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Cytotoxicity evaluation of eluates from universal adhesives by real-time cell analysis

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The aim of this study was to evaluate the cytotoxicity of universal adhesives on L929 mouse fibroblast cell line by using a real-time cell analysis. In order to obtain extract, six different cured dental adhesives were immersed in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C for 24 h. A real-time cell analysis system was used to assess cytotoxicity of the dental adhesives. After seeding 25,000 cells/300 μL/well cell suspensions into the wells of an e-plate, fibroblasts were exposed to extracts of tested adhesives at varying dilutions (1:1, 1:2, and 1:10) and observed at every 30 min intervals for 72 h. Three-way ANOVA one factor repeated measures were used to analyze the results (α=0.05). All tested adhesives induced cell viability loss, cell morphology alteration, and cell death depending on extract concentration and time. Cell viability of L929 cells to between 44 and 10% for 1:1 diluted extracts, at 72 h, when compared to the negative control.

Keywords: Cytotoxicity, Universal adhesives, Dental adhesives, Mouse fibroblast cells, Real time cell analysis

INTRODUCTION

Dental adhesive systems are solutions of resin monomers that create an interface between the restorative resin materials and tooth hard tissues. Dental adhesive systems are generally classified into two categories, total-etch (TE) and self-etch (SE) systems. TE systems are either 3-step or 2-step; SE systems are 2-step or 1-step[1,2]. SE adhesive strategy has become very popular due to the efficiency offered by the simplified bonding procedures[3]. In recent years, a new type of adhesive category, "universal" or "multi-mode", has been introduced. They are basically 1-step SE systems but can be applied in three different modes; TE, SE, or selective-etch[4]. Additionally they can be used to provide bond to various substrates besides tooth structure[5].

Phosphoric acid application to enamel and dentin which is employed in TE mode of universal adhesives, removes the smear layer from the dentin surface, resulting in opening of the dentin tubules, thus may severely increase the permeability of dentin[6]. Increased dentin permeability enhances the possibilities of pulpal insult, either by bacterial products[7] or by cytotoxic leachable components from the restorative materials[8,9]. It is known that some components of the adhesive systems are capable of penetrating dentin tubules up to 200–300 μm in pulpal direction[10,11].

Numerous investigators have focused on bonding strength of universal adhesives[12-14], or on their effects on microleakage[15]. Nevertheless, a critical concern for dentin adhesive systems is biologic compatibility since these adhesives generally remain in close contact with living dental tissues over a long period of time.

Studies investigating the biocompatibility of universal adhesives are still limited, and none have performed cytotoxicity testing by real-time cell analysis (RTCA). To this respect, the aim of this study was to evaluate the of cytotoxicity universal adhesives, with real-time and continuous monitoring of cell viability.

MATERIALS AND METHODS

Six different universal adhesives were evaluated in this study; Gluma Bond Universal (GBU; Heraeus Kulzer, Hanau, Germany), Prime&Bond Universal (PBU; Dentsply DeTrey, Konstanz, Germany), Clearfil Universal Bond Quick (CUB; Kuraray Noritake, Tokyo, Japan), All-Bond Universal (ABU; Bisco, Schaumburg, IL, USA), G-premio bond (GPB; GC, Tokyo, Japan), and Single Bond Universal (SBU; 3M ESPE, St Paul, MN, USA). The detailed composition and the manufacturers' instructions for curing of these adhesives are listed in Table 1.

Sample preparation

The extract medium of six adhesives investigated in this study was prepared as previously described[16]. One hundred microliters of each adhesive was placed in the center of each well of a 6-well plate with a micropipette, thinned with oil-free compressed air to completely cover the bottom of the well (well diameter: 34.8 mm) and create a uniform film thickness in order to maximize the standardization of the area in contact with the medium. Adhesives were then photo-polymerized with a LED curing light (Elipar DeepCure-S LED Curing Light, 3M ESPE) according to manufacturers' instructions. The

Color figures can be viewed in the online issue, which is available at J-STAGE.

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Table 1 Test materials and their composition according to manufacturers

Brand	Lot number	Chemical composition	pH	Recommended irradiation time (s)	Manufacturer
Gluma Bond Universal (GBU)	K010029	UDMA, MDP, 4-META, acetone, water	1.6–1.8	10	Heraeus Kulzer, Hanau, Germany
Prime&Bond Universal (PBU)	1711000026	UDMA, PENTA, Polymerizeable dimethacrylate resin, Polymerizeable trimethacrylate resin, acetone	2.5	10	Dentsply DeTrey, Konstanz, Germany
Clearfil Universal Bond Quick (CUB)	BV0101	Bis-GMA, HEMA, 10-MDP, Ethanol Hydrophilic amide monomers, Colloidal silica, Silane coupling agent, Sodium fluoride, dl-Camphorquinone, Water	2.3	10	Kuraray, Okayama, Japan
All-Bond Universal (ABU)	1600005763	MDP, Bis-GMA, HEMA, ethanol, water, initiators	3.2	10	Bisco, Shaumburg, IL, USA
G-premio bond (GPB)	1708162	10-MDP, 4-META, MDTP, methacrylate adic ester, distilled water, acetone, photo initiators, silica fine powder	1.5	10	GC, Tokyo, Japan
Single Bond Universal (SBU)	640012	MDP Phosphate Monomer, Dimethacrylate resins, HEMA, Vitrebond™ Copolymer, Filler, Ethanol, Water, Initiators, Silane	2.7	10	3M ESPE, St. Paul, MN, USA

UDMA: Diurethane dimethacrylate, MDP: 10-methacryloyloxydecyl dihydrogen phosphate, 4-META: 4-methacryloyloxyethy trimellitate anhydride, PENTA: Dipentaerythritol pentaacrylate phosphate, Bis-GMA: bisphenol A-glycidyl methacrylate, HEMA: 2-hydroxyethyl methacrylate, MDTP: 10-methacryoyloxydecyl dihydrogen thiophosphate

distal end of the curing tip was in contact with the plate bottom during polymerization.

After that, 2 mL of Dulbecco's Modified Eagle Medium (DMEM) was added per well as the extract medium. All test samples were prepared in a sterile laminar flow cabinet (Safe 2020, Thermo Fisher Scientific, Langenselbold, Germany). The plate was placed in incubator (Sanyo MCO-20AIC CO2 Incubator, SANYO Electric, Osaka, Japan) and left at 37°C for 24 h. All the adhesive samples were prepared and extracted in culture medium within the same day. After incubation, the extract medium was filtered through a sterile 0.22/μm syringe filter[16,17]. Original extracts were collected and then serially diluted in cell culture medium to obtain 1:2 and 1:10 before further testing. Cells in 2 mL of complete DMEM alone were used as a negative control.

Cell culture

L929 mouse fibroblast cells (ATCC CCL-1) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) containing Penicillin (100 Units/mL) and Streptomycin (100 μg/mL). Cells were cultured in T75 tissue culture flasks in a humidified incubator with a 5% CO_2 atmosphere at 37°C. When cells reached to 80% confluency, they were trypsinized. After the cells became detached, media was added to the flask. The cell resuspension was centrifuged at 400× g for 5 min. The pellet was resuspended with the same media and counting of the cells were performed by using a hemocytometer.

Cytotoxicity assay with real-time cell analysis

A RTCA system, iCELLigence (ACEA Biosciences, San Diego, CA, USA), was used to assess cytotoxicity of the dental adhesives according to the instructions of the manufacturer. Briefly, system uses specially designed disposable 8-well electronic microtiter plates (E-plate). On the bottom of the wells, gold microelectrode rows are embedded, and 4 of them are removed from the center of each well to allow monitoring of the cells by using microscope. Cellular content changes on these electrodes are detected by the system using the changes in electrical impedance of these sensor electrodes. Electrical impedance changes are converted to a unitless parameter called "Cell Index (CI)" by the iCELLigence software. Increasing of the attached cell number of E-plate surface will also increase the CI. As well as the cell number morphological parameters including cell size, shape and strength of cell adhesion will also affect the changes in CI.

According to the proliferation experiment, the optimum cell number of L929 cells for cytotoxicity experiment was determined as 25×10^3 cells/well and cells were seeded to each well of E-Plate. Then, the proliferation, attachment and spreading of the cells were monitored

every 15 min by the iCELLigence system. Twenty four hours after seeding, while cells were in log phase, they were exposed to extracts of adhesive materials. Data were taken every 30 min to confirm the proliferation percentages of the cells. Measurements were recorded for 72 h after the addition of the extracts. Untreated cells were set as the negative control. For data analysis, the baseline CI (B-CI) was determined by subtracting the CI of a well with only culture media from the CI for a cell-containing well. For the statistical evaluation of the results, five experimental tests were conducted independently.

Cell morphology analysis

The morphologic alteration of L929 cells was observed directly using an inverted microscope (Leica DMIL Inverted Fluorescence, Heerbrugg, Switzerland) at 200× magnification and photographed in 24 , 48 and 72 h with an attached camera (Leica MC170 HD).

Statistical analysis

Data was analyzed using 3-way ANOVA one factor repeated measures. Commercially available statistical analysis software (SAS University Edition 2019, SAS Institute, Cary, NC, USA) was used for all analyses (α=0.05).

RESULTS

Cell viability results

Cell viability results were normalized against the untreated cells, which were set as the negative control having 100% cell viability. All tested adhesives were found cytotoxic on L929 cells, but the extent of the effects varied between the materials depending on chemical composition and extract concentrations.

The results showed that, after exposure to the extract of GBU for 24 h at a dilution of 1:1, cell viability significantly reduced to 43% and this decrease is statistically significant compared to other tested adhesives (p<0.05). GBU, GPB, ABU and PBU shows most cytotoxic affects in L929 cells for 48 and 72 h at a dilution of 1:1, and no significant difference was observed between those adhesives (p>0.05, Table 2, Figs. 1 and 2). According to the analysis of 1:2 diluted extracts of universal adhesives, GBP, GBU and PBU showed most cytotoxic effects in L929 cells at 24 h (p<0.05), but there was no difference between other the tested adhesives and control (p>0.05). For 1:2 diluted extracts, SBU exhibited the least cytotoxic effects at both 24 and 48 h (p<0.05, Table 2, Figs. 3 and 4). For 1:10 diluted extracts cell viability decreased to 88, 75 and 65% for CUB, PBU and GBU respectively at 24 h, and were statistically significant compared to other tested adhesives (p<0.05). Other tested time points (48 and 72 h) PBU was the most cytotoxic one at a dilution of 1:10 (p<0.05, Table 2, Figs. 5 and 6).

Cell morphology results

L929 cells in the culture medium (negative control) showed typical fibroblastic morphology. Different levels of morphologic alterations were observed with the universal

Table 2 Mean (standard error) of CI values of tested adhesives

	Dilution								
	1:1			1:2			1:10		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control	0.531 (0.01)^{b}	0.876 (0.02)^{b}	1.937 (0.02)^{b}	0.547 (0.01)^{a}	1.147 (0.02)^{b}	2.173 (0.02)^{b}	0.587 (0.01)^{a}	1.126 (0.02)^{b}	1.928 (0.02)^{b}
ABU	0.270 (0.01)^{ad}	0.236 (0.02)^{a}	0.199 (0.02)^{a}	0.500 (0.01)^{a}	0.832 (0.02)^{ad}	1.648 (0.02)^{a}	0.547 (0.01)^{ab}	0.890 (0.02)^{a}	1.466 (0.02)^{a}
CUB	0.349 (0.01)^{c}	0.403 (0.02)^{cd}	0.644 (0.02)^{c}	0.512 (0.01)^{a}	0.892 (0.02)^{ae}	1.690 (0.02)^{a}	0.474 (0.01)^{bd}	0.877 (0.02)^{a}	1.459 (0.02)^{a}
GBP	0.334 (0.01)^{ac}	0.217 (0.02)^{a}	0.187 (0.02)^{a}	0.379 (0.01)^{b}	0.476 (0.02)^{c}	0.769 (0.02)^{c}	0.529 (0.01)^{ab}	1.047 (0.02)^{be}	1.660 (0.02)^{c}
GBU	0.239 (0.01)^{d}	0.327 (0.02)^{ac}	0.472 (0.02)^{d}	0.398 (0.01)^{b}	0.751 (0.02)^{d}	1.429 (0.02)^{d}	0.349 (0.01)^{c}	0.747 (0.02)^{c}	1.057 (0.02)^{d}
PBU	0.299 (0.01)^{acd}	0.257 (0.02)^{a}	0.210 (0.02)^{a}	0.357 (0.01)^{b}	0.392 (0.02)^{c}	0.374 (0.02)^{e}	0.420 (0.01)^{cd}	0.442 (0.02)^{d}	0.731 (0.02)^{e}
SBU	0.357 (0.01)^{c}	0.453 (0.02)^{d}	0.852 (0.02)^{e}	0.521 (0.01)^{a}	0.989 (0.02)^{e}	1.908 (0.02)^{f}	0.534 (0.01)^{ab}	0.988 (0.02)^{ae}	1.692 (0.02)^{c}

Means sharing a letter are not significantly different (p>0.05).
Letters compare means in each column (time periods).
Lower value indicates higher cytotoxicity.
GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

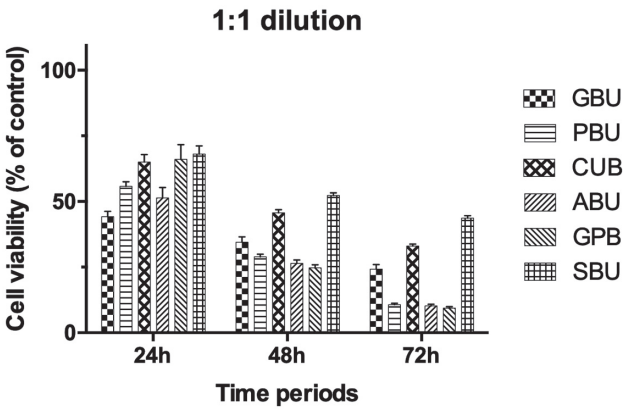


Fig. 1 Cellular viability of L929 mouse fibroblasts after treatment with 1:1 diluted extracts of tested adhesives at 24, 48 and 72 h time points.
Results were normalized with respect to negative control and given as mean. Error bars indicate standard deviation.

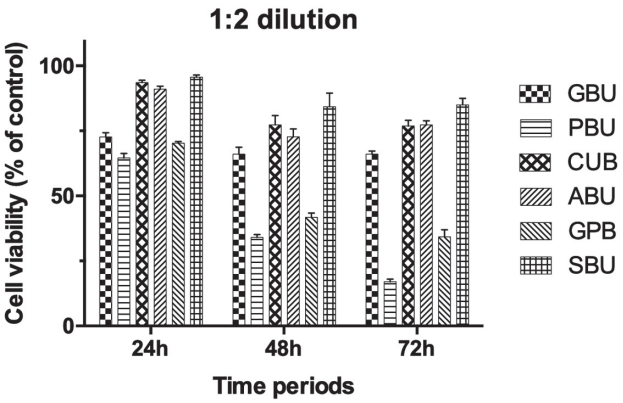


Fig. 3 Cellular viability of L929 mouse fibroblasts after treatment with 1:2 diluted extracts of tested adhesives at 24, 48 and 72 h time points.
Results were normalized with respect to negative control and given as mean. Error bars indicate standard deviation.

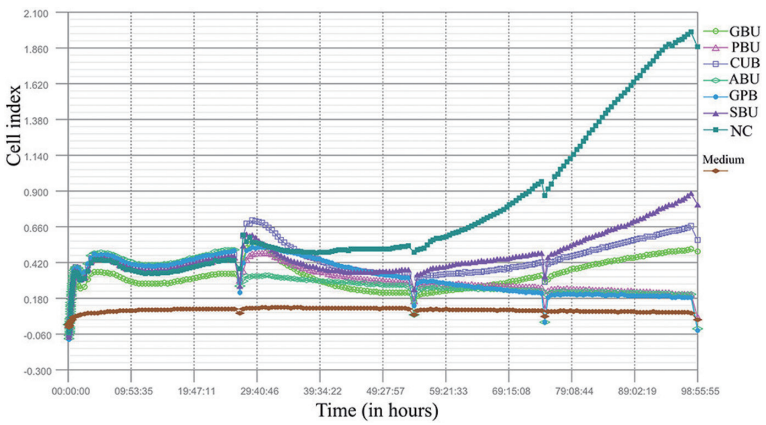


Fig. 2 Dynamic monitoring of treated with 1:1 diluted adhesive extracts in cultured L929 cells adhesion and cell proliferation. GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

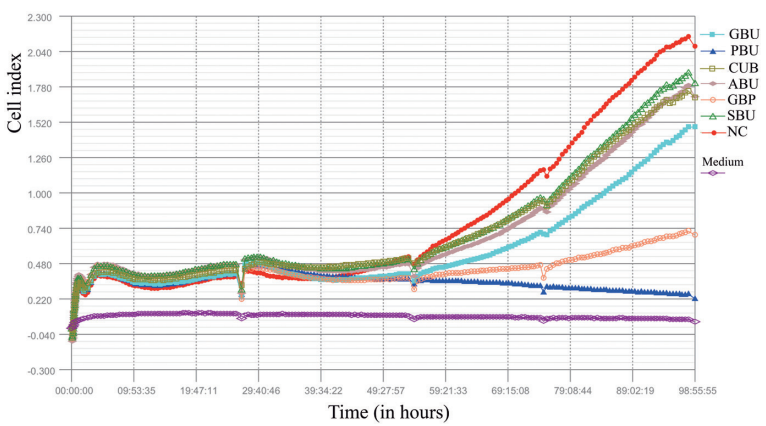


Fig. 4 Dynamic monitoring of treated with 1:2 diluted adhesive extracts in cultured L929 cells adhesion and cell proliferation. GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

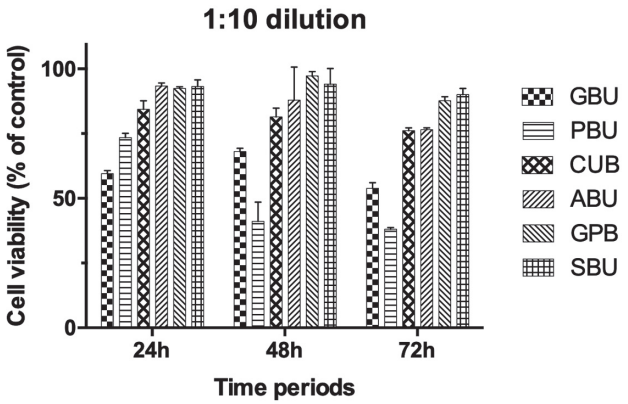


Fig. 5 Cellular viability of L929 mouse fibroblasts after treatment with 1:10 diluted extracts of tested adhesives at 24, 48 and 72 h time points.
Results were normalized with respect to negative control and given as mean. Error bars indicate standard deviation.

adhesives, depending on extract concentrations. One to one diluted extracts of CUB and SBU had little effects on the cell morphology compared to control at the 72 h; majority of the cells remained spindle shaped. However, 1:1 diluted extracts of other tested universal adhesives caused the greater part of the cells to become small, retracted and rounded, with condensed and fragmented nuclei morphology (Fig. 7). One to two diluted extracts of SBU, CUB and ABU had no obvious effect on the morphology of L929 cells, compared with to control at the 72 h. One to two diluted extracts of GPB and GBU had moderate effect on the morphology of cells; PBU showed the most dramatic effect on cell morphology. The effects of all tested universal adhesives on cell morphology were concentration dependent (Fig. 8). Accordingly, there was a decrease in the number of retracted round cells; most cells remained spindle shaped when 1:10 diluted extracts of universal adhesives were applied (Fig. 9). Cell morphology analysis confirmed the RTCA results. As the toxicity increased, the L929 cells become smaller,

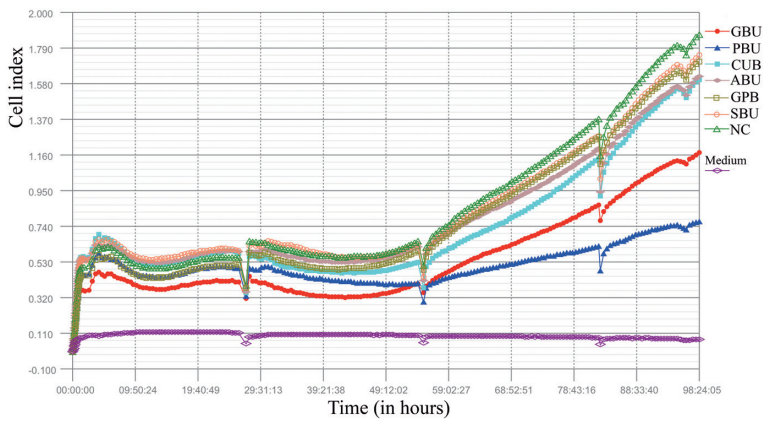


Fig. 6 Dynamic monitoring of treated with 1:10 diluted adhesive extracts in cultured L929 cells adhesion and cell proliferation.
GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

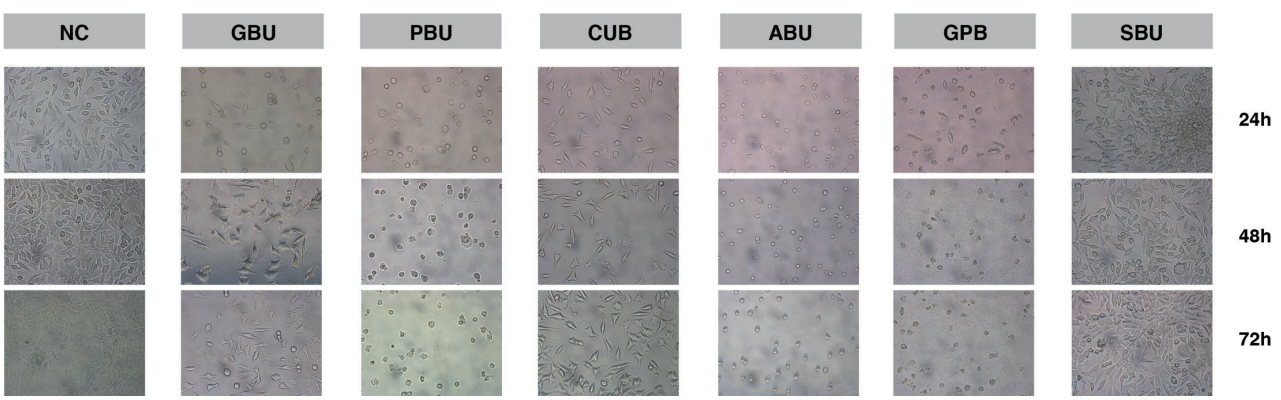


Fig. 7 Representative light microscope images of cellular morphology of L929 mouse fibroblasts cells after treatment with 1:1 diluted adhesive extracts.
Original magnification: 200×, GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

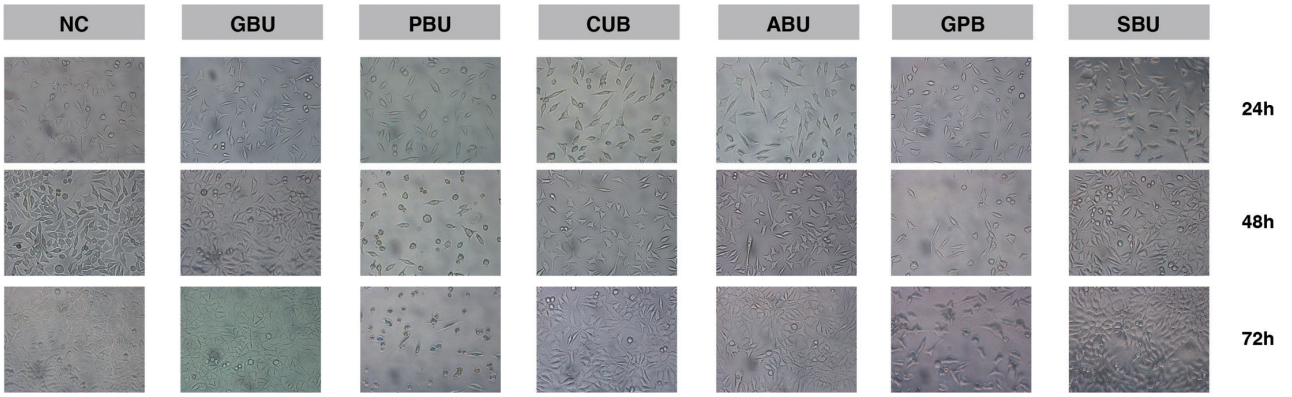


Fig. 8 Representative light microscope images of cellular morphology of L929 mouse fibroblasts cells after treatment with 1:2 diluted adhesive extracts.
Original magnification: 200×, GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

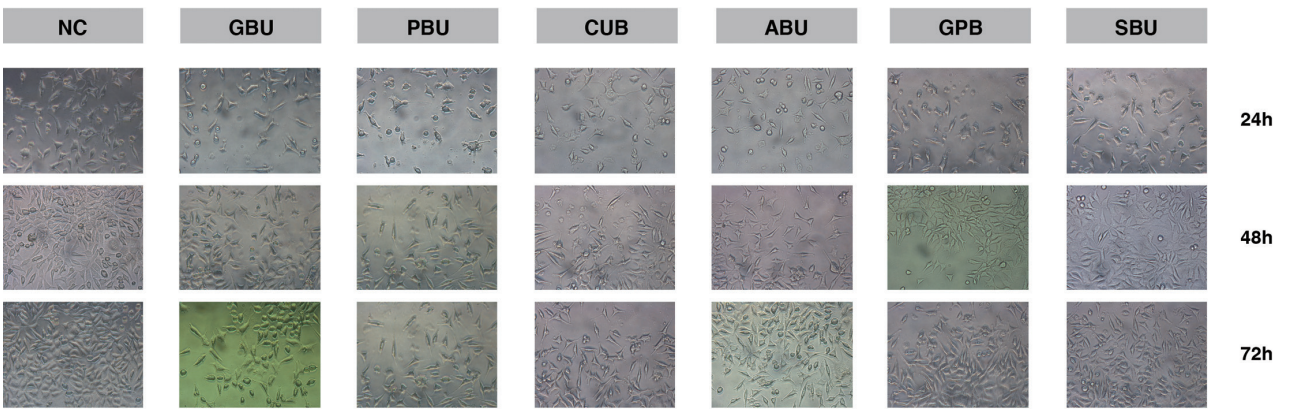


Fig. 9 Representative light microscope images of cellular morphology of L929 mouse fibroblasts cells after treatment with 1:10 diluted adhesive extracts.
Original magnification: 200×, GBU: Gluma Bond Universal, PBU: Prime&Bond Universal, CUB: Clearfil Universal Bond Quick, ABU: All-Bond Universal, GPB: G-premio bond, SBU: Single Bond Universal, NC: negative control

rounded and retracted, which is characteristic feature of cell death[18,19].

DISCUSSION

Traditionally, dental adhesives contain acrylic resin monomers such as bisphenol A-diglycidyl methacrylate (Bis-GMA), triethyleneglycol dimethacrylate (TEGDMA), urethane dimethacrylate or 1,6-di(methacryloyloxyethylcarbamoyl)-3,30,5-trimethylhexaan (UDMA), 2-hydroxyethyl methacrylate (HEMA), organic solvents such as water, acetone, ethanol, and initiators, inhibitors, additional filler particles[20]. It has been documented that resin monomers, such as Bis-GMA, UDMA, TEGDMA and HEMA causes significant cytotoxicity[21-23]. Bis-GMA may induce cytotoxicity and prostanoid production in pulp cells, leading to pulpal inflammation or necrosis with reactive oxygen species (ROS) production[24]. In addition to that, Bis-GMA induces apoptosis and causes substantial depletion of intracellular Glutathione (GSH) content in human gingival fibroblasts[25]. UDMA is another monomer frequently used in dental adhesives. Toxicity mechanism of UDMA is associated with ROS production, GSH depletion, cell cycle disturbance and cell apoptosis/necrosis[26]. Moreover, UDMA stimulates mRNA expression of cyclo-oxygenase-2, heme oxygenase-1 and carboxylesterase-2 in pulp cells[27]. TEGDMA is the co-monomer, which commonly used as diluent of many resin-based dental materials[28]. Due to its lipophilic character, TEGDMA can easily penetrate the cytosol and membrane lipid compartments of mammalian cells[29]. An adverse effect of TEGDMA on cell proliferation and exert proapoptotic and toxic effects on THP-1 cells were reported[30]. Furthermore, TEGDMA can induce decrease of intracellular GSH and cause DNA damage[31]. HEMA is one of the monomers in bonding agents that lead to certain safety concerns. HEMA can diffuse rapidly through dentin[32], and lead to toxicity on pulp cells[33]. It can induce growth suppression

and morphological changes of several kinds of cells[34-36]. It could also induce apoptosis through the topoisomerase I inhibitor topotecan[37]. Previous investigations showed that, Bis-GMA, is the most toxic, followed by UDMA, TEGDMA and HEMA[21,38].

Other than the conventional monomers present in the adhesive systems; the cytotoxicity of the acidic functional monomers is also an important issue. Various SE adhesives contain different acidic functional monomers such as 10-methacryloyloxydecyl dihydrogenphosphate (10-MDP), 2-(methacryloyloxyethyl)phenyl hydrogenphosphate (Phenyl-P) and 4-methacryloyloxyethyl trimellitate anhydride (4-META). 10-MDP is the most often used acidic monomer in recently marketed SE and universal adhesives, which contains a dihydrogen phosphate group for etching the tooth and a methacrylate group for cross-linking with other resin monomers[20]. 10-MDP promotes an inflammatory response and suppresses odontoblastic differentiation of human pulp cells[39]. In addition to its inhibitory effect on odontoblastic differentiation, 10-MDP is also thought to readily interact with calcium produced by odontoblast-like cells and directly depress mineralization[40]. 4-META is another functional monomer used in adhesives. Besides its self-etching properties, it provides adhesion to alloys[41] and is commonly added to universal adhesives. Although studies investigating the cytotoxicity of 4-META are limited, it was shown that 4-META resin containing luting material possessed high level biocompatibility with dental pulp cells[42,43]. Beyond the effects of any single monomer/chemical agent in any given adhesive system, the interaction among them is also important. Synergistic effects were detected when combinations of TEGDMA with UDMA or particularly with Bis-GMA were tested[21].

Apart from the potential cytotoxic effects of monomers in adhesive systems, the cytotoxicity of the photo-initiators should be of consideration. Conventionally, camphorquinone (CQ) or Diphenyl(2,4,6-trimethylbenzoyl) phosphine oxide (TPO) are added to adhesive systems as a photo-initiator[20]. CQ may elute from dental resin materials *in vitro*[44]. Previous studies have revealed that CQ and TPO exhibit cytotoxicity to many cells[45,46].

Besides the composition, pH is another significant factor that may affect the cytotoxicity of the adhesives. It is reported that the toxicity of the adhesives may increase with elevated acidity levels[47]. All of the adhesives used in this study have different levels of acidity (pH of GPB=1.5, GBU=1.6–1.8, CUB=2.3, PBU=2.5, SBU=2.7, ABU=3.2). However, it was not possible to evaluate the sole effect of acidity on cytotoxicity and it is beyond the scope of the current study.

Although universal adhesives have a similar composition to traditional one-step SE adhesives, some universal adhesives may contain silane in their composition to eliminate the silanization step when bonding to glass ceramics, hybrid materials and resin composites[4,48]. Little is known about the toxicity of this compound when incorporated into the adhesive system. Since it would be part of the hybrid layer and in close contact with the pulp-dentin complex it may be of issue; however, further research would be necessary to test this hypothesis.

Previous investigations have demonstrated that, depending on the remaining dentin thickness, monomers such as HEMA, CQ, Bis-GMA, 10-MDP and UDMA could be eluted from the dental materials and are able to diffuse through the dentin[49-51]. Therefore, biocompatibility of adhesives is a significant attribute since adhesives could be applied on deep dentin tissue near the pulp, where tubular density and diameter are greatest.

Commonly used methods and techniques to determine dental biomaterial-induced cytotoxicity are 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT) assay[52], 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzol-disulfonate (WST-1) assay[53], 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT)[38] and lactate dehydrogenase (LDH) assay[54]; and each method uses a different aspect of cellular metabolism as a means of quantifying live cells. However, all of these methods are single end-point qualitative measures of cell fitness[23]. Unlike above-mentioned endpoint approaches, real-time assay systems provide for the tracking of cellular growth over the entire time course of an experiment. RTCA was used by researchers to assess the cytotoxicity of different dental materials[23,55,56]. One of the RTCA devices is iCELLigence system (ACEA Biosciences, San Diego, CA, USA). This system uses microelectronic biosensor technology to do dynamic, real-time, label-free, and non-invasive analysis of cellular events, including cell number change, cell adhesion, cell viability, cell morphology, and cell motility[57]. Furthermore, the iCELLigence system provides for the calculation of time-dependent physiological EC50 values, which can be more illuminating than single EC50 end-points of classic toxicity testing. Moreover, the RTCA was proved to be effective to determine cell densities in small culture volumes and the reaction of live cells to chemical exposure[58]. Conversely, RTCA is not possible with the conventional end-point assays. The RTCA system we used in our study could therefore provide more useful data regarding cell viability.

In the aforementioned studies, the cytotoxicity of the monomers was evaluated by using end-point assays. However, very few studies evaluated cytotoxic effects of various dental materials and their various ingredients using RTCA. Other than real-time cytotoxicity assessment of temporary cements[56] resin cements[55], orthodontic resin composites[58] one specific study assessed cytotoxicity of frequently used monomers in dentistry, namely Bis-GMA, UDMA, TEGDMA and HEMA on human gingival fibroblasts with RTCA[23]. They compared the relative toxicities of these monomers against each other. While HEMA had a relative toxicity of 1, TEGDMA had 3, UDMA had 56 and Bis-GMA had 140. This indicates that Bis-GMA is highly toxic, especially when compared to TEGDMA and HEMA. However, many dental adhesives contain Bis-GMA in their formulations.

Two adhesives, CUB and ABU, evaluated in our study contain Bis-GMA, whereas GBU does not. These two adhesives showed varying levels of toxicity compared to the other adhesives. Therefore Bis-GMA alone may not be responsible for the overall toxicity of these adhesives. Manufacturers of other adhesives used in this study did not fully disclose the specific chemical name of their monomers; instead they used several general terms like methacrylate acidic ester, dimethacrylate resin, polymerizable dimethacrylate resin, and polymerizable trimethacrylate resin. Moreover, it is not viable to compare the cytotoxicity of adhesives with respect to a single monomer. As discussed above, adhesives are comprised of many chemicals and these may also affect the overall toxic potential of the adhesive. Authors of the above-mentioned study revealed a close match between the RTCA and end-point assay data[23]. They concluded that RTCA could be used as a rapid monitoring tool for cellular viability and be applied in toxicity testing of xenobiotics using in vitro cell cultures. This could indicate that RTCA could be an alternative to other assays.

In the current study the monomers in GBU were mainly composed of UDMA, MDP and 4-META. For PBU, monomers included UDMA, PENTA, polymerizeable dimethacrylate resin and polymerizeable trimethacrylate resin. CUB and ABU which contained similar monomers, such as Bis-GMA, HEMA and 10-methacryloyloxydecyl dihydrogen phosphate (MDP). MDP, 4-methacryloyloxyethy trimellitate anhydride (4-META), 10-methacryoyloxydecyl dihydrogen thiophosphate and methacrylate acidic ester were the main monomers in GBP. For SBU monomers included MDP, HEMA, dimethacrylate resins and Vitrebond™ Copolymer. In the present study, all the evaluated adhesive systems presented mild to severe toxicity, according to ISO norms 10993-5:2009. The reduction of cell viability was ranging between 60–90% when compared with the negative control at the 72 h for 1:1 diluted extracts. For the 1:1 diluted extracts at 72 h, GPB, ABU and PBU demonstrated the highest cytotoxic potential and SBU the least ($p<0.05$). Substantially, SBU induced the lowest toxic effect on L929 cells compared to other tested universal adhesives at all time intervals for 1:1 and 1:2 dilutions. Contrary to this, PBU showed the strongest cytotoxic effect at all time intervals for 1:2 and 1:10 dilutions. Nevertheless, all tested universal adhesives were cytotoxic against L929 cells. However, statistical analysis revealed different levels of toxicity. It seems logical to related these results to the differences in the composition of the monomers of the universal adhesives, since the previous studies have shown that residual monomers such as HEMA[59], TEGDMA[60] or Bis-GMA[61] may elute from polymerized dental adhesives. Moreover, the presence of acidic methacrylates in self-etch adhesives could also cause the reduction of cell culture medium pH resulting in cell damage. Organic and inorganic contents of the tested adhesive systems are different from each other, and there was no information available from the manufacturers on the percentage by weight of the matrix and the inorganic fillers; so, this aspect could not be discussed here.

Although there are many researchers studying the cytotoxicity of different generations of dental adhesives[8,16,62-65], studies on the cytotoxicity of universal adhesives are even limited[66,67]. In addition to that, despite the above-mentioned studies, to the authors knowledge, there are no studies that have evaluated the cytotoxicity of universal adhesives using real-time and continuous analysis of cell vitality. A study investigated cytotoxic effects of universal [Scothbond Universal (3M/ESPE, Seefeld, Germany)], SE [Adper Easy One (3M/ESPE), Clearfil SE Bond (Kuraray Medical, Okayama, Japan)] and TE [Adper Single Bond 2 (3M/ESPE Dental Products, St. Paul, MN, USA), Adper Scotchbond Multi-Purpose (3M/ESPE Dental Products)] adhesive systems with MTT assay on L929 mouse fibroblast cells at 24 h[66]. Authors concluded that all tested adhesive systems significantly reduced the cell metabolism, regardless of adhesive system. Universal adhesives had similar cytotoxic properties with other tested adhesive systems. They also demonstrated that the adhesives caused intense morphological alterations and cell membrane damage. Another study investigated cytotoxic effects of universal [Scotchbond Universal (3M ESPE), Ambar Universal (FGM, Joinville, SC, Brazil)], SE [OptiBond All-In-One (Kerr, Orange, CA, USA)] and TE [Adper Single Bond 2 (3M ESPE), Adper Scotchbond Multi-Purpose (3M ESPE), Ambar (FGM)] adhesive systems with MTT assay on 3T3 fibroblast cells[67], however, the authors have not provided information about the duration of exposure of the extracts to the cells. The authors concluded that all tested adhesive systems except universal adhesives, reduced cell viability between 26.04 and 56.57%. The universal adhesives Scotchbond Universal and Ambar Universal reduced cell viability to 2.13 and 3.57%, respectively. It is difficult to compare the results of the above-mentioned studies and the current study because of the many variations in experimental conditions such as cytotoxicity test method, cell type usage, method of obtaining elutes and exposure time.

This research has some limitations. Firstly, only single cell line was used for the assessment of cytotoxicity. The differences in cell lines and test models could also result in variability in cytotoxic responses. Cytotoxicity analysis using an established cell line, in this instance L929 mouse fibroblasts, would provide a general assessment. However, it was stated that toxic substances showed similar results on L929 fibroblasts and human gingival fibroblasts, suggesting that L929 fibroblasts assays may serve as sufficient screening models for *in-vitro* evaluation of cytotoxicity[68]. Further *in-vitro* investigations should focus on cytotoxic effect of these materials on human-derived cells as well as different testing models and methods. In this research, the curing light distance was standardized at 1 mm away from the samples. Clinically, this may be challenging to achieve because of depth of the cavity and the presence of matrix system in some occasions. Curing light-related parameters, including light type, distance, intensity

and curing modes may also influence the cytotoxicity of universal adhesives since they could have influence on monomer conversion and thus residual monomer. The absence of dentin barrier is another limitation of the present study, as it is well known that dentin reduces the adhesive's diffusion and consequent toxicity. Moreover, the acidic monomers are gradually buffered by the tooth substrate and they lose their ability to further etch dentin and therefore, one could expect less pronounced cytotoxicity *in-vivo*.

CONCLUSION

This preliminary study employing real-time cell viability analysis showed that all tested universal adhesives were cytotoxic to L929 cells at varying rates. Further studies should focus on more clinically relevant scenario in which dentin would be present as a barrier.

CONFLICT OF INTEREST

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article. There is no funding source for this research.

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