In vitro dentin barrier cytotoxicity testing of some dental restorative materials

R.D. Jiang[a], H. Lin[a,*], G. Zheng[a], X.M. Zhang[b], Q. Du[c], M. Yang[a]

[a] Dental Medical Devices Testing Center, Dental Materials Laboratory, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing 100081, China
[b] School of Stomatology, Jilin University, Changchun 130021, China
[c] Department of Stomatology, Beijing Hospital of Chinese Traditional and Western Medicine, Beijing 100039, China

ABSTRACT

Objectives: To investigate the cytotoxicity of four dental restorative materials in three-dimensional (3D) L929 cell cultures using a dentin barrier test.
Methods: The cytotoxicities of light-cured glass ionomer cement (Vitrebond), total-etching adhesive (GLUMA Bond5), and two self-etching adhesives (GLUMA Self Etch and Single Bond Universal) were evaluated. The permeabilities of human dentin disks with thicknesses of 300, 500, and 1000 μm were standardized using a hydraulic device. Test materials and controls were applied to the occlusal side of human dentin disks. The 3D-cell scaffolds were placed beneath the dentin disks. After a 24-h contact with the dentin barrier test device, cell viabilities were measured by performing MTT assays. Statistical analysis was performed using the Mann–Whitney *U* test.
Results: The mean (SD) permeabilities of the 300-μm, 500-μm, and 1000-μm dentin disks were 0.626 (0.214), 0.219 (0.0387) and 0.089 (0.028) $\mu l\, min^{-1}\, cm^{-2}\, cm\, H_2O^{-1}$. Vitrebond was severely cytotoxic, reducing the cell viability to 10% (300-μm disk), 17% (500 μm), and 18% (1000 μm). GLUMA Bond5 reduced the cell viability to 40% (300 μm), 83% (500 μm), and 86% (1000 μm), showing moderate cytotoxicity (300-μm) and non-cytotoxicity (500-μm and 1000-μm). Single Bond Universal and GLUMA Self Etch did not significantly reduce cell viability, regardless of the dentin thicknesses, which characterized them as non-cytotoxic.
Conclusions: Cytotoxicity varied with the materials tested and the thicknesses of the dentin disks.
Clinical significance: The tested cytotoxicity of materials applied on 300-, 500-, and 1000-μm dentin disks indicates that the clinical use of the test materials (excepting self-etching adhesives) in deep cavities poses a potential risk of damage to the pulp tissues to an extent, depending on the thickness of the remaining dentin.

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1. Introduction

Biocompatibility has been described as the reaction of a living system to exogenous materials [1]. It is necessary to evaluate the biocompatibility of dental materials for safety before clinical use, and the main means for biocompatibility testing are in vitro, animal, and clinical tests [1]. Cytotoxicity tests are convenient, provide repeatable results, do not require hurting animals, and are commonly used in vitro; however, the testing procedures of some traditional in vitro cytotoxicity tests, such as the agar or filter diffusion tests or extract tests, do not mirror clinical practice [2]. In these traditional tests, dental cements, adhesives, and composite resins have been reported to elicit varying degrees of chemical toxicity to cultured cells, which mainly depends on the content of unpolymerized monomers, such as bis-GMA, HEMA, TEGDMA, UDMA, glutaraldehyde, and camphorquinone [3–9]. However, some of these materials were reported to have no effect on pulp tissue in vivo [10–12]. This discrepancy may have arisen because traditional methods combined with two-dimensional (2D) mono-layer cell culture behave too sensitively, in comparison to complex in vivo conditions.

The dentin barrier test was developed to screen for chemical toxicity to pulp tissue by dental restorative materials, especially those used in direct contact with dentin [13–16]. This method is used instead of traditional in vitro models, as it closely mimics clinical practice and provides results that more accurately reflect in

* Corresponding author at: Peking University School and Hospital of Stomatology, 22 Zhongguancun Avenue South, Haidian District, Beijing 100081, China.
E-mail addresses: honglin196@126.com, daisyjrd@163.com (H. Lin).

vivo conditions [13,15,16]. The design of this method, which simulates the contact process after materials are applied to teeth, enables the implementation of various other clinical procedures, such as etching, desensitization, and laser treatment, allowing it to replace animal experiments [1,17,18].

Diffusion of substances from dental materials to the pulp is influenced by dentin thickness due to the diffusion process through the dentinal tubules and the adsorption on dentin components [19]. Consequently, the permeability of dentin can strongly affect the transfer of cytotoxic components from dental materials [19,20]. The permeability of human dentin varies greatly, as human teeth develop independently and can be affected by various factors such as aging, carious lesions, and other external stimuli [21]. This heterogeneity may interfere with test results in a dentin barrier test. Many in vitro studies have employed bovine dentin instead of human dentin [14–16,22,23]. Although bovine dentin is thought to show less permeability variation than human dentin [24], human dentin disks better resemble the characteristic of human target tissue in vivo than does bovine dentin. In addition, the permeability of dentin has been shown to be inversely related to its thickness [19]. However, to our knowledge, no study has investigated the effect of the thickness of human dentin disks on the cytotoxicity of materials in a dentin barrier test, and only one study was conducted to investigate this effect with bovine dentin disks, which showed that the cytotoxicity of materials decreased when increasing thickness of the bovine dentin disk; however, this effect was not always observed and was material-dependent [25]. Many dentin barrier cytotoxicity studies have used 500-μm-thick dentin disks to represent the remaining dentin beneath a deep cavity [14–16,18]. Thinner or thicker slices may be used to stimulate deeper or shallower clinical cavities [26].

It is important to use a dentin barrier testing method based on conditions that are as similar as possible to clinical conditions when evaluating the cytotoxicity of currently used dental restorative materials, especially those used in direct contact with dentin such as cements and adhesives, so as to help determine the chemical toxicity of these materials to pulp tissue in specific application in a cavity. Such a method should incorporate human dentin thickness as a critical parameter for simulating clinical cavities of various depths. Standardization of human dentin permeability is required for this testing method to produce consistent results [2,17,27].

The aim of this study is to evaluate, in vitro, the cytotoxicity of four dental restorative materials to three-dimensional (3D) cultures of fibroblasts in a dentin barrier test device, using human dentin disks of varying thickness.

2. Materials and methods

This study was approved by the Institutional Review Board of Peking University School and Hospital of Stomatology. Informed consent from patients was not required by the ethics committee.

2.1. Materials and sample preparation

The materials used in this study are listed in Table 1. All materials were applied directly to the dentin, according to the manufacturer's instructions. Non-toxic medical-grade silicone blocks, Φ 6 mm × 2 mm, were used as the negative control (100% cell viability).

2.2. Dentine disks

In total, 120 human third molars collected from adult patients aged 18–40 years were used. After extraction for orthodontic reasons, the teeth were cleaned by removing the debris and soft tissues, stored in 0.5% chloramine T solution in deionized water at 4 °C, and used within 2 months. Before use, the teeth were soaked in 70% ethanol for 15 min.

Dentin disks were obtained by cutting the teeth perpendicular to the long axis using a low-speed saw (Isomet-Buehler, Lake Bluff, IL, USA). The first cut was made at the cementoenamel junction to remove the root. Using the same cutting angle, disks of (300 ± 50)-μm, (500 ± 50)-μm, and (1000 ± 50)-μm thicknesses were obtained after removal of the entire pulp cavity, including the pulp horn. Only the disk next to the pulp cavity was used and, therefore, only one disk was sampled from each crown.

2.3. Assessment of dentin permeability

The hydraulic conductance of the dentin disks was measured before use in cytotoxicity tests. The equipment, made in-house according to the hydraulic-conductance model described by Outhwaite and Pashley [28,29], consisted of a water bath, a steel chamber, and a micropipette (Fig. 1). The water bath, filled with deionized water, provided a pressure of 32 cm H_2O (3.14 kPa) [30] to the pulp side of the dentin disk. To remove the smear layer, the dentin disks were acid-etched on both sides with 35% phosphoric acid for 30 s, rinsed with deionized water, and cleaned in an ultrasonic cleanser (Kudos, Shanghai, China) at 53 kHz for 5 min. The dentin disks were fixed in the middle of the chamber by the steel inserts, with pressure applied from the pulp side to the occlusal side. A measurement area of 0.28 cm² was delineated by a pair of rubber "O" rings with an inner diameter of 6 mm, and only the area in the middle of the disks was used. In the dentin barrier test, the materials were applied to the same area. After the equipment chamber was filled with deionized water from the water bath, it was sealed, and then the whole system was filled

Table 1
Materials used in the study.

| Material	Classification	Manufacturer	Lot. No.	Main components
Vitrebond	Light-cured glass ionomer cement	3M EPSE Dental Products	N621444	Powder: glass powder, diphenyliodonium chloride Liquid: copolymer of acrylic and itaconic acid, water, HEMA
GLUMA Bond5	Total-etching adhesive	Heraeus Kulzer GmbH	010301	UDMA, 4-meta, HEMA, glutaraldehyde (trace), silica (trace), ethanol, camphorquinone (trace), water
GLUMA Self Etch	Self-etching adhesive	Heraeus Kulzer GmbH	010705	Acetone, water, UDMA, 4-meta, camphorquinone (trace), silica (trace)
Single Bond Universal	Self-etching adhesive	3M EPSE Dental Products	528361	HEMA, bis-GMA, ethyl alcohol, MDP, silanized silica, water, camphorquinone
Positive control		Peking University School of Stomatology		Powder: glass powder, polyacrylic acid, diphenyliodonium chloride Liquid: camphorquinone, ethyl-4-dimethylaminobenzoate, HEMA, water
Negative control		Ji'nan Medical Silicone Rubber Products Factory	050701	Medical-grade silicone

Water Bath
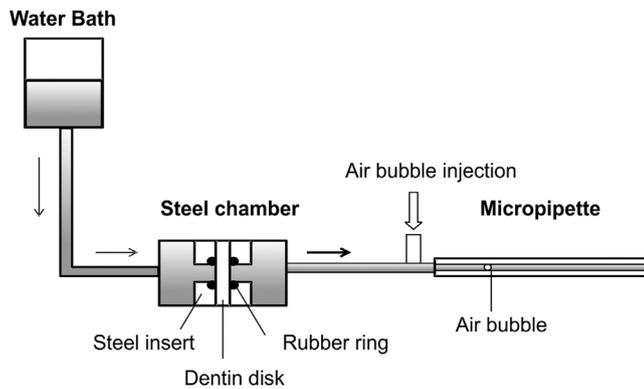
Fig. 1. The hydraulic permeability device.

with deionized water. A small air bubble was introduced into the micropipette. Experiments were performed at room temperature after the air bubble showed stable motion for 5 min. Before every measurement, a glass disk with a size similar to that of the dentin disks was tested to ensure a good seal. The scale of the micropipette was 100 μl and the division value was 5 μl.

The volume of water filtering through the dentin disk was measured by displacement of the air bubble over a defined period of certain time (10 min for the 300-μm and 500-μm disks, 20 min for the 1000-μm disks). The dentin permeability was calculated using the following equation:

$$L_p = J_v / (A \times t \times P)$$

where L_p is the hydraulic conductance of dentin ($\mu l\ min^{-1}\ cm^{-2}\ cm\ H_2O^{-1}$), J_v is the volume of water filtering through the dentin disks during the observation time (μl), A is the measurement area (cm^2), t is the observation time (min), and P is the pressure applied to the dentin disks (cm H_2O).

Thirty dentin disks with closer permeability to the mean value (n = 40) were selected from 40 dentin disks for each thickness and grouped randomly into four test material groups and two control groups (five disks per group). Before the application of cements and self-etching adhesives for the dentin barrier test, the smear layer of the dentin disks was rebuilt by grinding the disks with 400-grit sandpaper for 15 s under the same pressure. The prepared dentin disks were sterilized by soaking in 70% ethanol for 15 min and then thoroughly rinsed with deionized water, as described in the guidelines published by the International Organization for Standardization [26]. The disks were stored in 0.9% sodium chloride solution at 4 °C and used within one week.

2.4. Three-dimensional cell culture

L929 mouse fibroblasts (ATCC CCL1) were maintained in minimum essential medium (MEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 100 IU/ml penicillin, 150 μg/ml streptomycin, and 2.2 mg/ml sodium bicarbonate. Cells at the end of the exponential growth phase were used.

Polystyrene scaffolds with 4 fiber layers (8-mm diameter, 3D Biotek, New Jersey, USA), a 150-μm fiber diameter, and 150-μm fiber spacing were used for 3D cell culture. The scaffolds were placed on the inserts of 6-well tissue culture plates with 2 ml of growth medium per well and seeded with 40 μl of L929 cell suspension (1.5×10^5 cells/ml). After a 48-h incubation (37 °C, 5% CO_2), the scaffolds were transferred to 24-well plates and incubated for 14 ± 2 d. The growth medium was changed three times per week, and the plates were changed once per week.

2.5. Dentin barrier test

After 14 ± 2 d, the scaffolds were introduced into a cell culture perfusion system (3D Biotek), which was partially customized. The polycarbonate split chamber, the main component of this system, was comprised of a cylindrical cavity with an inner diameter of 6 mm and a height of 25 mm (Fig. 2). The dentin disks were placed on top of the scaffolds (occlusal side facing upward), such that the chamber was separated into two compartments by the dentin disks. The upper compartment simulated the tooth cavity, and the lower one simulated the pulp cavity.

The lower compartment was perfused with 0.3 ml of assay medium (growth medium with 6 g/l HEPES buffer) per h for 24 h at 37 °C. The assay medium was pumped into the chamber inlet and out via the outlet. In the lower compartment, the fluid covered the cell scaffolds to just below the dentin disks. After 24 h, perfusion was stopped and the test materials were placed into the upper compartment in direct contact with the occlusal side of the dentine disk. This treatment lasted for 24 h at 37 °C. Cell viability was determined by performing an MTT assay.

The scaffolds were removed from the split chambers and placed into 24-well plates containing 1 ml of pre-warmed MTT solution (Sigma, St. Louis, MO, USA; 1 mg MTT/ml in MEM without phenol red). The scaffolds were incubated with MTT at 37 °C under 5% CO_2 for 2 h and washed two times with phosphate-buffered saline solution. The blue formazan precipitate was extracted by adding 0.5 ml of dimethyl sulfoxide and then shaking the plates at room temperature for 30 min. This solution (200 μl) was transferred to a 96-well plate, and the absorbance at 540 nm (OD_{540}) was determined spectrophotometrically.

2.6. Statistical analysis

Five replicates were used for each dental material and control, and each test was performed in duplicate. The results are expressed as a percentage of the negative control. The non-parametric Mann–Whitney U test ($\alpha = 0.05$) was performed for statistical comparisons between groups, using SPSS software, version 20.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Dentin permeability

The mean (SD) values of dentin permeability for the 300-μm, 500-μm, and 1000-μm dentin disks (n = 30) used in the dentin barrier test were 0.626 (0.214), 0.219 (0.0387), and 0.089 (0.028) $\mu l\ min^{-1}\ cm^{-2}\ cm\ H_2O^{-1}$, respectively.

3.2. Dentin barrier test

The experimental results and statistical analysis are summarized in Fig. 3. The medians of OD_{540} readings for the test materials
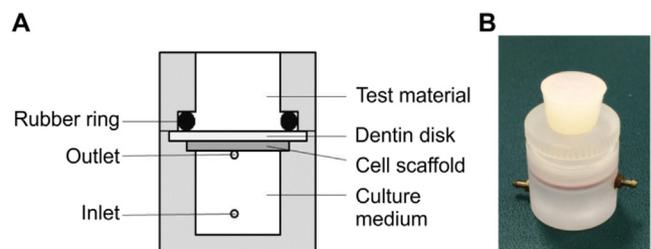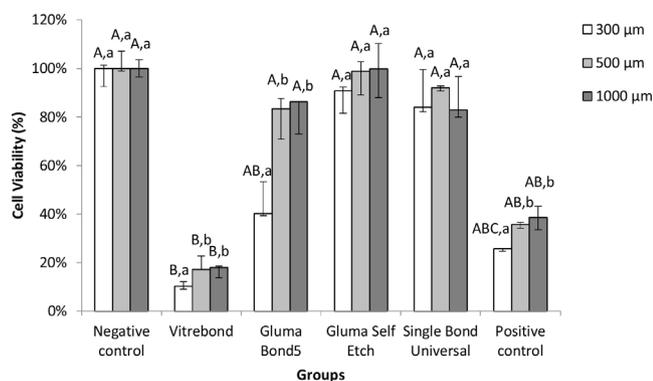

Fig. 2. Diagram (A) and photograph (B) of the split chamber.

Fig. 3. Effect of test materials on 3D cultures of L929 cells over a 24-h incubation period. The results for each test material and positive control are expressed as the percent cell viability compared with the negative control (medical silicone = 100%). The indicated values are the median, 25%, and 75% percentiles. At each dentin thickness, different upper-case letters indicate statistically significant differences between the groups (p < 0.01). For each group (p < 0.01), different lower-case letters indicate statistically significant differences between the dentin thicknesses.

and controls are given in Table 2. Vitrebond reduced the cell viability to 10%, 17%, and 18% when using 300-μm, 500-μm, and 1000-μm dentin disks, respectively. The cell viability observed with Vitrebond was the lowest for all test materials (even lower than the positive control); thus, it showed severe cytotoxicity. GLUMA Bond5 decreased the cell viability to 40% with the 300-μm dentin disks. It was significantly different from both negative and positive control groups (p < 0.01), which could be considered as a moderately cytotoxic material when used with 300-μm dentin disks. The cell viability decreased to 83% and 86% when GLUMA Bond5 was used with the 500-μm and 1000-μm dentin disks, respectively, and did not differ significantly from that of the negative control (p ≥ 0.05), indicating that GLUMA Bond5 was non-cytotoxic when used with 500-μm and 1000-μm dentin disks. Regarding the two self-etching adhesives, GLUMA Self Etch and Single Bond Universal showed cell viabilities ranging from 91% to 100% and 83% to 92%, respectively, for the 300- to 1000-μm dentin disks. For dentin disks of all thicknesses, the cell viabilities associated with either self-etching adhesive did not differ significantly from the negative control (p ≥ 0.05), indicating that both materials were non-cytotoxic. The positive control group differed significantly from all the other groups, and the negative control group showed statistical differences compared to the Vitrebond (all thicknesses of dentin) and GLUMA Bond5 (300-μm dentin disk) groups only (p < 0.01).

Thickness-dependent responses in cell viability were observed for Vitrebond and GLUMA Bond5. For these two materials, the cell viability was significantly higher with 1000-μm dentin disks than the 300-μm disks (p < 0.01).

Table 2
Median values of OD$_{540}$ readings for test materials and controls.

Group	Dentin thickness		
	300 μm	500 μm	1000 μm
Vitrebond	0.138	0.214	0.228
GLUMA Bond5	0.542	1.038	1.092
GLUMA Self-Etch	1.221	1.232	1.266
Single Bond Universal	1.129	1.146	1.052
Positive control	0.344	0.445	0.489
Negative control	1.344	1.245	1.267

4. Discussion

Human dentin functions as a barrier that can prevent substances from diffusing to the pulp, thereby decreasing the cytotoxicity of dental restorative materials [19,25,31]. Thus, the dentin barrier test is a suitable in vitro method of cytotoxicity screening for dental restorative materials. This study used a modified dentin barrier test method for the evaluation of test materials.

The total-etching adhesive GLUMA Bond5 was found to be the most cytotoxic of the three adhesives tested. It significantly decreased cell viability when used with 300-μm dentin disks. Significant cytotoxicity of total-etching adhesives when used with thin dentin disks (e.g. ≤300 μm) was reported in previous studies using similar methods [17,22]. However, GLUMA Bond5 had little effect on 3D cell cultures with 500-μm and 1000-μm dentin disks, which showed that a sufficiently thick layer of dentin can protect cells from cytotoxic components. The dentin barrier test results for GLUMA Bond5 were also consistent with clinical findings; total-etching adhesives are seldom applied directly to deep cavities in a clinical setting to avoid pulp tissue irritation, as demonstrated previously [10].

The present data show that the self-etching adhesives GLUMA Self-Etch and Single Bond Universal did not significantly decrease cell viability, even when applied to 300-μm dentin disks. This is consistent with the results for other self-etching adhesives obtained using a similar method [22,25,32] and in vivo tests [12], but is contradictory to the results of several traditional in vitro studies [7,33]. Furthermore, our results are contrary to those of a dentin barrier experiment performed without a 3D cell culture system [17,27]. These data highlight the protective function of dentin, as well as the advantages of the 3D cell culture system used in this study.

Dentin appeared to have a better protective effect against self-etching adhesives than against total-etching adhesives, which can be partially explained by the relatively gentle action of self-etching adhesives. As self-etching adhesives only dissolve 5 μm of the smear layer [34], the remaining smear layer and smear plugs still block the dentinal tubules, preventing penetration of cytotoxic components and acids. Although self-etching adhesives usually have relatively low pH values due to their acidic components, the neutralization reaction that occurs between acid and hydroxylap-atite, the main mineral in dentin, can help to decrease the acidic stimuli to cells during application [25,35].

The light-cured glass ionomer cement Vitrebond exhibited severe cytotoxicity to cells grown on any thickness of dentin disk, which is consistent with results of several in vitro studies, some of which used the dentin barrier method [4,15,16]. Vitrebond even killed all cells when using a dentin barrier test with 2D-cultured L929 cells [14]. Diphenyliodonium chloride, the photoinitiator of Vitrebond, and HEMA were previously found to be the main contributors to the high cytotoxicity [36,37]. Conversely, in vivo, Vitrebond does not produce clear pulp damages, even when used in deep cavities [10,38]. However, Galler et al. [25] have speculated that these in vivo results do not accurately reflect clinical situations where Vitrebond is applied to impaired teeth with damaged pulp, in contrast to the healthy pulp used in experiments. Based on our results, this kind of material is not recommended in direct contact with human dentin, especially in deep cavities, and a pulp-capping procedure is necessary to avoid potential damage to pulp tissue.

In this study, 3D cell cultures grown in dynamic culture conditions were used to mimic pulp tissue structures with blood flow, in vivo. Three-dimensional cell cultures more accurately reflect the physiological and morphological characteristics of in vivo cells than do 2-D cell cultures; thus, 3D models could better mimic drug metabolism in vivo [15,16,39]. Conventional glass

ionomer cement and zinc oxide-eugenol cement were previously found to have higher cytotoxicities in a dentin barrier test in 2D cell cultures [14] than in 3D cell cultures [16] or in vivo [40–42]. A similar discrepancy was observed in this study.

Although the dentin barrier test method has long been used to study the cytotoxicity of dental restorative materials [13–17], this test has not been routinely used as it still has some limitations in practical applications. The most important limitation is that dentin shows remarkable variation in permeability, which directly affects the quantity of cytotoxic materials that reach the pulp [19]; this variation is caused by individual and regional differences in the structure of human dentinal tubules [19,21,24]. Significant regional differences in the permeability of the occlusal coronal dentin have been found in humans [43]. The dentin covering the pulp horns shows higher permeability than that covering the central pulp cavity [19,43]. Thus, the dentin area selected for permeability measurements and the dentin barrier tests should not be limited to a small area that does not represent the average cavity size or dentin permeability observed in clinical settings. In this study, occlusal human dentin was sampled from near the pulp to mimics the in vivo target tissue. Dental restorative materials were applied to a defined area ($0.28 \, cm^2$) that included the locus over the pulp horns. The permeability of the dentin disks was standardized before they were used in the dentin barrier test to confirm the minimal variability, thus ensuring the accuracy and repeatability of the results.

The dentin disk thickness, an important parameter in this study, is directly related to dentin permeability [19]. Galler et al. [25] speculated that the thickness of bovine dentin disks may affect the cytotoxicity of materials in dentin barrier tests. A similar finding was obtained in this study.

In addition to thickness, reactions between the constituents of dentin and dental materials may influence the diffusion of cytotoxic components; for example, hydroxylapatite can neutralize acids, as mentioned above [19,25,35]. Some ingredients of adhesives, such as glutaraldehyde, can cross-react with the collagen in dentin [44], which helps to decrease the cytotoxicity of these adhesives [25,44]. However, it is noteworthy that the collagen in dentin may be destroyed during preparation, such as during sterilization procedures. The dentin disks used in this study were disinfected with 75% alcohol, which may have had less of an effect on their organic components than the autoclaving used in several other studies. Although both sterilization methods are recommended for the dentin barrier test by the International Organization for Standardization [26], autoclaving is expected to change the organic components of dentin to a greater extent than is 75% alcohol, as it has stronger protein denaturation characteristics [45]. Type I collagen, which is the main component of dentin collagen, is thermally denatured at 41 °C [45]. Collagen maintained in dentin may have been responsible for the protective effect of dentin against the glutaraldehyde-containing adhesive, such as GLUMA Bond5, in this study. Although autoclaving sound teeth was previously shown not to affect dentin permeability [46], the effect of autoclaving of incised dentin disks on dentin permeability remains unclear. After autoclaving, coagulated collagen on the surfaces of dentin disks might collapse and block the dentinal tubular orifices, reducing permeability. This may explain the higher cell viability observed for Vitrebond in other similar studies, wherein the dentin was autoclaved [16,25].

Previously, the application of test materials on dentin disks, including the materials' form and the obturation of occlusal side of dentin, was found to potentially affect the cells beneath the dentin [47]. In this study, solid reference materials were selected as appropriate control materials based on the application of test materials used, which is consistent with other similar studies [22,25]. The components of the positive control were in accordance with ISO standard [26]. Non-toxic medical-grade silicone, which is generally considered to have good biocompatibility, was selected as the negative control to assess the negative response in this test system.

5. Conclusions

Based on the products tested, the light-cured glass ionomer cement and the total-etching adhesive produced significant cytotoxicity, although to different extents depending on the dentin thickness used. In contrast, the self-etching adhesives were non-cytotoxic for all dentin thicknesses tested. The thickness of human dentin disks had a clear effect on cytotoxicity, except when using non-cytotoxic materials. The experimental design in this study mimics clinical settings to a great extent, except for the use of mouse fibroblasts. Three-dimensional cell culture may be particularly useful for performing dentin barrier tests. With the aim of better simulating in vivo conditions, further studies should be performed to apply this method to cell with properties more closely resembling human pulp cells.

Conflict of interest statement

The authors have no competing interests to declare.

Acknowledgments

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