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Cytotoxic effects of contemporary bulk-fill dental composites: A real-time cell analysis

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The aim of this study was to evaluate the cytotoxicity of contemporary flowable and paste-like bulk-fill dental composites by using a real-time cell analysis. In the present paper, cytotoxicity levels of five flowable, five paste-like bulk-fill composite materials and one conventional flowable, one conventional paste-like resin composite were examined on L929 mouse fibroblast cell line. After seeding 25,000 cells/300 μL/well cell suspensions into the wells of an E-plate, test materials were added and observed at every 30 min intervals for 72 h. Kruskal Wallis H and Mann Whitney *U* multiple comparison tests were used to analyze the results. Pre-reacted glass-ionomer (PRG) containing bulk-fill composites were severely toxic at all time points (24, 48 and 72 h, $p<0.05$). None of the tested composites demonstrated high cell viability (>70%) at 48 and 72 h. Flowable and paste-like composites of the same brand exhibited similar cytotoxic properties ($p>0.05$).

Keywords: Bulk-fill composites, Cytotoxicity, Real-time cell analysis, iCELLigence system

INTRODUCTION

The use of resin composite have become routine in dental practice for restoration of posterior teeth due to the increasing aesthetic expectations of patients and concerns regarding the possible side effects of amalgam restorations. Although composite resins are considered to be more aesthetic in comparison with amalgam restorations, they have disadvantages such as sensitivity in technical implementation, exhibiting polymerization shrinkage, having limited depth of cure, and lower physico-mechanical properties compared to amalgams[1-3]. Conventional composites are usually placed in 2 mm increments to ensure proper degree of conversion. This leads to prolongation of the restoration placement duration and increases the risk of air bubble formation and moisture contamination between layers[4,5]. These limitations led to development of a new class of composite resin type known as "bulk fill" and sub-classified as either "paste-like (full body)" or "flowable (base)"[6]. According to manufacturers' these materials can be properly photo-polymerized even when they are applied in thick layers up to 4–5 mm, while exhibiting low polymerization stresses[7,8].

Monomer conversion is the major contributing factor to the properties of the final polymer network. During the light polymerization of the composite resins, not all of the monomers participate in the polymer network and remain as unreacted free monomers. Cytotoxicity mechanisms are predominantly associated with the leaching of these residual monomers formed during monomer-polymer conversion[9]. For bulk-fill composites a conversion rate higher than 55% is in the clinically acceptable range but is still lower than that of conventional composites[10].

In addition to leaching of unreacted monomers, cytotoxicity may also arise from the release of initiators from the organic resin or metal ions from the inorganic filler[11]. Such cytotoxic restorative materials can induce short-term and long-term adverse tissue reactions varying from postoperative sensitivity to irreversible pulp damage[12].

Studies investigating the cytotoxicity of bulk-fill composites are still limited[13-15] and none have compared the cytotoxicity of the flowable and paste-like bulk-fill composite of the same brand. Moreover, to date there is no study investigating the cytotoxicity of bulk-fill composites using a real time cell analysis. Therefore, this study aimed to evaluate the cytotoxicity of contemporary flowable and paste-like bulk-fill composites, utilizing real-time and continuous monitoring of cell vitality.

MATERIALS AND METHODS

Five flowable bulk-fill composite materials [Venus Bulk Fill-VBF (Heraeus Kulzer, Hanau, Germany), Filtek Bulk Fill Flowable-FBF Restorative (3M Dental Products, St. Paul, MN, USA), Smart Dentin Replacement-SDR (Dentsply DeTrey, Konstanz, Germany), x-tra base-XTB (VOCO, Cuxhaven, Germany), Beautifil-Bulk Flowable-BBF (Shofu, Kyoto Japan)]; five paste-like bulk-fill composite materials [Tetric EvoCeram Bulk Fill-TEB (Ivoclar Vivadent, Schaan, Liechtenstein), Filtek Bulk Fill Posterior Restorative-FBR (3M Dental Products),

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Table 1 Composite materials and their composition

Material Type	Material	Shade	Lot number	Matrix composition	Filler % by weight	Recommended thickness (mm)	Recommended curing time and light intensity for each layer	Number of layer of samples	Manufacturer
Bulk-fill flowable	Venus Bulk Fill (VBF)	Universal	10108	UDMA, EBPADMA, Ba-Al-F silicate glass, YbF_3, SiO_2	65	4	20 s, >500 mW/cm^2	1 (4 mm)	Heraus Kulzer, Hanau, Germany
	Filtek Bulk Fill Flowable (FBF)	Universal	N875811	Bis-GMA, UDMA, bis-EMA, Procrylat resin, YbF_3, zirconia filler, silica	64.5	4	10 s, ≥1,000–2,000 mW/cm^2	1 (4 mm)	3M Dental Products, St.Paul, MN, USA
	Surefil SDR Flow (SDR)	Universal	8103	Modified UDMA, EBPADMA, TEG-DMA, Ba-Al-F-B, silicate glass, Sr-Al-F silicate glass, camphorquinone, photo-accelerator, BHT, UV stabilizer, titanium dioxide, iron oxide pigments, fluorescent agent	68	4	20 s, ≥550 mW/cm^2	1 (4 mm)	Dentsply DeTrey, Konstanz, Germany
	x-tra base (XTB)	Universal	1649539	Bis-EMA, aliphatic dimethacrylate, Ba-Al-Si-glass, YbF_3, fumed SiO_2	75	4	10 s, ≥800 mW/cm^2	1 (4 mm)	VOCO, Cuxhaven, Germany
	Beautifil-Bulk flowable (BBF)	Universal	61508	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG filler based on fluoroboroaluminosilicate glass, polymerization initiator, pigments, and others	73	4	10 s, ≥1,000 mW/cm^2	1 (4 mm)	Shofu, Kyoto, Japan
Conventional flowable	Clearfil Majesty Flow (CMF)	A2	CM0038	TGDMA, Hydrophobic aromatic dimethacrylate Silanated barium glass filler Silanated colloidal silica dl-Camphorquinone Accelerators Pigments	81	1.5	20 s, >300 mW/cm^2	3 (1.5+1.5+ 1 mm)	Kuraray, Okayama, Japan
	Tetric EvoCeram Bulk-fill (TEB)	IVA (Universal)	T39247	Bis-GMA, UDMA (%17–18), Barium glass, YbF_3, prepolimer, additives, catalysts, stabilizers, and pigments	76–77	4	10 s, ≥1,000 mW/cm^2	1 (4 mm)	Ivoclar Vivadent, Schaan, Liechtenstein
	Filtek Bulk Fill Posterior Restorative (FBR)	A2	N719050	AUDMA, AFM, UDMA,DDDMA, YbF_3, silan treated ceramic/slica/zirconia, EDMAB, Benzotriazol, Titanium dioxide,water	76.5	5	10 s, ≥1,000–2,000 mW/cm^2	1 (4 mm)	3M Dental Products
Bulk-fill paste-like	QuiXfil, Quixx Posterior (QXF)	Universal	1505000543	UDMA, TEG-DMA, dimethacrylate and trimethacrylate resins, carboxylic acid, modified dimethacrylate resin, BHT, UV stabilizer, camphorquinone, ethyl-4-dimethylaminobenzoate, silanated strontium aluminum sodium fluoride phosphate silicate glass	77	4	10 s, ≥1,000 mW/cm^2×3	1 (4 mm)	Dentsply, Konstanz, Germany
	x-tra fill (XTF)	Universal	1749292	Inorganic filler in a methacrylate matrix (Bis-GMA, UDMA, TEG-DMA)	86	4	10 s, ≥800mW/cm^2	1 (4 mm)	VOCO
	Beautifil-BulkRestorative (BBR)	Universal	071406	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG filler based on fluoroboroaluminosilicate glass, polymerization initiator, pigments, and others	87	4	10 s, 1,000 mW/cm^2	1 (4 mm)	Shofu
Conventional paste-like	Clearfil Majesty Esthetic (CME)	A2	4H0173	Bis-GMA, Hydrophobic aromatic dimethacrylate, Hydrophobic aliphatic methacrylate, Silanated barium glass filler, Pre-polymerized organic filler, dl-Camphorquinone,Initiators, Accelerators, Pigments, Others	—	1.5	20 s, >300 mW/cm^2	3 (1.5+1.5+ 1 mm)	Kuraray

UDMA: urethane dimethacrylate, EBPADMA: ethoxylated bisphenol-A-dimethacrylate, Bis-GMA: bisphenol-A-glycidyl methacrylate, Bis-EMA: ethoxylated bisphenol-A-glycol dimethacrylate, TEGDMA: triethyleneglycol dimethacrylate, Bis-MPEPP: 2,2-bis[4-(2-methacryloxy)ethoxy)phenyl]propane, S-PRG: Surface Pre-Reacted Glass, AUDMA: aromatic urethane dimethacrylate AFM: addition-fragmentation monomers, DDDMA: 1,12-dodecane dimethycrylate, EDMAB: ethyl 4-dimethyl aminobenzoate, BHT: butylated hydroxy toluene

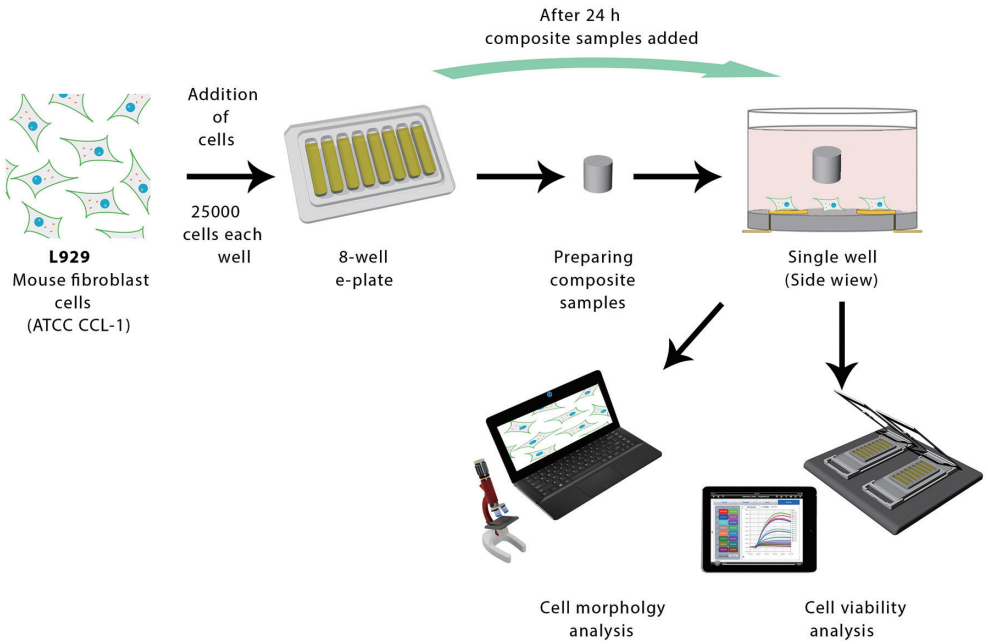


Fig. 1 Study design of cytotoxicity assay.

QuiX Fill Posterior Restorative-QXF (Dentsply DeTrey), x-tra fill-XTF (VOCO), Beautifil-Bulk Restorative-BBR (Shofu)]; one conventional flowable resin composite (Clearfil Majesty Flow-CMF, Kuraray Noritake Dental, Okayama, Japan), and one conventional paste-like composite (Clearfil Majesty Esthetic-CME, Kuraray Noritake Dental) as control, were used. Properties and details of the test materials are presented in Table 1. Study design was described in Fig. 1.

Specimen preparation

Bulk-fill composite materials were applied as one layer and conventional composites (CME and CMF) were applied as three horizontal layers into the custom made cylindrical teflon molds (height: 4 mm, diameter: 4 mm) according to manufacturers' instructions, than covered with Mylar strip and 1 mm thick glass plate. Glass plate was then pressed to the height of the mold to extrude excess material. Each layer was cured with a LED light-curing unit (SDI Radii Plus, SDI, Australia) according to the manufacturers' instructions. Light intensity was assured to be equal or higher than 1,000 mW/cm^2 which was measured using a radiometer (Hilux Ledmax curing lightmeter, Benlioglu Dental, Ankara, Turkey). All test samples were prepared in a sterile laminar flow cabinet (Safe 2020, Thermo Fisher Scientific, Langenselbold, Germany) and sterilized under UV light for 20 min. After light curing, the composite samples were stored at 37°C in the dark for 24 h in a disinfected glass container.

Cell culture

L929 mouse fibroblast cells (ATCC CCL-1) were maintained in Dulbecco's modified eagle's medium (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.

iCELLigence system

The iCELLigence system (Roche Applied Science, Penzberg, Germany - ACEA Biosciences, San Diego, CA, USA) was used to assess the cytotoxicity of tested composite resins 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.

Cell proliferation optimization using iCELLigence system

The day before to the experiment, passage of the L929 cells was confirmed and when reached 80% confluency

in a T75 flask, the cells were treated with 0.05% trypsin/EDTA. Flasks were leaved in a 37°C incubator for 1–5 min. After detached cells were observed under a microscope, trypsinization was stopped with adding 5 mL media to the flask. The cell resuspension was centrifuged at 400× g for 5 min. The pellet was resuspended with 5 mL media, and the cells were counted under a microscope using a hemocytometer and the concentrations of the cell suspensions were adjusted. Before spreading the cells to the wells of E-plate, a standard background was measured by transferring 50 µL of pre-warmed media to each well. Then E-plate was placed into the iCELLigence station and initiated the software to perform background measurement. After first step cells were seeded as 6,300, 12,500, 25,000 and 50,000 cells per well of E-plate, the total volume of wells was adjusted to 300 µL with media. E-Plate was placed on iCELLigence station located in the cell culture incubator and cells were monitored every 15 min for 48 h.

Cytotoxicity assay with iCELLigence system

According to the previous 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. Approximately 24 h after seeding, while cells were in log phase, they were exposed to composite 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 composite materials. 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 directly observed using an inverted microscope (Leica DMIL Inverted Fluorescence, Heerbrugg, Switzerland; 200×) and photographed with a camera (Leica MC170 HD).

Statistical analysis

The collected data from all groups were imported to Statistical Package for Social Sciences (SPSS) for Windows software, version 16.0 (SPSS, Chicago, IL, USA). Because the distribution of the data did not meet the requirements for normality and homogeneity of variances assumptions, the nonparametric Kruskal-Wallis one way analysis of variance by ranks and Mann-Whitney *U* tests were used for the multiple and pairwise comparisons, respectively. The confidence interval was set to 95% and $p<0.05$ was considered statistically significant.

RESULTS

Optimal density for cell proliferation and viability

Before cytotoxicity experiments, firstly, the system needs optimized seeding densities for the cells. To determine the optimum seeding density of L929 cells used in the experiments, cells were seeded into the E-plates in four different densities; 6.3×10^3, 12.5×10^3, 25×10^3 and 50×10^3 cells/well. The optimal seeding density of L929 cells for this study was determined to be 25×10^3 cells/well.

Cell viability analysis

All the tested composites were found cytotoxic on L929 cells, but the extent of the effects varied between the materials (Figs. 2 and 3). Cell viability results were normalized against the untreated cells, which are set as the negative control with 100% cell viability.

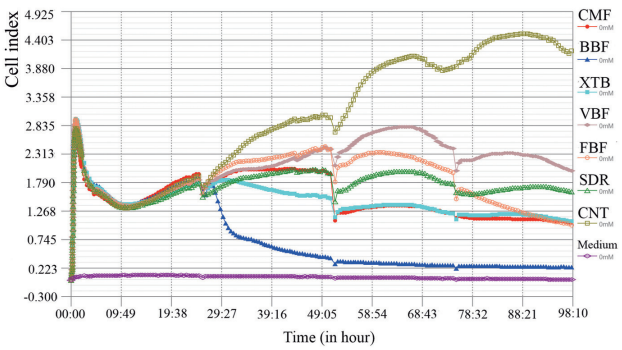


Fig. 2 Dynamic monitoring of treated with flowable composites in cultured L929 cells adhesion and cell proliferation.
CMF: Clearfil Majesty Flow, BBF: Beautifil-Bulk Flowable, XTB: x-tra base, VBF: Venus Bulk Fill, FBF: Filtek Bulk Fill Flowable Restorative, SDR: Smart Dentin Replacement, CNT: Control

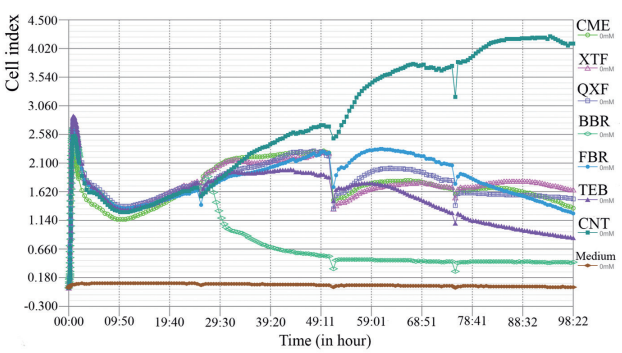


Fig. 3 Dynamic monitoring of treated with paste-like composites in cultured L929 cells adhesion and cell proliferation.
XTF: x-tra fill, QXF: QuiX Fill Posterior Restorative, BBR: Beautifil-Bulk Restorative, FBR: Filtek Bulk Fill Posterior Restorative, TEB: Tetric EvoCeram Bulk Fill, CNT: Control

When compared to other flowable composites, BBF was the most toxic one with a cell viability rate of 15% after 24 h ($p<0.05$). The cell viability reduced to 80% for VBF, 79% for FBF, 77% for SDR, 77% for CMF, and 71% for XTB at 24 h. Cell viability at 48 h was found to be 64% for VBF, 52% for FBF, 47% for SDR, 32% for XTB, 32% for CMF and 6% for BBF. At 72 h, cell viability was 49% for VBF, 39% for SDR, 26% for XTB, 26% for CMF, 25% for FBF and 5% for BBF (Table 2). Measurements carried at 72 h showed the lowest number of live cell count in all flowable composite groups, except the BBF group, which exhibited similar decline in CI at 48 h ($p>0.05$, Fig. 2).

For the paste-like bulk-fill composites BBR significantly reduced the cell viability to 20% at 24 h and this result is statistically significant compared to other tested paste-like composites ($p<0.05$). The cell viability was reduced to 85% for QXF, 85 % for CME, 83% for FBR, 83% for XTF, and 71% for TEB, at 24 h, respectively. Cell viability at 48 h were found to be 57% for FBR 50% for QXF, 47% for XTF, 46% for CME, 37% for TEB and 12% for BBR. At 72 h, cell viability was 41% for XTF, 37% for QXF, 34%, for CME, 32% for FBR, 21% for TEB and 10% for BBR (Table 2). Measurements carried out on the 72 h showed the lowest CI values for all paste-like composite groups and it's significantly different from 24 and 48 h CI values ($p<0.05$, Fig. 3).

The cytotoxicity data of flowable and paste-like composites of the same brand (CMF/CME, FBF/FBR, SDR/QXF, XTB/XTF, BBF/BBR) at 24, 48 and 72 h showed no statistically significant differences ($p>0.05$). However, BBF/BBR group had the most dramatic cytotoxic effect on L929 cells (Figs. 4–8).

Cell morphology analysis

Untreated L929 cells (negative control) were spindle shaped in appearance with extended cellular processes, filopodi, and lamellipodia. Different levels of morphologic alterations were observed with the various bulk-fill and conventional composites, depending on chemical

Table 2 Statistical analysis of cytotoxicity of tested composite materials

Time (h)	Cytotoxicity of flowable bulk-fill composites (From most to least cytotoxic)
24th	BBF>XTB>CMF, SDR>FBF, VBF>Control
48th	BBF>XTB, CMF>SDR, FBF>VBF>Control
72nd	BBF>XTB, CMF, FBF>SDR>VBF>Control
Time (h)	Cytotoxicity of paste-like bulk-fill composites (From most to least cytotoxic)
24th	BBR>TEB, XTF, FBR, CME, QXF>Control
48th	BBR>TEB>XTF, FBR, CME, QXF>Control
72nd	BBR>TEB>XTF, FBR, CME, QXF>Control

Results of Kruskal Wallis H and Mann Whitney *U* multiple comparison tests ($p<0.05$). >indicates statistical significance.
CMF: Clearfil Majesty Flow, BBF: Beautifil-Bulk Flowable, XTB: x-tra base, VBF: Venus Bulk Fill, FBF: Filtek Bulk Fill Flowable Restorative, SDR: Smart Dentin Replacement, XTF: x-tra fill, QXF: QuiX Fill Posterior Restorative, BBR: Beautifil-Bulk Restorative, FBR: Filtek Bulk Fill Posterior Restorative, TEB: Tetric EvoCeram Bulk Fill

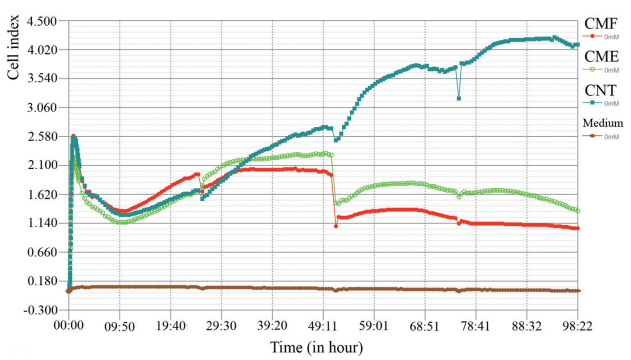


Fig. 4 Dynamic monitoring of treated with same brand (CMF/CME) flowable and paste-like composites in cultured L929 cells adhesion and cell proliferation.
CME: Clearfil Majesty Esthetic, CMF: Clearfil Majesty Flow, CNT: Control

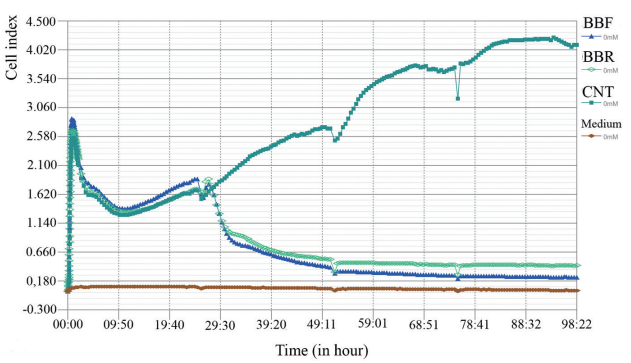


Fig. 5 Dynamic monitoring of treated with same brand (BBF/BBR) flowable and paste-like composites in cultured L929 cells adhesion and cell proliferation.
BBF: Beautifil-Bulk Flowable, BBR: Beautifil-Bulk Restorative, CNT: Control

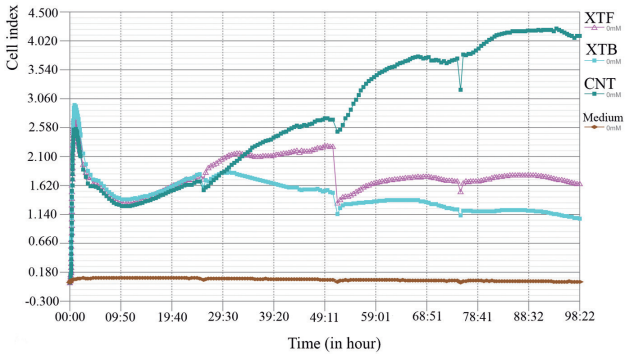


Fig. 6 Dynamic monitoring of treated with same brand (XTF/XTB) flowable and paste-like composites in cultured L929 cells adhesion and cell proliferation.
XTF: x-tra fill, XTB: x-tra base, CNT: Control

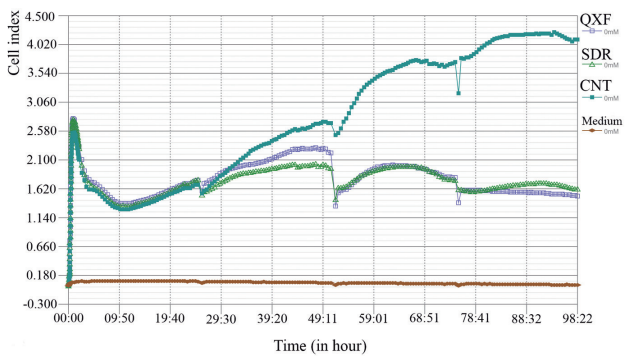


Fig. 7 Dynamic monitoring of treated with same brand (SDR/QXF) flowable and paste-like composites in cultured L929 cells adhesion and cell proliferation.
QXF: QuiX Fill Posterior Restorative, SDR: Smart Dentin Replacement, CNT: Control

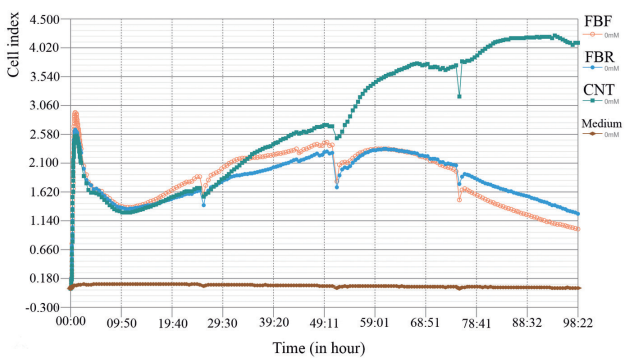


Fig. 8 Dynamic monitoring of treated with same brand (FBF/FBR) flowable and paste-like composites in cultured L929 cells adhesion and cell proliferation.
FBF: Filtek Bulk Fill Flowable Restorative FBR: Filtek Bulk Fill Posterior Restorative, CNT: Control

composition. When the morphological changes at 72 h were evaluated, VBF had moderate effects on the morphology of L929 cells; approximately 50% of cells remained spindle shaped. XTB, CMF, SDR, FBF, TEB, XTF, FBR, CME and QXF had severe effects on the morphology of tested cells; less than 40% of cells remained unaffected. BBF and BBR caused the

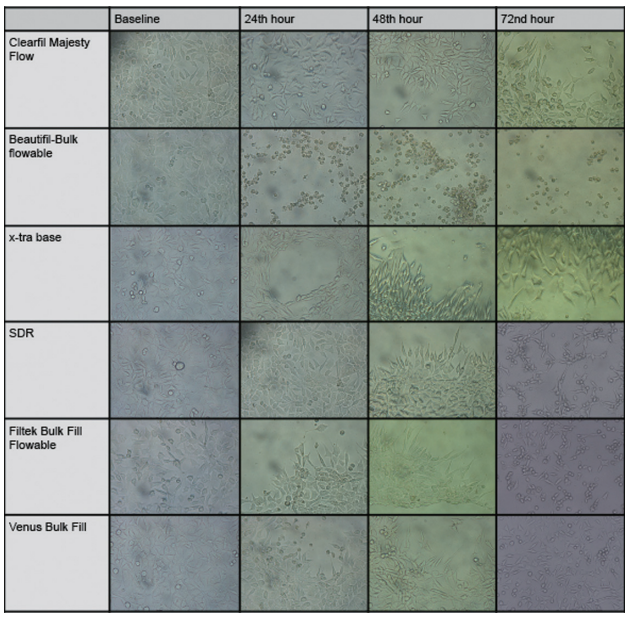


Fig. 9 Representative images of cellular morphology of L929 mouse fibroblasts cells after treatment with flowable resin composites.

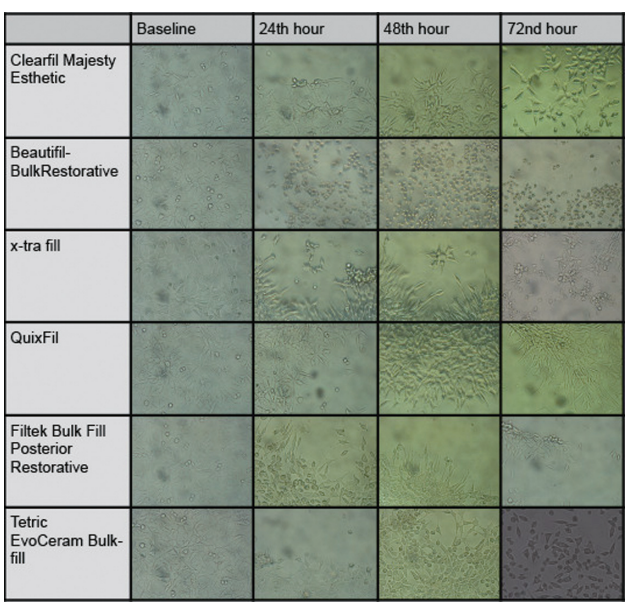


Fig. 10 Representative images of cellular morphology of L929 mouse fibroblasts cells after treatment with paste-like resin composites.

dramatically morphological change on L 929 of cells and nearly all of the cells become smaller, rounded and retracted, with condensed and fragmented nuclei morphology (Figs. 9 and 10).

Cell morphology analysis confirmed the real-time cell analysis results. As the toxicity increased, the cells had a rounded shape, which indicated that they were no longer alive. The most severe morphological changes were observed especially in the most cytotoxic BBF/BBR groups.

DISCUSSION

Bulk-fill resin composites have gained popularity among clinicians, since they allow the placement of thicker layers and thus provide simplified and time-saving restorative procedures. While their physico-mechanical properties, curing characteristics and wear performance have been extensively investigated[16-21], scientific data on the biocompatibility of bulk-fill composite materials are still limited[13-15].

Dental biomaterials should ideally be nontoxic to both hard and soft oral tissues. Nevertheless many *in vitro* investigations have indicated that the polymerization reaction that generates the cross-linked polymer matrix from the dimethacrylate resin monomer is never complete and cytotoxic reactions occur due to the release of unpolymerized monomers[10,22-24].

To date, several *in vitro* test designs have been recommended by the International Standards Organization (ISO) 10993 protocols for assessing the cytotoxicity of dental biomaterials (ISO 10993-5:2009, ISO 10993-12:2012) These include the direct contact test model where dental material and cell lines are placed in direct contact; the indirect contact test model, where a barrier is placed between the dental material and cell layer; and finally the extract test model, where the cells are exposed to the extract of the dental material[13,14]. In this study, tested composite resin discs were placed in plates wherein the cells were incubated so as to provide direct contact between the cells and composite samples. This was done to observe the effects of the components leaking from the composites on the cells.

Cell types used for *in vitro* cytotoxicity studies could play a significant role in the outcomes. Pulp fibroblasts, gingival fibroblasts, cells obtained from cattle dental papilla, odontoblast-like cells collected from mice and human dental pulp stem cells are most commonly used in studies investigating the cytotoxicity of dental materials[9,13-15,24-26]. In the present study, L929 cells were selected, as they are easily available and have efficient in-vitro growth due to their homogenous morphology and growth characteristics[9].

Several techniques and methods such as 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT) assay[9], 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzol-disulfonate (WST-1) assay[14], lactate dehydrogenase (LDH) assay[27] are used to assess the dental biomaterial-induced cytotoxicity with each of them using different aspects of cellular metabolism as a means of quantifying live cells. However, all of these methods are single end-point qualitative measures of cell fitness[28]. Unlike end-point approaches, real-time assay systems provide the tracking of cellular growth over the entire time course of an experiment. One of the real time assays is iCELLigence system RTCA (Roche Applied Science). This system was used by researchers to assess the cytotoxicity of different dental materials[25,29,30]. It uses microelectronic biosensor technology to perform 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[31]. The system also 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, real-time analysis is effective for determining cell densities in small culture volumes and observing the reactions of live cells to chemical exposure which is not yet possible with current end point assays[30]. In addition to this advantages, researchers[32] found strong correlations with conventional methods and real-time assay systems. For these reasons real-time and continuous analysis of cell vitality were performed to evaluate the cytotoxicity of bulk-fill composites in this study.

Cytotoxic effects of resin based dental materials may depend on their organic ingredients[24,26,27]. Residual monomers, such as triethyleneglycol dimethacrylate (TEGDMA), bisphenol-A-diglycidyl methacrylate (Bis-GMA), ethoxylated bisphenol-A-glycol dimethacrylate (Bis-EMA), ethylene glycol dimethacrylate (EGDMA) and 2-hydroxyethyl methacrylate (HEMA) might leak from bulk-fill composite resins in different quantities[22,33]. Previous investigations showed that, Bis-GMA, among the components usually present in the polymeric resin matrix, is the most toxic one, followed by UDMA (urethane dimethacrylate), TEGDMA and HEMA[34,35]. Although the mechanisms of the cytotoxicity induced by these monomers were not completely explained, the cytotoxic effects of these monomers at different levels may be related to their different molecular weights. Among these monomers, Bis-GMA has the highest molecular weight while HEMA has the lowest one[34]. A suggested mechanism of monomer cytotoxicity is the alteration of the lipid layers of cell membranes caused by these monomers, which can influence the permeability of the membrane[36]. Based on this theory, Issa *et al*.[37] have attributed higher toxicity of Bis-GMA to its liposolubility effect on the membrane. Studies related to the mechanism of cytotoxicity associated with resin-based dental materials, have concentrated on reactive oxygen species (ROS) and glutathione depletion. ROS are described as molecules that destabilize cellular redox equilibrium, leading to apoptosis-induced cell death[38]. Monomers, such as TEGDMA, HEMA, and Bis-GMA, induced ROS production in different cell lines[26,27,39,40]. However, beyond the effects of the single monomer, the interaction among them is also important. Synergistic effects were detected when combinations of TEGDMA with UDMA or particularly with Bis-GMA were tested[34].

Even if it is difficult to achieve exact information from manufactures regarding the chemical composition and proportion of various molecular constituents of their products, significant differences in polymer matrix composition of composite materials can be underlined. In light of this information, one of the possible reasons for the various cytotoxic behaviors of the tested composite materials in this study may be related their different organic compositions.

In this study, significantly reduced cell viability was observed for both BBF and BBR throughout the entire duration of the experiment. These composite resins contain pre-reacted glass-ionomer (PRG) filler in a resin matrix, unlike other tested dental composites. The fluoro-alumino-silicate glass has been pre-reacted with polyacid to form a glass ionomer matrix structure and then blended with resin. PRG filler containing resin based dental materials provide higher fluoride release than compomers due to the glass ionomer hydrogel matrices[41]. Consistent with the results of this study, in a previous study by Toh *et al.*[15] BBR and BBF were found to be more cytotoxic than other tested bulk-fill composites. They speculated that the cytotoxic effects of BBR and BBF might be due to release of fluoride and other ions, such as PRG fillers include aluminum, boron, sodium, silicon, strontium, and zinc. In addition, monomers such as TEGDMA and Bis-GMA in the resin matrix, which are also present in other resin-based composites, may also be responsible for this cytotoxic effect[28].

VBF showed the least cytotoxic effect among the flowable bulk-fill composites tested ($p<0.05$). This result could be related to the monomer composition, because it contains neither TEGDMA nor Bis-GMA in contrast to the other flowable bulk-fill composites tested in the study. For paste-like composite materials, all tested paste-like composites show similar cytotoxic properties, except for BBR. Again, with the exception of BBF and BBR, tested bulk-fill composites generally have comparable or higher cell viability than the conventional composites.

Time is also a significant factor, which can have an influence on cytotoxicity. Although Ferracane and Condon[42] reported that residual monomer release occurs 24 h after polymerization, that released materials after 24 h may be neglected, and that composite materials show cytotoxic effects on tissues within the first 24 h, on the contrary, more recent researches[10,22] show that, the amount of the eluted residual monomer from bulk-fill composites increased with time. Even though, in this study all the tested composites except BBF and BBR, demonstrated acceptable cell viability (>70%) based on the ISO cutoff (ISO 10993-5:2009) at 24 h., the cell viability rates for all composites decreased under 70% at the end of 72 h. When the significant relationship between elution of residual monomers from composite resins and cytotoxic effect[23] is considered, this decrease might be associated with the increase in release of cytotoxic substances over time.

In the current investigation, when the cytotoxic effects of the flowable and paste-like forms of the composites belonging to the same brand were compared, no statistically significant difference was found. This result was unexpected, as some studies have shown that the flowable composite materials were more toxic than their paste-like forms, because of the increase in monomer leach[15,43]. On the other hand the great majority of organic composition of flowable composites are comprised of lower molecular weight monomers such as TEGDMA, which are less cytotoxic[34,35]. This may be the reason why flowable and paste-like composites of the same brand exhibit similar cytotoxic effects, in this study. Nevertheless, since this study did not evaluate leaching of monomers from resin composites, no definitive results could be drawn regarding the different compositions of the composites tested.

Only a few studies evaluated the cytotoxicity of bulk-fill dental composite materials[13-15] but it is difficult to compare these studies' results because of the many variations in experimental conditions such as cytotoxicity test method, cell type usage, cell–material contact method and exposure time. Lim *et al.*[13] investigated the cytotoxic effects of SDR and a conventional composite resin (Spectrum TPH, Dentsply Caulk) with different cytotoxicity test models (direct contact, indirect contact and extract tests) on L929 mouse fibroblast cells and at the end of the 24 h they found that SDR is less cytotoxic, independently of the test method. Conversely, in the current study SDR demonstrated a decrease in CI compared to the conventional composite resin (CMF) at the end of the experiment. This result may be due to several reasons. The first of these may be related to the duration of the experiment. In the study by Lim *et al.*[13], the experiment was carried out for 24 h, in our study, the cytotoxicity of the tested composites was evaluated for 72 h. The cytotoxicity of SDR may have increased over time and may have led to this result. In support of this inference, SDR and CMF exhibit similar CI at the end of the 24 h in our study. Another reason for the differences between the results of the two studies may be that the organic compositions of the composites used as the control group are different. In addition, the difference in the test methods used to evaluate cytotoxicity may account for the differences in results.

Şişman *et al.*[14] investigated the cytotoxic effects of five bulk-fill composites (SDR, TEB, XTF, FBF and Sonic Fill) with WST-1 assay on human dental pulp stem cells on the 1st, 7th, 14th, and 21st days. Researchers found higher cell viability for FBF group compared to the positive control (no composite disc containing steam cells) on the first day. The SDR group showed the least number of live cells on the 7th day. Researchers also detected least cell viability in XTF group on day 14. At the end of the 21st day, they reported an increase in cell viability for all test groups except SDR. Similarly, FBF found to be one of the least cytotoxic flowable composites at the end of 24 h, in our study. However, it was not possible to compare the results at other time intervals, as the experimental periods between the two studies were different.

Toh *et al.*[15] investigated the cytotoxic effects of two conventional [Filtek Z350 XT universal restorative and

Filtek Z350 XT universal flowable (3M ESPE)] and five bulk-fill composites [BBR, BBF, SDR, EverX Posterior (GC Europe, Lueven, Belgium), Tetric N-Ceram Bulk-Fill (Ivoclar Vivadent)] with MTT assay on L929 mouse fibroblast cells for 24 h. Similar to the results of current investigation, BBR and BBF were significantly more cytotoxic than the other tested composite resins in both 2 and 4 mm thickness, and they also demonstrated that all the tested dental composites showed different levels of cytotoxicity depending on chemical composition, specimen thickness, and testing concentrations of extracts. The result of the current study showed all the tested composites were cytotoxic on L929 cells at different levels. In general, these results are consistent with above-mentioned studies[13-15] Slight differences between previous studies and this study may result from the methodological variations.

This research has several limitations. Firstly, only single cell line and test models were used for the assessment of cytotoxicity. The differences in cell lines used 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, Schedle *et al.*[44] 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. 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 study, the polymerization of composite resins was carried out according to the manufacturers' instructions, and recommended curing time for the tested materials were between 10–60 s depending on the particular composite. For this reason, as another limitation of this study, samples were exposed to curing light for different periods. Researchers have indicated that a longer light application than recommended by the manufacturer will not affect the cytotoxic properties of dental composite materials[45]. However, de Souza Costa *et al.*[46] suggested that, when resin materials are irradiated for a short time or with low intensity light, cytotoxicity increases. Polymerization of the tested composite samples in equal period may provide an easier comparison, however it is preferred to cure in accordance with the manufacturer's instructions to simulate the clinical conditions. Further *in-vitro* investigations may focus cytotoxic effects of curing light-related parameters, including light type, distance, intensity and curing modes on bulk-fill composites.

CONCLUSIONS

Within the limitations of this *in-vitro* study, the flowing conclusions were drawn:

1. Different resin composites exhibited varying degrees of cytotoxicity on L929 cells and the cytotoxic effects of composite resins on cell viability paralleled with the changes in cell morphology.
2. The cytotoxicity results of flowable and paste-like composites of the same brand showed no statistically significant differences between groups per brand at all tested time intervals.
3. The cytotoxicity of all tested composite resins increased over time, except BBF, which exhibited similar decline in CI at 48 and 72 h.
4. Among the tested composite materials, BBR and BBF have significantly higher cytotoxicity.

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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.

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