ORIGINAL ARTICLE

Cytotoxicity test of dentin bonding agents using millipore filters as dentin substitutes in a dentin barrier test

Mi-joo Kim · Kyoung-nam Kim · Yong-keun Lee · Kwang-mahn Kim

Abstract

Objectives The purpose of this study was to find proper dentin substitute for standardized dentin barrier test and perform the cytotoxicity test of commercial bonding agents with the proper substitute.

Materials and methods The three-dimensional cells attached to dentin disc or millipore filters as the dentin substitute were tested in a dentin barrier test by perfusion. MTT assay was performed as an evaluation method for the cell survival rate. The cytotoxicity test of serial phenol dilution by bovine dentin disc was done to determine a standard toxic material, and the test of this proper phenol by using various millipore combinations was performed to find the suitable dentin substitute. Also, the cytotoxicity test of bonding agents was performed by this standardized substitute. The cell viability was expressed as percentages of untreated group.

Results Phenol concentration of 0.05 % was selected as the standard toxic material. The different combinations of millipore filters—two sheets of 0.45 μm, two sheets of 0.22 μm, and the combination of 0.65, 0.45, and 0.22 μm—showed similar cytotoxicity to natural dentin discs by 0.05 % phenol ($p>0.05$). The millipore combination of 0.65, 0.45, and 0.22 μm that had structural similarity to natural dentin discs was used as the substitute for cytotoxicity test of bonding agents. The toxic level of Adper Prompt L-Pop using the selected substitute was significantly the highest among four kinds of dentin bonding agents ($p<0.05$). Also, the dentin barrier test by the substitute showed constant results compared with the one by the natural dentin disc.

Conclusions The millipore filter combination of 0.65, 0.45, and 0.22 μm could be used as the substitute for the cytotoxicity test of materials applied to dentin.

Clinical relevance Dentin barrier test by standardized substitutes would be helpful for considering the potential toxicity of dentin bonding agents prior to clinical adaptations and reducing the variations of natural bovine dentin that has individually different characteristics.

M.-j. Kim · K.-n. Kim · Y.-k. Lee · K.-m. Kim (✉)
Department and Research Institute of Dental Biomaterials and Bioengineering, College of Dentistry, Yonsei University, 50 Yonsei-ro, Seodaemun-gu,
Seoul 120-752, Republic of Korea
e-mail: kmkim@yuhs.ac

M.-j. Kim · K.-n. Kim · K.-m. Kim
Research Center for Orofacial Hard Tissue Regeneration, College of Dentistry, Yonsei University,
50 Yonsei-ro, Seodaemun-gu,
Seoul 120-752, Republic of Korea

Introduction

Increased life expectancy and concerns about oral health have created demands for professional knowledge and improved clinical techniques among dentists, and have spurred the development of innovative biomaterials in dental departments worldwide. However, most biomaterials used in dentistry contain harmful ingredients that can potentially cause cell injury or tissue inflammation, either directly or indirectly. Therefore, proper biocompatibility testing tools are needed to evaluate the toxicity of dental materials before clinical adaptations.

There are some limitations in correlating in vitro tests with clinical studies because eluted dental materials may not necessarily be cytotoxic in vivo. However, the in vitro cytotoxicity test is important in understanding the biologic risk of these materials during the initial development stages [1].

Several in vitro methods have been used to evaluate the toxicity of dental biomaterials, including the agar diffusion test, filter diffusion test, and pulp and dentin usage test [2–6]. Among in vitro methods, a dentin barrier test system for evaluating cytotoxicity mimics a clinical situation and is

more desirable than the direct cell–material contact in vitro methods. It also has the potential to replace the animal experimentation [7, 8].

In the dentin barrier test, natural bovine teeth are generally used according to the ISO 7405. Bovine teeth are easy to obtain, their size allows for ease of handling, and their dentinal tubules have similar size, morphology, and density to human teeth [9–11]. In this test, a permeability of dentin plays an important role in determining the toxicity of materials by allowing increased diffusion of the released components through dentin to the pulp. However, this reportedly varies three- to tenfold over just a few millimeters. Therefore, it seems very difficult to obtain standardized dentin slices [12]. This can induce experimental differences among tests by diverse dentin discs. Thus, it is needed to identify the dentin substitute with characteristics similar to natural bovine dentin that can provide consistent results in a dentin barrier test.

Therefore, the aim of this study was to compare the experimental results of dentin barrier test using dentin substitutes with those using natural bovine dentin in order to find proper standard barrier and perform the cytotoxicity test of dentin bonding agents by selected substitute.

Materials and methods

Dentin disc and dentin substitute preparation for three-dimensional cell culture

L-929 mouse fibroblasts were cultured in growth medium RPMI1640 supplemented with 10 % fetal bovine serum, 150 IU/mL penicillin, 150 μg/mL streptomycin, 0.125 μg/mL amphotericin B, and 0.1 mg/mL geneticin (Gibco, Grand Island, NE, USA). During perfusion, 5.96 g/l HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA) was added to the growth medium.

Dentin of bovine incisors was sectioned longitudinally and polished with #1200 grit sandpaper. Finally, the incisors were made into 500-μm-thick, 13-mm-diameter discs. One side of each disc was etched with 50 % citric acid for 30 s. The discs were autoclaved before the experiment, as described previously [13], and soaked in Hank's balanced salt solution (Gibco, Grand Island, NE, USA).

Millipore filters (Millipore, Billerica, MA, USA) of 13 mm diameter were used as dentin substitutes. These membrane filters are made from biologically inert mixtures of cellulose acetate and cellulose nitrate and are widely used in analytical and research applications. Pore sizes used in this study were 0.05, 0.10, 0.22, 0.45, 0.65, and 0.80 μm in diameter. The number of overlapped filters varied from one to four sheets.

Polyamide mesh (Sefar Medifab, Heiden, Swiss) was used to partially simulate the three-dimensional cellular structure of natural teeth. Polyamide mesh combined with the filters or dentin disc were inserted into Minusheet (Minucells and Minutissue, Bad Abbach, Germany), a ring-shaped carrier for the barrier and three-dimensional cultured cells. Mesh was coated with 0.03 mg/mL fibronectin (Sigma-Aldrich, St. Louis, MO, USA) in sterile water and dried for 2 h in a dry cabinet to allow for improved cell adhesion. Table 1 lists the properties of the millipore filters and polyamide mesh.

The coated accessories were inserted into a six-well tissue culture plate, and 40 μL of L-929 cell suspension (2.5×10^5 cells/mL) was seeded on the polyamide mesh combined with the overlapped filters or bovine dentin disc. Two milliliters of growth medium was then added to each well after cell adhesion. The cells were cultured for 14 days at 37 °C in a humidified atmosphere containing 5 % CO_2 with changes of media every other day.

Cytotoxicity test by in vitro perfusion chamber system

After 14 days, the filters or dentin disc with three-dimensional cultivated mesh were transferred to a perfusion device. The commercial perfusion cell culture system (Minucells and Minutissue, Bad Abbach, Germany) was connected to a perfusion pump (Ismatek, Devon, England) capable of adjusting

Table 1 Properties of the millipore filters and polyamide mesh

Millipore filters	Pore size (μm)	Thickness (μm)	Water flow rate (mL/min/cm²)	Air flow rate (l/min/cm²)	Porosity (%)	Lot no.
	0.05	105	0.74	0.25	72	R0EA36164
	0.10	105	1.5	0.4	74	R0DA28545
	0.22	150	18	2	75	R9EN97146
	0.45	150	60	4	79	R0AA84944
	0.65	150	140	9	81	R0AA84940
	0.80	150	190	16	82	R0DA27020
Polyamide mesh	Mesh opening (μm)	Thickness (μm)	Lot no.			
	200	150	03-200/47			

media flow. The mesh was set downward (on the "pulpal" side), and millipore filters or dentin disc attached to the mesh were placed upward (on the "cavity" side) when transferred to a perfusion chamber. All culture chambers were set on a 37 °C warm plate with their own water bath system that could maintain a constant temperature. Figure 1 shows this experimental design schematically.

After applying a flow rate of 0.3 mL/h for 24 h, the upper surface of a combination was cleaned with a cotton pellet soaked in water. First, 20 μL of toxic agents (1.00, 0.50, 0.10, 0.05, or 0.01 % phenol) was applied gently. Subsequently, the flow rate was increased to 2 mL/h to simulate the in vivo environment for 24 h. This was to determine the proper phenol concentration, as one of the representative toxic agents recommended in ISO 10993-12 to determine suitable dentin substitute. The determined dilution combined with proper millipore filters should decrease cell viability by approximately 50 %.
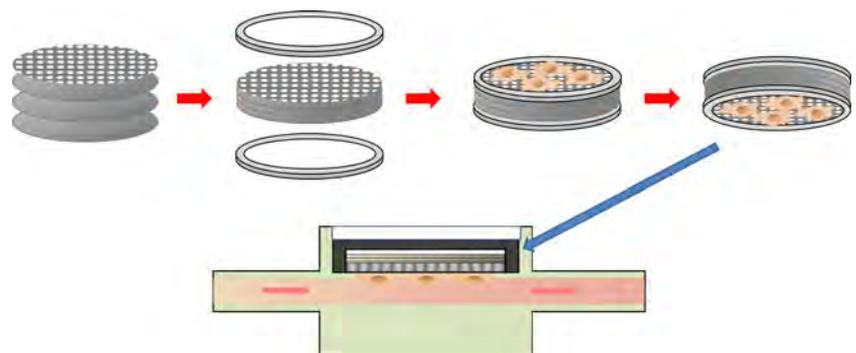
Next, the following millipore filter combinations were tested using the selected phenol dilution, one to four sheets each of 0.45, 0.22, 0.10, and 0.05 μm, 0.80+0.65+0.45 μm, 0.65+0.45+0.22 μm, 0.45+0.22+0.10 μm, and 0.22+0.10+ 0.05 μm filter combinations. Cytotoxicity results by each dentin substitute combination were compared with those by the natural dentin disc group, and suitable dentin substitutes were selected through this procedure.

Twenty microliters of each dentin bonding agent was also applied with perfusion to the selected proper millipore combination that had similar permeable and structural characteristics to dentin revealed in the previous experiment. Bonding materials are the followings: Mac-bond II (Tokuyama Dental Corp., Tokyo, Japan), Clearfil SE bond (Kuraray Medical Inc., Okayama, Japan), One-step (Bisco Inc., Schaumburg, Il, USA), and Adper Prompt L-Pop (3 M ESPE, St. Paul, MN, USA). Polymerization of bonding agents was done by a light-curing unit—Elipar Freelight 2 (3 M ESPE, St. Paul, MN, USA, 1,200 mW/cm^2)—according to the manufacturer's instructions to mimic clinical situations.

Untreated fibroblasts attached to polyamide mesh with dentin or millipore filters served as negative controls, while groups treated by 50 % phenol in sterile water were used as positive ones representing 100 % cytotoxicity.

MTT assay was performed to evaluate cell viability by filters or dentin disc combinations. An absorbance at 540 nm was determined spectrophotometrically. Cytotoxicity test results of toxic materials by filters or dentin disc were expressed as percentages of cell viability of untreated filter or dentin disc groups, respectively. Diverse kinds of the specimens with specific toxic materials were tested at a time under same conditions, and this procedure was repeated five times independently.

Statistical analysis

Statistical analysis of groups was performed by the nonparametric Mann–Whitney U test ($p > 0.05$). A p value < 0.05 was considered significant.

Results

Cytotoxicity test of five different phenol dilutions by natural bovine dentin

Figure 2 summarizes the results of dentin barrier tests with natural bovine dentin using five different phenol dilutions. Each box in the boxplot graph shows the maximum, 75 %; median, 25 %; and minimum values. Groups with different letters above the data bar are statistically significant ($p < 0.05$). Control means untreated dentin disc group. Phenol concentration of 1.00 and 0.50 % made the lowest cell viability, and they were not significantly different ($p > 0.05$). Cell viabilities with 0.10, 0.05, and 0.01 % dilution increased stepwise and showed significant differences ($p < 0.05$). Among them, 0.05 % phenol was the closest one to the desired 50 % viability and was thus chosen as the suitable standard material for subsequent testing.

Cytotoxicity test of the selected phenol by different numbers of same size, overlapping filters

Phenol dilution determined in Fig. 2—0.05 %—was used for the test with various kinds of millipore filter combinations, and

Fig. 1 Experimental scheme of perfusion system setting. Overlapped millipore filters combined with polyamide mesh were inserted into the Minusheet rings. After cell seeding, it was cultivated for 14 days and placed upside down. Adaptations of toxic materials were done on the upper compartment of chamber with flowable media in a perfusion system

the results were described in Fig. 3. Control means untreated dentin disc or millipore filter group. It had a tendency that more overlapped filters resulted in reduced cell viability in the same sized filter ($p<0.05$). Dentin group by phenol was a standard, and cell viability with two sheets of 0.45 μm or 0.22 μm was similar to that of natural bovine dentin discs ($p>0.05$). The gaps between the maximum and minimum value were significantly larger in the dentin standard group than in the filter test groups ($p<0.05$).

Cytotoxicity test of the selected phenol by various kinds of filter combinations

The 0.05 % phenol made statistically significant cell viability among the millipore combinations in Fig. 4 ($p<0.05$). Cell viability by 0.65+0.45+0.22 μm filter combination was not significantly different from that by the dentin disc as a standard ($p>0.05$), and the box size of dentin groups was significantly larger than that of the other filter groups ($p<0.05$).

Cytotoxicity test of commercial dentin bonding agents by proper dentin substitutes

The 0.65+0.45+0.22 μm filter combination that showed similar permeable and structural characteristics to natural dentin was utilized for the application of commercial dentin

bonding agent listed in Table 2. Dentin groups were shown by the left six boxplots, and the 0.65+0.45+0.22 μm filters were shown by the ones on the right hand side in Fig. 5. They showed the differences among dentin bonding agents by the dentin or substitute. Adper Prompt L-Pop was significantly the most toxic material among the four kinds of bonding agents in both dentin and filter groups ($p<0.05$). One-step and Mac-bond II were not significantly different from each other ($p>0.05$), and they showed higher cell viability compared with the other test groups ($p<0.05$). Especially, the standard deviations of dentin groups were significantly larger than those of the filter groups except for the two controls ($p<0.05$).

Discussion

Among many different cytotoxicity testing methods, dentin barrier test is an effective way to assay the cytotoxicity of dental biomaterials using 500 μm natural dentin discs as a permeable barrier. This test is generally performed in a static state. In the current study, we employed a perfusion cell culture system in order to simulate the in vivo environment.

The causes of unfavorable pulp responses to toxic dental materials are controversial. Some suggest that these
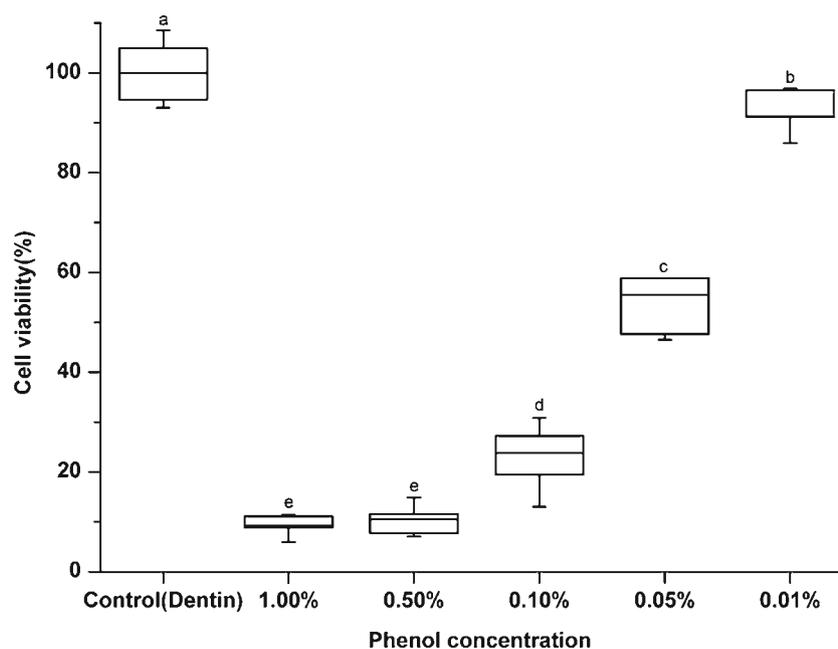
Fig. 2 Cytotoxicity test of five different phenol dilutions by natural bovine dentin. Figure 2 shows the cytotoxicity test results of five different phenol dilutions by 500 μm thickness of natural bovine dentin disc as a barrier. The untreated group was set to 100 % cell viability, and the results of test groups were expressed as percentages of those of the untreated ones. *Each box* in the boxplot graph shows the maximum, 75 %; median, 25 %; and minimum values. Phenol concentration of 1.00 and 0.50 % made the lowest cell viability ($p<0.05$), and they were not significantly different ($p>0.05$). Each cell viabilities of 0.10, 0.05, and 0.01 % dilution increased gradually ($p<0.05$). The 0.05 % phenol was the closest one to the desired 50 % viability. Groups with *different letters* above the *data bar* are statistically significant ($p<0.05$)
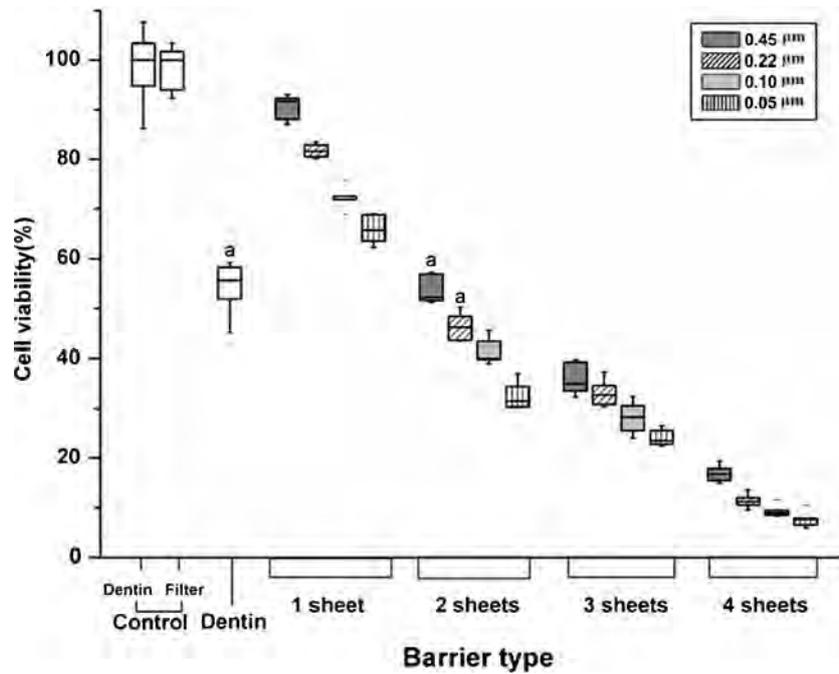
Fig. 3 Cytotoxicity test of the selected phenol by different numbers of same size, overlapping filters. Figure 3 shows the test of the selected phenol—0.05 %—by different numbers of same size, overlapping filters. *Each box* in the boxplot graph shows the maximum, 75 %; median, 25 %; and minimum values. The control is untreated dentin or filter, and the cell viability of dentin disc plus 0.05 % phenol was set as a standard for finding a suitable barrier. More overlapped filters

resulted in reduced cell viability in the same sized filter ($p < 0.05$). And the test results with two sheets of 0.45 or 0.22 μm was similar to those of natural bovine dentin discs ($p > 0.05$). The standard deviations were significantly larger in the dentin test group than in the filter test groups ($p < 0.05$). Groups with *same letters* above the *data bar* mean no statistical differences ($p > 0.05$)

responses are due to bacterial invasion secondary to cellular damage and microleakage [14, 15], while others state

that they are due to chemical toxicity of the materials [16, 17]. In this study, we focused on the latter phenomenon by

Fig. 4 Cytotoxicity test of the selected phenol by various kinds of filter combinations. The cytotoxicity test of determined phenol—0.05 %— was performed by various kinds of filter combinations. *Each box* in the boxplot graph shows the maximum, 75 %; median, 25 %; and minimum values. The control group of filters had shorter box height compared with that of dentin. Based on the dentin with phenol, cytotoxicity test result by filter combination 0.65+0.45+ 0.22 μm was similar to that by the dentin discs ($p > 0.05$). The standard deviation in the dentin group was significantly larger than that in the other filter groups ($p < 0.05$). Groups with *different letters* above the *data bar* are statistically significant ($p < 0.05$)
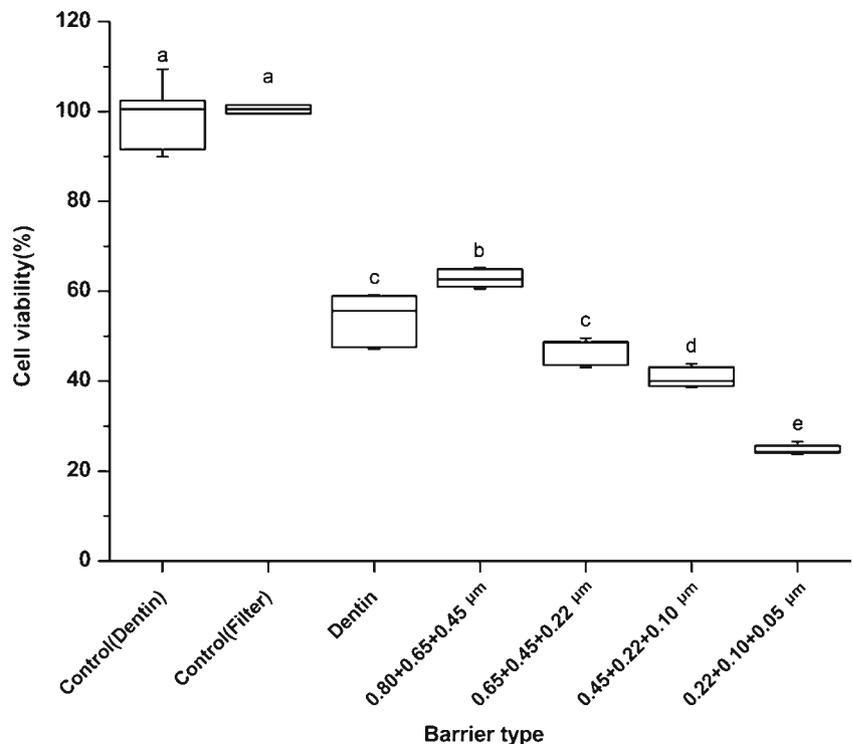
Table 2 Dentin bonding agents applied to the dentin substitutes

Product	Manufacturer	Composition	Application method	Lot no.
Mac-bond II	Tokuyama Dental Corp., Tokyo, Japan	Primer: A: MAC-10, HEMA, isopropyl alcohol, acetone, phosphate monomer; B: ethanol, water	1. Mix equal amounts of bond agents A and B 2. Apply to dentin (20 s)	006E79
		Bond: MAC-10, HEMA, Bis-GMA, TEGDMA	3. Adhesive application, light-curing (10 s)	
Clearfil SE bond	Kuraray Medical Inc., Okayama, Japan	Primer: MDP, HEMA, water, camphorquinone	1. Apply primer with disposable applicator and leave it undisturbed for 20 s 2. Air-thin primer with mild stream of air	51846
		Bond: MDP, Bis-GMA, HEMA, hydrophobic dimethacrylate	3. Apply bonding resin with disposable applicator 4. Air-thin adhesive 5. Light-activate adhesive (10 s)	
One-step	Bisco Inc., Schaumburg, Il, USA	HEMA, BPDM, initiator, acetone	1. Acid etching of 32 % phosphate acid (15 s) 2. Rinsing (15 s), air-dry (5 s)	0900000448
			3. Application of 2 consecutive coats of One-step (14 s)	
			4. Air-dry (10 s), light-curing (10 s)	
Adper Prompt L-Pop	3 M ESPE, St. Paul, MN, USA	Methacrylated phosphoric acid esters, Bis-GMA, water, HEMA, camphorquinone	1. Mixture of Adper Prompt L-Pop and application with pressure (15 s) 2. Air-dry (10 s), light-curing (10 s)	A50801239125

MAC-10 11-methacryloxy-1, 1-undecan dicarboxylic acid, *HEMA* 2-hydroxyethyl methacrylate, *Bis-GMA* bisphenol A diglycidylmethacrylate, *TEGDMA* triethyleneglycol di-methacrylate, *MDP* 10-methacryloxydecyl dihydrogen phosphate, *BPDM* biphenyl dimethacrylate

analyzing the toxicity of phenol as a representative dental biomaterial.

In a previous study, various formulations of dentin discs were provided: dentin slices from the third human molars, bovine dentin discs, or pressed dentin powder chips [9, 18–20]. Millipore filters that are usually applied to the study of filtration, air or particle monitoring, etc. are used in this study because they can be obtained easily, stabilized biologically, and provided in various sizes of paper-like material. Even proper pore size selection can make indirect cell to toxin contacts mimicking in vivo. Wennberg et al. already used the millipore filters for cell cultures with agar media. By separating the cells from test specimens by a millipore filter rather than by an agar layer in an agar overlay test, a more adequate cell–material contact might be obtained [21]. But an agar as a solid state media is different from that of the liquid nutritional one; therefore, a perfusion cell culture system with flowable media was used in the present study. Moreover, various kinds of millipore filters were overlapped to mimic the dentinal permeability. They were able to simulate porous morphological features of dentin by using 0.05 to 0.80 μm filters considering the size of natural human dentin [22, 23].

It is known that the filters are made from biologically inert mixtures of cellulose acetate and cellulose nitrate with sterilization and that each sheet of filters is resistant to chemical materials like ethanol [24]. Also, the cell viability of each bonding material by the dentin disc or millipore filter was not significantly different, respectively, as seen in

Fig. 5 (*p*>0.05). Hence, the filters could be used as substitutes for the dentin discs without chemical interferences.

Filter composition is different from that of the natural teeth. To our knowledge, the only index by which to investigate the suitability of millipore filters for the study of cell viability was via comparison to natural teeth. The most important reason to utilize bovine teeth rather than human dentin is that the bovine tooth has transdentinal permeability similar to that of coronal human root dentin [25, 26]. That is, the permeability is a critical feature in diffusing materials from dentin to pulp. Therefore, use of the dentin substitutes corresponding partially to the anatomical structure of natural dentin could be meaningful when compared to other studies. Furthermore, the dentin substitutes must have structures and components similar to natural teeth, which will be the focus of a subsequent study.

Dental materials such as dentin bonding agents, resin cements, endodontic sealer, or other agents can harm the teeth and the surrounding soft tissues and give hypersensitivity or other symptoms when applied clinically. First, in the present study, dilutions of phenol and water recommended by ISO 10993-12 were tested as a standard positive control prior to testing the dental bonding agents. Based on our findings, dentin barrier tests for commercial products can be performed using properly standardized dentin substitutes—0.65+0.45+0.22 μm, which has the most structural similarity to the natural dentinal tubules. And in case of clinical adaptations, among four kinds of dentin bonding agents, Adper
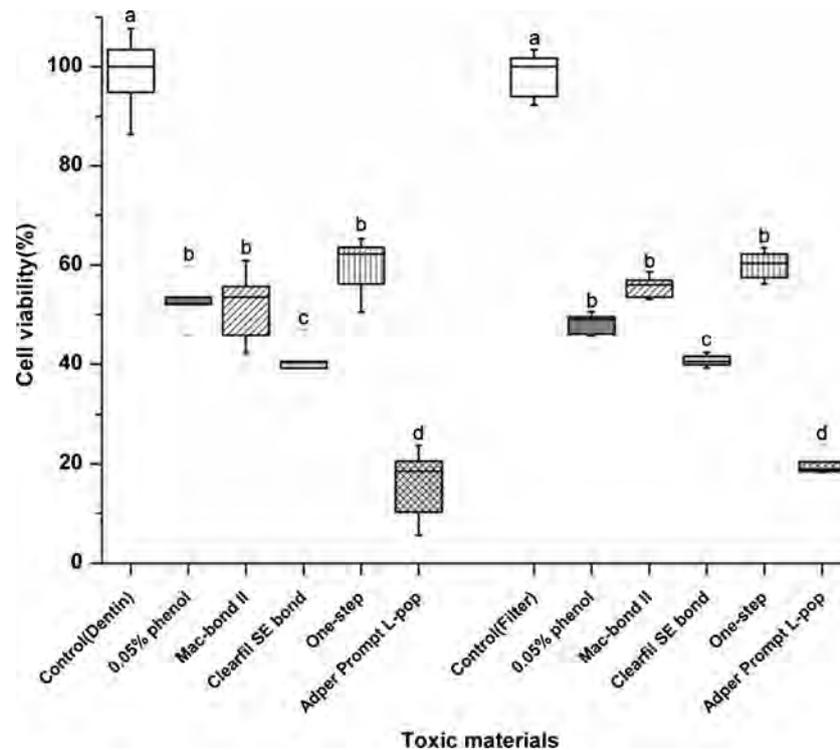
Fig. 5 Cytotoxicity test of commercial dentin bonding agents by proper dentin substitutes. The figure shows the cytotoxicity test of commercial dentin bonding agents by $0.65+0.45+0.22$ μm filter combination (dentin groups—*the left six boxplots*, substitute groups—*the right ones*). *Each box* in the boxplot graph shows the maximum, 75 %; median, 25 %; and minimum values. Among toxic agents, 0.05 % phenol, Mac-bond II, and One-step showed no statistical differences with both barriers ($p > 0.05$). Adper Prompt L-Pop was significantly the most toxic material in both dentin and millipore groups ($p < 0.05$). One-step and Mac-bond II tested by the dentin or substitute barrier showed higher cell viability compared with the other test groups ($p < 0.05$). The gap of standard deviations by Adper Prompt L-Pop with dentin and substitute was the largest among the groups. Groups with *different letters* above the *data bar* are statistically significant ($p < 0.05$)

Prompt L-Pop showed the lowest cell viability, and One-step and Mac-bond II were higher than the other groups ($p < 0.05$). These results reflect the differences between bonding systems, application methods, and compositions including monomers, solvents (ethanol or acetone), etc.

Pulpal blood flow is one of the most important factors in diluting leachable toxic substances from exposed sites. In a previous study, testing was performed with perfusion at a rate of 0.3 to 5 mL/h or without perfusion to determine the appropriate perfusion rate for cells [8]. Perfusion rate of 5 mL/h led to a general decrease in cell viability, and the rate of 0.3 mL/h showed similar results to the static condition. A rate of 2 mL/h increased cell viability and simulated the pulp flow system. Among many suggestions for the pulp flow rate [27–29], 2 mL/h was chosen as a suitable flow to mimic the in vivo environment as described previously [30]. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) of 25 mM was used to balance the pH outside the incubator for 24 h during changes of perfusion rates.

Increasing number of overlapping filters of the same size resulted in reduced cell viability in the same sized filter ($p < 0.05$). However, in another study, cytotoxicity of dental adhesives decreased when dentin disc thickness increased gradually from 100 to 500 μm [31]. This difference may be due to the fact that filters used in the present study were flexible and fragile rather than brittle. This could increase an undercurrent of toxic materials inside gaps if the substitutes had more overlapped filters. In this way, the filters could act as a storehouse, steadily emitting toxic components. By contrast, natural dentin has a stable and brittle structure that allows toxic materials to penetrate through the long tubular-shaped channels.

The following dentin substitutes showed similar cell viability to bovine dentin discs ($p > 0.05$): two sheets of 0.45 μm, two sheets of 0.22 μm, and $0.65+0.45+0.22$ μm. But when considering the structural characteristics of dentin, $0.65+0.45+0.22$ μm reflecting the changes of dentinal tubular sizes was chosen as the proper dentin substitute. And this test using the substitute could be done for evaluating the toxicity of materials applied to dentin. The development and evaluation of materials with inner structures and compositions that are more similar to natural dentin will be needed for better experimental consistency in future studies. Despite the fact that this experimental design had such shortcomings, this study has a meaning that the standardized test method with commercial millipore

filters can give us much more constant cytotoxicity results than the test with natural bovine dentin.

Conclusions

Cytotoxicity tests by two sheets of 0.45 μm, two sheets of 0.22 μm, and 0.65, 0.45, and 0.22 μm filter combination were not significantly different from those by natural bovine dentin discs ($p > 0.05$). Among them, the millipore combination of 0.65, 0.45, and 0.22 μm would be the best choice for substituting for the dentin discs in a dentin barrier test in terms of structural similarity to dentin. It could be applied for testing biocompatibility of dental materials contacting to the dentin directly such as dentin bonding agents.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Ergun G, Egilmez F, Uctasli MB, Yilmaz S (2007) Effect of light curing type on cytotoxicity of dentine-bonding agents. Int Endod J 40:216–223
2. Camps J, Tardieu C, Dejou J, Franquin JC, Ladaique P, Rieu R (1997) In vitro cytotoxicity of dental adhesive systems under simulated pulpal pressure. Dental Mater: Off Publ Acad Dental Mater 13:34–42
3. Wang RR, Li Y (1998) In vitro evaluation of biocompatibility of experimental titanium alloys for dental restorations. J Prosthet Dent 80:495–500
4. Ignatius AA, Schmidt C, Kaspar D, Claes LE (2001) In vitro biocompatibility of resorbable experimental glass ceramics for bone substitutes. J Biomed Mater Res 55:285–294
5. Murray PE, Garcia-Godoy F (2007) Comparison of the clinical and preclinical biocompatibility testing of dental materials: are the ISO usage tests meaningful? J Biomed Mater Res A 81:51–58
6. Li Y, Wong C, Xiong J, Hodgson P, Wen C (2010) Cytotoxicity of titanium and titanium alloying elements. J Dent Res 89:493–497
7. Schmalz G, Schuster U, Thonemann B, Barth M, Esterbauer S (2001) Dentin barrier test with transfected bovine pulp-derived cells. J Endod 27:96–102
8. Schmalz G, Schuster U, Nuetzel K, Schweikl H (1999) An in vitro pulp chamber with three-dimensional cell cultures. J Endod 25:24–29
9. Nakamichi I, Iwaku M, Fusayama T (1983) Bovine teeth as possible substitutes in the adhesion test. J Dent Res 62:1076–1081
10. Haapasalo M, Orstavik D (1987) In vitro infection and disinfection of dentinal tubules. J Dent Res 66:1375–1379
11. Laurance-Young P, Bozec L, Gracia L, Rees G, Lippert F, Lynch RJ, Knowles JC (2011) A review of the structure of human and bovine dental hard tissues and their physicochemical behaviour in relation to erosive challenge and remineralisation. J Dent 39:266–272
12. Pashley DH, Andringa HJ, Derkson GD, Derkson ME, Kalathoor SR (1987) Regional variability in the permeability of human dentine. Arch Oral Biol 32:519–523
13. Schmalz G, Garhammer P, Schweiki H (1996) A commercially available cell culture device modified for dentin barrier tests. J Endod 22:249–252
14. Brannstrom M, Nyborg H (1972) Pulpal reaction to composite resin restorations. J Prosthet Dent 27:181–189
15. Bergenholtz G, Cox CF, Loesche WJ, Syed SA (1982) Bacterial leakage around dental restorations: its effect on the dental pulp. J Oral Pathol 11:439–450
16. Qvist V, Stoltze K, Qvist J (1989) Human pulp reactions to resin restorations performed with different acid-etch restorative procedures. Acta Odontol Scand 47:253–263
17. Tagami J, Sugizaki J, Hosoda H (1990) Effect of various pretreatments for dentin bonding on dentin permeability. Shika zairyo, kikai = J Japan Soc Dental Mater Devices 9:240–246
18. Hume WR (1988) Methods of assessment in vitro of restorative material cytotoxicity using an intact human dentine diffusion step. Int Endod J 21:85–88
19. Hanks CT, Diehl ML, Craig RG, Makinen PK, Pashley DH (1989) Characterization of the "in vitro pulp chamber" using the cytotoxicity of phenol. J Oral Pathol Med 18:97–107
20. Tyas MJ (1977) A method for the in vitro toxicity testing of dental restorative materials. J Dent Res 56:1285–1290
21. Wennberg A, Hasselgren G, Tronstad L (1979) A method for toxicity screening of biomaterials using cells cultured on millipore filters. J Biomed Mater Res 13:109–120
22. Holland GR (1976) An ultrastructural survey of cat dentinal tubules. J Anat 122:1–13
23. Brannstrom M, Garberoglio R (1972) The dentinal tubules and the odontoblast processes. A scanning electron microscopic study. Acta Odontol Scand 30:291–311
24. Weiss AJ (2012) Overview of membranes and membrane plates used in research and diagnostic ELISPOT assays. Methods Mol Biol 792:243–256
25. Reeder OW Jr, Walton RE, Livingston MJ, Pashley DH (1978) Dentin permeability: determinants of hydraulic conductance. J Dent Res 57:187–193
26. Tagami J, Tao L, Pashley DH, Horner JA (1989) The permeability of dentine from bovine incisors in vitro. Arch Oral Biol 34:773–777
27. Hashimoto M, Ito S, Tay FR, Svizero NR, Sano H, Kaga M, Pashley DH (2004) Fluid movement across the resin-dentin interface during and after bonding. J Dent Res 83:843–848
28. Sauro S, Pashley DH, Montanari M, Chersoni S, Carvalho RM, Toledano M, Osorio R, Tay FR, Prati C (2007) Effect of simulated pulpal pressure on dentin permeability and adhesion of self-etch adhesives. Dental Mater: Off Publ Acad Dental Mater 23:705–713
29. Yoon MJ, Kim E, Lee SJ, Bae YM, Kim S, Park SH (2010) Pulpal blood flow measurement with ultrasound Doppler imaging. J Endod 36:419–422
30. Ulker HE, Sengun A (2009) Cytotoxicity evaluation of self adhesive composite resin cements by dentin barrier test on 3D pulp cells. Eur J Dent 3:120–126
31. Galler K, Hiller KA, Ettl T, Schmalz G (2005) Selective influence of dentin thickness upon cytotoxicity of dentin contacting materials. J Endod 31:396–399