid microbial contamination. Immediately after the adhesive procedures, the IVPC were stored again in the incubator for additional 24 h.

Analysis of cell viability (SDH production - MTT assay)

Cell metabolism was evaluated using the methyltetrazolium (MTT) assay. This method determines the activity of succinic dehydrogenase (SDH) enzyme, which is a measure of cellular (mitochondrial) respiration, and can be considered as the metabolic rate of cells. Eight out of 10 discs were used for this analysis 24 h following application of EASs. The eluates (culture medium containing the EAS components that diffused through the dentin discs) were collected for subsequent analysis of type of cell death by flow cytometry. After the 24-hour incubation, the discs were removed from the IVPCs and placed in new 24-well plates with the pulpal side containing the MDPC-23 cells turned upwards. For each disc, the culture medium was aspirated and replaced by 900 μ L of fresh DMEM plus 100 μ L of MTT solution (Sigma-Aldrich, St Louis, MO, USA) (5 mg/mL sterile PBS). The cells in contact with the MTT solution were incubated at 37°C for 4 h. Thereafter, the culture medium with the MTT solution was aspirated and replaced by 400 μ L of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the violet formazan crystals producing a homogeneous bluish solution resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. After agitation and confirmation of the homogeneity of the solutions, three 100 μ L aliquots of each well were transferred to a 96-well plate (Costar Corp., Cambridge, MA, USA). Cell metabolism was evaluated by photometry as being proportional to the absorbance measured at 570 nm wavelengths with an ELISA plate reader (Thermo Plate, Nanshan District, Shenzhen, Gandong, China). The values obtained from the three aliquots were averaged to provide a single value for each sample. The first three wells of the plate were filled with 100 μ L of the 0.04 N acidified isopropanol solution to determine the value corresponding to total light transmission, that is, the maximum value for decrease of cell metabolism. The absorbance was expressed in numerical values, which were subjected to statistical analysis to determine the effect of the EAS components on the mitochondrial activity of the cells.

Analysis of Cell Morphology by Scanning Electron Microscopy (SEM)

The other two discs were used for SEM analysis 24 h following application of EASs. As described above, the eluates were collected for subsequent analysis of type of cell death by flow cytometry.

After the 24-hour incubation period, the cells were fixed in 1 mL of 2.5% glutaraldehyde (Sigma-Aldrich) in PBS for 1 h at room temperature. Then, the glutaraldehyde was aspirated and the cells were washed with PBS and post-fixed with 1% osmium tetroxide (Electron Microscopy Science, Fort Washington, PA, USA) for 1 hour at room temperature. The cells that remained adhered to the dentin substrate were washed again with PBS and then dehydrated in a series of increasing ethanol concentrations (30, 50 and 70%, one time for 30 min each; 95 and 100%, two times for 60 min each). The cells were immersed for 60 minutes (three 20-minute changes) in 1,1,1,3,3,3-hexamethyldisilazane (HMDS, ACROS Organics, Morris Plains, NY, USA). Finally, the specimens were mounted on metallic stubs and then stored in a desiccator for 24 hours, and sputter-coated with a gold layer (SDC 050; Bal-Tec AG, Balzers, Germany), and their morphology was examined with a scanning electron microscope (DSM 960, Carl. Zeiss Inc., Oberkochen, Germany)

Flow cytometry Analysis

The type of cell death was determined by the propidium iodide (PI) flow cytometry assay. The cells were analyzed by fluorescence-activated cell sorting (FACS) in a flow cytometer

(FACSCanto; BD Biosciences, San Jose, CA, USA) equipped with argon laser and Cell Quest software (BD Biosciences, San Jose, CA, USA). For identification of the type of cell death, MDPC-23 cells (3×10^4 cells/cm²) were cultured in 24-well plates for 48 h. Next, the culture medium was replaced by the eluates collected prior to the MTT assay and SEM analysis. After 24 h in contact with the test eluates, the cells were harvested with trypsin, centrifuged at 5,000 rpm for 2 min to remove the supernatant and resuspended in 300 μ L of ligation buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1mM MgCl₂ and 1.8 mM CaCl₂. For acquisition of cells stained positively for apoptosis, an aliquot of 300 μ L of cell suspension of each experimental condition was treated with annexin-V (Institute of Biomedical Sciences, São Paulo, SP, Brazil) at a concentration of 1:500 for 20 min in the dark. The samples were acquired in the FL-1 channel of the flow cytometer. Acquisition of cells stained positively for necrosis was made immediately after the addition of 3 μ g/mL of propidium iodide (Sigma-Aldrich Corp., St. Louis, MO, USA) at a concentration of 100 μ g of reagent per mL of buffering solution. The samples were acquired in the FL-2 channel of the flow cytometer. Data were obtained as percentage of the number of cells stained for each reagent from the total number of MDPC-23 cells recognized by the flow cytometer. One group (R+) served as an internal control of the method for identification of necrotic cell death, in which the cells were placed in contact with 29% hydrogen peroxide for 30 min before flow cytometry analysis. Data for statistical analysis were obtained as percentage of the number of cells stained by the fluorochrome (annexin or propidium iodide) in relation to the percentage of the total number of MDPC-23 cells identified by the flow cytometer.

Analysis of Degree of Monomer Conversion

The degree of monomer conversion (%DC) was determined using a Fourier transform infrared (FTIR) spectrometer (Spectrum 100 Optica; PerkinElmer, MA, USA) equipped with an attenuated total reflectance (ATR) device, which is composed of a horizontal selenide zinc (SeZn) crystal (Pike Technologies, Madison, WI, USA). The %DC was calculated using previously established methods [26], based on the ratios between the peaks 1638 cm^{-1} (carbon double-bonds in aliphatic chains) and 1608 cm^{-1} (aromatic chains of carbon atoms that originate the benzene rings of monomer molecules, which are used as a reference since their intensity remains unaltered during the polymerization process). Three microliters of each EAS were applied directly to the horizontal face of the crystal and coated with a polyester strip to prevent the formation of an oxygen inhibited layer. The graphs for calculation of baseline data (non-polymerized sample) were constructed and photoactivation ($510 \pm 10\text{ mW/cm}^2$, Optilux 500, Kerr Company, Orange, CA, USA) of specimens was performed for 10 or 20 s, generating the graph representative of the values used for calculating the degree of monomer conversion. Five repetitions were made for each EAS and photoactivation time.

Statistical Analysis

Data for each response variable (SDH enzyme production, apoptotic and necrotic cell death, and degree of monomer conversion) did not fulfill the requisites of normal distributions and homoscedasticity. Kruskal-Wallis and Mann-Whitney non-parametric tests for pairwise comparisons of groups were used, in comparing the variables factors “EAS”, “saturation solvent” and “photoactivation time”. The significance level was set at 5%.

RESULTS

Analysis of cell viability – (SDH production - MTT assay)

SDH production by the MDPC-23 cells after application of the different EASs on the occlusal side of 0.4-mm-thick dentin discs etched with phosphoric acid and saturated with

water or ethanol is presented in Table 2. Dentin saturation with ethanol, with and without EAS application, did not exert any toxic effect to the MDPC-23 cells as the SDH production values did not differ significantly from those obtained with water saturation. However, application of R3 and R4 to ethanol-saturated acid-demineralized dentin increased cell metabolism by 36.4% and 106.6%, respectively, compared with the values obtained for the same EASs applied to water-saturated dentin. In the water saturation condition, none of the EASs presented cytotoxicity to the MDPC-23 cells, as the SDH production values did not differ from those obtained for R0 (no adhesive system). Comparison among the EASs showed that, for both saturation solvents, R4 presented significantly higher SDH production than R2 ($p < 0.05$), which, in turn, was not significantly different from R0 ($p > 0.05$).

Type of cell death (apoptosis or necrosis)

Necrotic cell death was the predominant type for all adhesive systems (Figure 1). Except for R3, no statistically significant difference was found among the EASs with respect to apoptotic cell death induced by the two saturation solvents (Figure 1a). Apoptotic and necrotic cell death ratios ranged from 3–10% and 12–47%, respectively. A significant increase ($p < 0.05$) in necrotic cell death ratio was observed for R3, R4 and R5 when dentin was saturated with ethanol (Figure 1b).

Analysis of cell morphology (SEM)

For R0 (control), the groups in which dentin was saturated with water and ethanol presented a similar number of cells adhered to the substrate and they exhibited typical morphology of the MDPC-23 cell line, which includes abundant cytoplasm with multiple small cytoplasmic projections and a flattened cytoskeleton (Figures 2a and 2a'). For R2, the most hydrophobic resin blend, a smaller number of cells were observed in the group bonded to dentin saturate with ethanol (Figure 2b'), and several cells exhibited a round morphology. Application of R3 on ethanol-saturated dentin (Figure 2c') resulted in the formation of cell agglomerates with consequent exposure of underlying dentinal tubules, and several cells exhibited a round morphology in the same way as observed for R2. For R4, both the number and the morphology MDPC-23 cells were very similar between the groups saturated with water or ethanol (Figures 3a and 3a'), while the application of R5 on ethanol-saturated dentin resulted in a smaller number of cells adhered to the substrate, leaving large cell-free areas (Figure 3b').

Degree of monomer conversion (%DC)

The %DC of the EASs photoactivated for 10 and 20 s are presented in Table 3. Overall, an increase in %DC occurred with increasing the degree of hydrophilicity, for both photoactivation times. A significant positive correlation was found between the degree of hydrophilicity (Hoy's δ_h) of the EAS and the DC ($p < 0.05$ in both cases). That is, the R^2 of DC versus δ_h of the resins was 0.70 after 10 s of photoactivation and 0.80 for 20 s (Figure 4). Photoactivation for 20 s increased significantly the %DC of all EASs compared with 10 s (Table 3).

DISCUSSION

Under clinical conditions, residual (unreacted) monomers can diffuse through dentin, penetrate the pulp chamber and reach the pulp cells [17]. As odontoblasts are organized in a monolayer that underlies the dentin and lines externally the pulp tissue, they are the first cells to encounter potentially cytotoxic compounds released from dental materials that cross the dentin barrier and reach the pulp. In an attempt to approximate *in vivo*/*in vitro* results, the present study used IVPCs containing dentin discs, in which the adhesive procedures were performed on the occlusal side of dentin disks seeded with odontoblast-like MDPC-23

cells on the pulpal side. The cells were exposed to potential irritation by compounds released from the EASs for 24 h, which is sufficient time for a complete transdental diffusion of monomers [27], permitting the immediate analysis of the cytotoxic effects of the tested materials.

The %DC increased with the increase of degree of hydrophilicity and the photoactivation time of the EASs. In spite of this, the highest values obtained (*ca.* 59%), indicate that a large amount of unreacted monomers was close to pulpal cells. The alterations in cell morphology and metabolism observed in the present study could be attributed to the transdental diffusion of these monomers. Commercially available adhesive systems have been reported to produce %DC between 20 and 50%, and this wide range is influenced by factors inherent to the composition of the adhesive systems, such as the amount of solvent in the adhesive, type of photoactivation source (halogen or LED), intensity (light-curing unit power) and photoactivation time [28,29]. The polymerization reaction of each monomer depends on the mobility and density of the crosslinking reagents [16]. Therefore, a higher BisGMA concentration in the adhesive system increases its viscosity, reducing the velocity of the reaction and consequently the %DC [30].

The use of 100% ethanol for saturation of demineralized dentin did not cause cytotoxic effects to the odontoblast-like cells. However, a significant increase in necrotic cell death ratio was observed for three of the four EASs applied to dentin saturated with ethanol compared with water. From this result, it could be inferred, indirectly, that greater transdental monomer diffusion occurred in the presence of ethanol. Further research might determine which monomers were favored by dentin saturation with ethanol, as such information is not yet available in the literature. Other studies, however, have demonstrated better dentinal seal [31] and long-term stability of resin-dentin bond [12] when dentin is saturated with ethanol followed by application of hydrophobic adhesive systems such as R2 (Table 1). As no significant difference was found among R3, R4 and R5 with respect to %DC, the increase of necrotic cell death ratio in the presence of ethanol cannot be attributed only to the diffusion of high molecular weight hydrophobic monomers with accentuated toxicity [32,33], as R3 contains 70% of BisGMA while R4 and R5 contain only 40% of this monomer.

Cell metabolism increased by 34 and 106%, respectively, when R3 and R4 were applied to ethanol-saturated dentin. However, these adhesive systems were also associated with an increase of the necrotic cell death ratio, which could be explained by the fact that some monomers present in their composition, i.e., BisGMA, TEGDMA and HEMA, can induce oxidative stress and cytotoxicity in pulpal cells [34] (Demirci et al., 2008). Additionally, R4 and R5 were the only EASs with an acidic pH, *ca.* 4 and 3, respectively. It may be assumed that, apart from demineralization by phosphoric acid etching, these acidic resins caused an additional demineralization of the dentinal tissue. Such demineralized dentin can release several proteins, such as collagenases, gelatinases (MMPs) and growth factors (BMP and TGF- β), which were imprisoned by the mineral involucres. Once released, they have the potential to stimulate cellular functions namely increase of mitochondrial respiration and alkaline phosphatase synthesis and activity, among others [35]. SEM is a descriptive and qualitative analytical method with different applications. In the present study, SEM was used to identify alterations on the phenotypical characteristics of cells and the increase or reduction of the number of cells adhered to dentin. The SEM findings illustrated the results of the *in vitro* cell viability and cell death determination assays, as some cells presented cytoskeletal alterations and the number of cells decreased with consequent increase in the cell-free areas, when dentin was saturated with ethanol prior to EAS application.

The identification of cell death type is important for analysis of the cytotoxicity of adhesive systems because necrotic cell death is considered more aggressive because it causes cell membrane disruption. This causes exposure of all organelles essential for cell survival, which might activate an inflammatory tissue cascade. On the other hand, apoptosis is a physiological process of 'programmed' cell death, which occurs normally during development and aging or when cells are damaged by pathologies or noxious agents [36]. Induction of apoptosis seems to be associated with the production of reactive oxygen species (ROS), which is the main event of immediate cell response to exposure to monomers. Some authors [37,38] have affirmed that resin monomers such as BisGMA, TEGDMA and HEMA can affect the regulation of cytokine production, which reduce the expression of the antioxidant glutathione (GSH), increasing intracellular ROS levels and leading to apoptotic cell death. Another pathway of apoptotic cell death has as its main component cytochrome C, a hemoprotein from the intramembrane space of mitochondria, which is converted into peroxidase upon ROS generation. Lipid peroxidation of phosphatidylserine by cytochrome C produces recognition signals that induce phagocytosis of the apoptotic bodies by macrophages [39]. Although apoptotic cell death has been observed for all EASs, necrotic cell death was more prevalent. Flow cytometry analysis revealed that, in addition to monomers that diffused through dentin, the eluates placed in contact with the MDPC-23 cells also contained metabolites and dead cell rests. When a cell dies due to necrosis it releases Ca^{2+} ions and cellular rests that can induce the production of inflammatory cytokines, which, in large amounts, can induce the increase of necrotic cell death ratios.

Dentin has a protective role in response to potential irritating agents to pulp tissue [23,40]. Although the results of the present study demonstrated potential cytotoxicity of EASs, these cytopathic effects can be considered less severe when compared to the direct effects of resin monomers on odontoblast-like cells (data not shown), as the dentin barrier can attenuate the aggressive potential of components of adhesive systems due to intrinsic characteristics of this tissue, such as buffering capacity and hydraulic conductance [41,42]. Therefore, the transdental diffusibility of monomers varies according to parameters such as thickness of remaining dentin, dentin permeability and alterations promoted by the defense mechanisms of the pulpodentinal complex [43].

Saturation of phosphoric acid-demineralized dentin with ethanol increased the cytotoxicity of the EASs (except for R2), but no direct correlation was observed between their degree of hydrophilicity and this cytopathic effect. Despite these unfavorable results, from the methodology used in the present study, one cannot affirm that the EASs will have the same biological behavior in vivo. This in vitro evaluation can only predict that the pulp tissue would potentially better accept certain materials, as other major factors that influence the defense mechanism and tissue repair act in vivo, such as inflammatory reaction, immunological system and lymphatic system. In this context, other levels of investigation, such as preclinical and clinical studies, should be conducted to evaluate the biosecurity of the adhesive protocol involving dentin saturation with ethanol and infiltration with hydrophobic monomers, as encouraging outcomes as to the bond strength and long-term stability of resin-dentin bonds have already been described [14–16].

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Figure 1.

Percentage of (a) apoptotic (% of MDPC-23 cells anexin V-stained cells) and (b) necrotic (% of MDPC-23 cells stained by propidium iodine) cell death according to the saturation solutions and EASs. In both graphs, letters permit comparison of the saturation solutions for the same EAS; same letters indicate no statistically significant difference (Mann-Whitney, $p > 0.05$). C Pos = positive control, 29% hydrogen peroxide; R0 = control, no adhesive system; R1-R4 = EASs in an increasing order of hydrophilicity.

Figure 2.

Composite figure of SEM micrographs representative of MDPC-23 cells on dentin saturated with water (left column) or ethanol (right column) prior to application of the EASs, (a) and (a') R0 (Control, no adhesive system): Typical morphology of MDPC-23 cells, that is wide cytoplasm with numerous small cytoplasmic projections and flattened cytoskeleton covering most part of dentin surface; (b) and (b') R1: Smaller number of cells and altered cell morphology when occlusal dentin was saturated with ethanol; (c) and (c') R2: When dentin was saturated with ethanol, agglomerates of cell with round morphology were formed with consequent exposure of dentin substrate. x500.

Figure 3.

Composite figure of SEM micrographs representative of MDPC-23 cells on dentin saturated with water (left column) or ethanol (right column) prior to application of the EASs, (a) and (a'). R3: Reduction of the number of cells adhered to dentin when dentin was saturated with ethanol, though without alteration of the morphology of the cells that remained adhered to the dentin substrate; (b) and (b') R4: Reduction of the number of cells and alteration of cell morphology when dentin was saturated ethanol. x500.

Figure 4.

Hoy's solubility (δ_h) plotted against the percentage conversion of resins 2-5 after 10 and 20 s of light-activation. For both lengths of light-activation there was a significant regression ($p < 0.05$), accounting for 70 and 80% of the observed data (R^2) for 10 and 20 s, respectively.

Table 1

Composition, H₂O solubility parameters^a, and pH of the experimental adhesive systems (EASs)

EAS Composition ^a	Solubility parameter Non-solvent adhesive				pH
	δ _d	δ _p	δ _h	δ _t	
70.0% BisGMA					
28.5% TEGDMA					
R2 1.0% EDMAB	15.9	12.4	6.9	21.2	~ 7
0.25% CQ					
70.0% BisGMA					
28.74% HEMA					
R3 1.0% EDMAB	15.6	13.0	8.5	22.1	~ 7
0.25% CQ					
40.0% BisGMA					
30.0% TCDM					
R4 28.75% HEMA	16.2	13.5	9.0	23.0	~ 4
1.0% EDMAB					
40.0% BisGMA					
30.0% BisMP					
R5 28.75% HEMA	15.1	13.5	11.1	23.1	~ 3
1.0% EDMAB					

^aCadenaro *et al.* [20] Effect of adhesive hydrophilicity and curing time on the permeability of resins bonded to water vs. ethanol-saturated acid etched dentin.

E-BisGMA = ethoxylated bisphenol A diclycidyl ether dimethacrylate; BisGMA = 2,2-bis[4-(2-hydroxy-3-methacryloylpropoxy)]-phenyl propane; TEGDMA = triethyleneglycol dimethacrylate; HEMA = 2-hydroxyethyl methacrylate; TCDM = di(hydroxyethylmethacrylate)ester of 5-(2,5-dioxotetrahydrofuryl)-3-methyl-3-cyclohexane-1,2-dicarboxylic acid; CQ = camphoroquinone; EDMAB = 2-ethyl dimethyl-4-aminobenzoate; BisMP = Bis[2-(methacryloyloxy)ethyl] phosphate

Table 2

Succinic dehydrogenase (SDH) enzyme production (optical density) by the MDPC-23 cells after application of different experimental adhesive systems (EASs) on acid-etched dentin saturated with water or ethanol.

EAS	Saturation Solvent	
	Water	Ethanol
R0 (control)	0.307 (0.240–0.397) ^{*a. AB}	0.272 (0.221–0.342) ^{a. B}
R2	0.261 (0.181–0.296) ^{a. B}	0.290 (0.277–0.332) ^{a. B}
R3	0.282 (0.264–0.328) ^{b. AB}	0.375 (0.315–0.495) ^{a. AB}
R4	0.393 (0.317–0.495) ^{b. A}	0.562 (0.371–0.657) ^{a. A}
R5	0.310 (0.268–0.353) ^{a. AB}	0.359 (0.313–0.395) ^{a. AB}

* Values represent medians (percentile 25–percentile 75), n=8.

^{A, a} Lowercase letters permit comparisons in rows and uppercase letters permit comparisons in columns. Groups identified with same letters do not differ significantly (Mann-Whitney, $p > 0.05$). R0 = control, no EAS; R1–R4 = EASs in an increasing order of hydrophilicity (i.e. δ_H in Table 1).

Table 3

Degree of monomer conversion (%DC) of experimental adhesive systems (EASs) photoactivated for 10 or 20 s.

EAS	Photoactivation time	
	10 s	20 s
R2	50.0 (49.8–50.0) ^{*B. b}	52.7 (51.7–52.8) ^{a. A}
R3	53.7 (53.6–53.7) ^{b. AB}	55.7 (55.6–55.7) ^{a. A}
R4	56.5 (56.2–56.5) ^{B. a}	58.5 (58.4–58.6) ^{a. A}
R5	56.1 (56.1–56.4) ^{B. a}	58.9 (58.6–59.5) ^{a. A}

* Values represent medians (percentile 25–percentile 75), n=5.

^{A, a} Uppercase letters permit comparisons in the rows and lowercase letters permit comparisons in columns. Medians identified with same letters do not differ significantly (Mann-Whitney, $p > 0.05$). R1–R4 = experimental adhesive systems in an increasing order of hydrophilicity.