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In Vitro Biocompatibility of Contemporary Bulk-fill Composites

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Clinical Relevance

Despite manufacturers' claims, not all bulk-fill resin-based composites are biocompatible at 4 mm thickness. Bulk-fill composites based on pre-reacted glass ionomer (PRG) technology may be less biocompatible than non-PRG materials.

SUMMARY

This study evaluated the biocompatibility of contemporary bulk-fill resin-based composites (RBCs) including PRG (pre-reacted glass ionomer) materials based on the International Organization for Standardization 10993. In addition, the effect of composite thickness on cytotoxicity was also assessed. Two standard composites, two bulk-fill PRG RBCs, and three bulk-fill non-PRG RBCs were investigated. Block-shaped specimens of 2-mm and 4-mm thickness were cured with an irradiance of 700 mW/cm^2 for 20 seconds with a light-emitting diode curing light and eluted with culture medium at 37°C for 24 hours. L929 mouse fibroblasts were exposed to extracts at varying dilutions (1:1, 1:2, and 1:10) for 24 hours. Analyses were performed to assess cytotoxicity, phase contrast microscopy, and quantitative cell viability. Among the bulk-fill RBCs, extracts of PRG materials resulted in the lowest cell viability. At 4-mm thickness, undiluted extracts of bulk-fill non-PRG RBCs had significantly higher cell viability than the standard composites. Chemical composition, specimen thickness, and testing concentrations of extracts had significant effects on cell viability and morphology. Cytotoxic effects of composites on cell viability were parallel with cell morphologic changes. Not all bulk-fill RBCs demonstrated high cell viability (>70%) at 4-mm thickness despite manufacturers' recommendations of bulk placement and curing.

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INTRODUCTION

Amalgam has been the traditional material for restoring posterior teeth because of its effectiveness and cost.[1] In recent years, the use of amalgam has declined because of increased patient esthetic demands, fear of mercury toxicity, and environmental concerns after disposal. Resin-based composites (RBCs) are an esthetic alternative to amalgam. RBCs, however, have several disadvantages, including technique sensitivity, polymerization shrinkage, limited depth of cure, and lower physicomechanical properties compared with amalgam. The aforementioned may account for the higher failure rates and secondary caries associated with RBCs compared

with amalgam.[1] RBCs are usually placed in increments of less than 2 mm to ensure adequate light curing and reduce polymerization shrinkage.[2] Several manufacturers have introduced innovative bulk-fill RBCs that can be placed in a single increment to reduce the time and effort required for layering and adapting posterior composites.

An ideal bulk-fill RBC should have low polymerization shrinkage, high degree of conversion, superior depths of cure, and ample mechanical properties, and it should be biocompatible.[3-7] Through the use of novel proprietary resins, special modulators, unique fillers, and filler control, bulk-fill RBCs are said to have lower polymerization shrinkage and depths of cure up to 4 mm. At such cavity depths, curing light penetration may be compromised, thus leading to reduced monomer to polymer conversion, leaching of unreacted monomers, and biocompatibility issues. The latter may be more problematic with low viscosity flowable bulk-fill materials in view of their higher resin content.

Biocompatibility is the ability of materials to coexist with living tissues without causing harm. Nonbiocompatible or cytotoxic (ie, toxic to cells) restorative materials can cause short-term and long-term adverse tissue reactions ranging from postoperative sensitivity to irreversible pulp damage.[8] RBCs alone contributed to more than 12% of adverse reactions to dental materials.[9] In addition to the leaching of unreacted monomers, cytotoxicity can also be caused by the release of initiators and other additives from the organic resin as well as metal ions from the inorganic fillers. Proper curing of RBCs is important to ensure adequate mechanical properties and biocompatibility.[10,11]

Studies investigating the cytotoxicity of bulk-fill RBCs are still limited, and none have performed cytotoxicity testing of the recently launched bulk-fill PRG pre-reacted glass ionomer (PRG) RBCs. The latter, also known as giomers, are based on PRG technology in which acid-reactive fluoride containing glass is reacted with polyacids in the presence of water, freeze-dried, milled, silanized, ground, and used as fillers. In addition to fluoride release and demineralization inhibition, these materials also possess antiplaque formation properties.[12-15] Bulk-fill PRG RBCs are available in both regular (Beautifil bulk restorative [BBR], Shofu, Kyoto, Japan) and low (Beautifil bulk flowable [BBF], Shofu) viscosities. Recent studies have indicated cytotoxicity of fluoride to tissues by multiple mechanisms, including inhibition of enzyme activity, generation of reactive oxygen species (ROS), impairment of the antioxidant defense system, induction of inflammation, and apoptosis.[16,17] Kanjevac and others[18] have also reported positive correlation of cytotoxicity of glass ionomer cements with their fluoride release.

The objective of this study was to evaluate the biocompatibility of contemporary bulk-fill RBCs, including PRG materials based on the International Organization of Standardization (ISO) 10993.[19-21] The effect of material thickness (2 mm versus 4 mm) and extract dilutions were also investigated.

METHODS AND MATERIALS

Materials selected for this study included two standard composites (Filtek Z350 XT universal restorative [ZFR] and Filtek Z350 XT universal flowable [ZFF], 3M ESPE, St Paul, MN, USA), two bulk-fill PRG RBCs (BBR and BBF), three bulk-fill non-PRG RBCs (Smart Dentin Replacement bulk-fill flowable [SDR], Dentsply Caulk, Milford, DE, USA), EverX posterior (EXP) (GC Europe, Lueven, Belgium), and Tetric N-Ceram bulk-fill (TNC) (Ivoclar Vivadent, Schaan, Liechtenstein). The technical profiles and composition of the materials are shown in Table 1. L-929 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) high glucose and fetal bovine serum (FBS) were acquired from Biowest SAS (Nuaillé, France), 0.05% trypsin/ethylenediaminetetraacetic acid and penicillin/streptomycin (P/S) were obtained from Life Technologies (Singapore), and an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell viability assay kit was procured from Promega (Madison, WI, USA).

A custom-made black polyvinyl mold with a recess 4 mm long by 4 mm wide and variable depths of 2 mm and 4 mm was fabricated for specimen preparation. Four specimens per material were fabricated for each bulk-fill and standard RBC (n=4 per group). The mold was filled using a single increment, and excess material was removed by compressing the mold between two glass slides (1-mm thick). The specimens were then cured for 20 seconds through the glass slide using a BlueShot light-emitting diode (LED) curing light (Shofu) with an irradiance of 700 mW/cm^2 and an exit window of 8-mm diameter. Before each use, the intensity of the curing light was verified using an LED radiometer (Demetron LED radiometer; Kerr Corporation, Middleton, WI, USA). The cured specimens were sterilized by swabbing briefly with 70% ethanol before they were immersed in cell culture medium (DMEM-high glucose supple-

Table 1: *Composite Materials and Their Composition*

Material	Abbreviation	Shade	Matrix Composition	Filler % by Weight	Recommended Thickness (mm)	Recommended Curing Time and Light Intensity
Filtek Z350 XT universal restorative	ZFR	A2	Bis-GMA, UDMA, TEGDMA, PEGDMA, Bis-EMA resin, silica filler, zirconia/silica cluster filler	78.5	2	20 s $\geq$400 mW/cm^2
Filtek Z350 XT flowable restorative	ZFF	A2	Bis-GMA, TEGDMA, procrylat resins, ytterbium trifluoride filler, silica filler, zirconia/silica cluster filler	65	2	20 s 400-1000 mW/cm^2 10 s 1000-2000 mW/cm^2
Beautifil bulk restorative	BBR	A	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG filler based on fluoroboroaluminosilicate glass, polymerization initiator, pigments, and others	87	4	10 s $\geq$1000 mW/cm^2
Beautifil bulk flowable	BBF	Universal	Bis-GMA, UDMA, Bis-MPEPP, TEGDMA, S-PRG filler based on fluoroboroaluminosilicate glass, polymerization initiator, pigments, and others	73	4	10 s $\geq$1000 mW/cm^2
SDR posterior bulk-fill flowable base	SDR	Universal	Barium-alumino-fluoro-borosilicate glass, strontium alumino-fluoro-silcate glass, modified UDMA resin, EBPADMA, TEGDMA, camphorquinone photoinitiator, photoaccelerator, BHT, ultraviolet stabilizer, titanium dioxide, iron oxide pigments, fluorescing agent	68	4	20 s $\geq$550 mW/cm^2
EverX Posterior	EXP	Universal	Bis-GMA, TEGDMA, silicon dioxide, barium glass, glass fiber, polymethylmethacrylate, photo initiator	74.2	4	20 s $\geq$700 mW/cm^2 10 s $>$1200 mW/cm^2
Tetric N-Ceram Bulk-Fill	TNC	IVA (Universal)	Dimethacrylates, barium glass, ytterbium trifluoride, mixed oxide, additives, catalysts, stabilizers, and pigments	75-77	4	20 s$\geq$500 mW/cm^2 10 s $\geq$1000 mW/cm^2)

Abbreviations: BHT, butylated hydroxyl toluene; Bis-EMA, bisphenol A polyethylene glycol diether dimethacrylate; Bis-GMA, bisphenol A glycidyl methacrylate; Bis-MPEEP, 2,2,bis (4-methacryloxypolyethoxyphenyl) propane; EBPADMA, ethoxylated bisphenol A dimethacrylate; PEGDMA, polyethylene glycol dimethacrylate; S-PRG, surface pre-reacted glass ionomer; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate.

mented 10% FBS and 1% P/S) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. The extracts of the composite specimens were prepared at a surface area to volume ratio of 3 cm^2/mL culture medium following the guidelines of ISO10993.[19-21] All the composite specimens were prepared and extracted in culture medium within the same day. After incubation, the original extracts (1:1) were collected and then diluted in cell culture medium to obtain 1:2 and 1:10 dilutions before further testing. At least two independent experiments were performed for each test material.

The L929 mouse fibroblasts were seeded at a density of 2×10^4 cells per well in 96-well plates at 37°C in 5% CO_2 for 24 hours. The cells were allowed to attach for at least 15-18 hours before exposure to the composite extracts. The plating medium was removed and 100 μL of the extracts were added into each well for further incubation at 37°C for 24 hours. After incubation, cell morphologic changes

Table 1: *Composite Materials and Their Composition (ext.)*

Manufacturer	Batch Number
3M ESPE (St Paul, MN, USA)	N454576
3M ESPE (St Paul, MN, USA)	N452481
Shofu Inv (Kyoto, Japan)	51441
Shofu Inc (Kyoto, Japan)	71404
Dentsply Caulk (Milford, DE, USA)	1402000759
GC Europe (Lueven, Belgium)	1311301
Ivoclar Vivadent (Schaan, Liechtenstein)	R65894

were observed by phase contrast microscopy (DMI3000B, Leica, Wetzlar, Germany). Cell viability was assessed by MTS assay following the manufacturer's protocol. Briefly, 20 μL of MTS reagent was added to each well. The cells were incubated at 37°C in CO_2 for 2 hours, and the absorbance readings were taken at 490 nm and 650 nm (reference) using an Infinite 2000 plate reader (Tecan, Männedorf, Switzerland). Untreated cultures without any material served as a negative control. Percentage cell viability was calculated by normalization of the absorbance readings against that of the negative control (set as 100%).

Cytotoxicity testing of the materials was performed in quadruplicates (n=4 per material) and at least two independent experiments were performed to confirm the results. Cell viability data was analyzed using one-way analysis of variance and Scheffe's *post hoc* testing at a significance level of 0.05. The StatView software version 5.0 (SAS Institute Inc, Cary, NC, USA) was used to perform the statistical analysis.

Table 2: *Statistical Analysis of Cytotoxicity of Various Composite Materials* [a]

Dilution	Thickness (mm)	Cytotoxicity (From Most to Least Cytotoxic)
1:1	2	BBF > BBR, ZFR, TNC, ZFF, SDR, EXP
		BBR > ZFR, TNC, ZFF, SDR, EXP
		ZFR > SDR, EXP
		TNC > EXP
	4	BBR = BBF > ZFF, ZFR, TNC, EXP, SDR
		ZFF = ZFR > TNC, EXP, SDR
		TNC > SDR
		EXP > SDR
1:2	2	BBF > ZFR, BBR, SDR, ZFF, TNC, EXP
		ZFR > EXP
		BBR > EXP
	4	BBF > BBR, ZFR, ZFF, SDR, EXP, TNC
		BBR > ZFR, ZFF, SDR, EXP, TNC
		ZFR = ZFF > SDR, EXP, TNC
1:10	2	BBF = TNC = SDR = ZFR = EXP = BBR = ZFF
	4	BBF > BBR, SDR, ZFR, TNC, ZFF, EXP

Abbreviations: BBF, Beautifil bulk flowable; BBR, Beautifil bulk restorative; EXP, EverX Posterior; SDR, SDR posterior bulk-fill flowable base; TNC, Tetric N-Ceram bulk-fill; ZFR, Filtek Z350 XT universal restorative; ZFF, Filtek Z350 XT flowable restorative.

[a] *Results of one-way analysis of variance/Scheffe's* post hoc *($p<0.05$). > indicates statistical significance.*

RESULTS

Cell Morphology Analysis

Untreated L929 cells (negative control) were spindle shaped in appearance with extended cellular processes, filopodi, and lamellipodia (Figure 1). Varying degrees of morphologic alterations were observed with the various bulk-fill RBCs and standard composites, depending on chemical composition, specimen thickness, and extract concentrations (Figure 1). Undiluted extracts of ZFR and ZFF cured at 2-mm thickness had no obvious effect on the cell morphology compared with the negative control (Figure 1A). However, at 4-mm thickness, undiluted

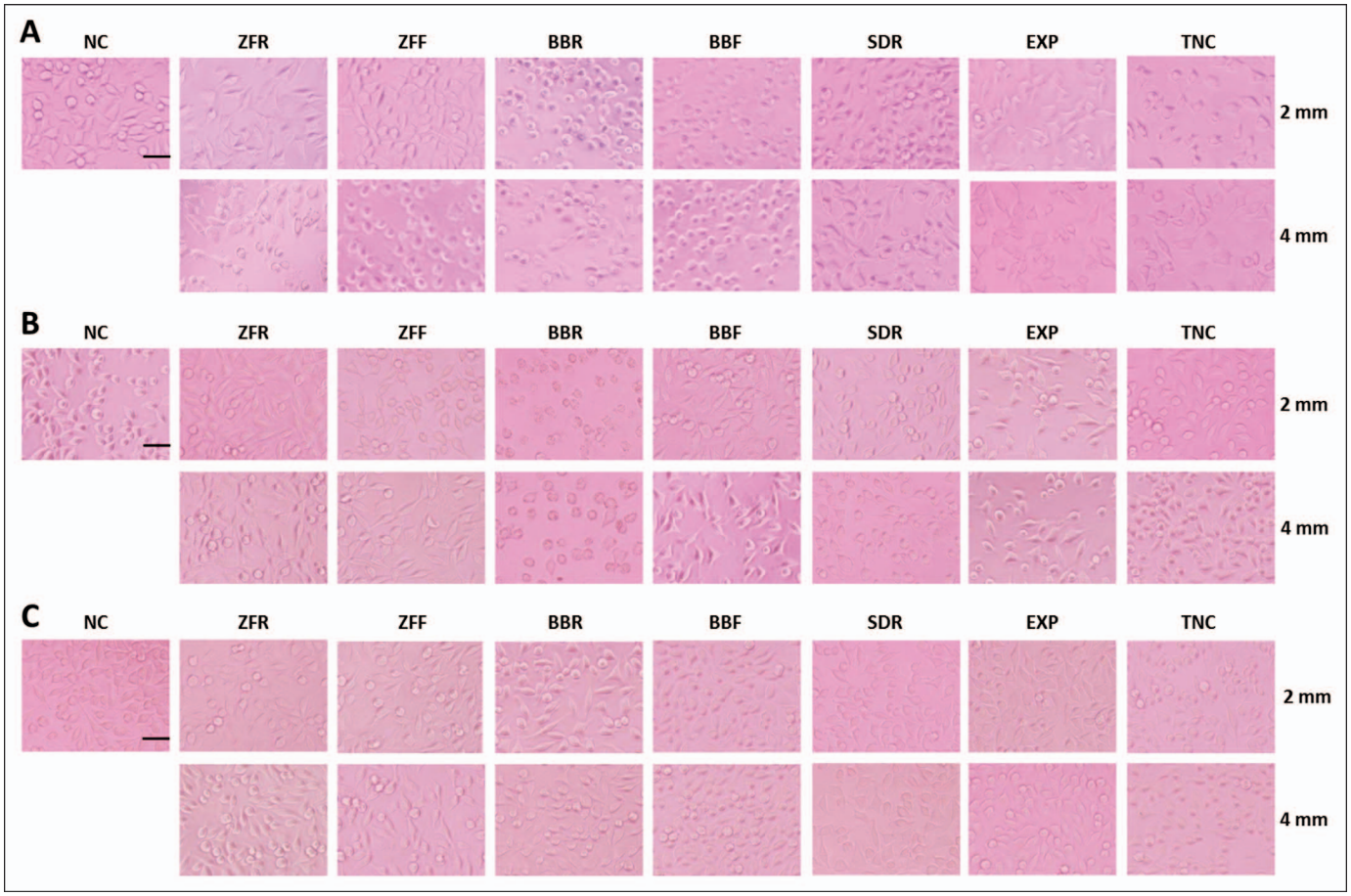

Figure 1. *Cellular morphology of L929 mouse fibroblasts after treatment with extracts of various resin composites. The cell cultures were exposed to (A): original extracts (1:1), (B): 1:2 diluted extracts, and (C): 1:10 diluted extracts at 37 °C for 24 hours, and cell morphology of treated and untreated cell cultures (negative control, NC) were observed under phase contrast microscopy. Representative images from four replicate cultures (n=4) were presented. Scale bar = 100 μm.*

extracts of ZFR and ZFF resulted in significant retraction and rounding of the cells.

Among the bulk-fill RBCs, undiluted extracts of bulk-fill PRG RBCs (BBR and BBF) at both 2-mm and 4-mm thickness caused the majority of cells to become small, retracted, and rounded, with condensed and fragmented nuclei morphology. The effects of BBR and BBF on cell morphology were concentration dependent (Figures 1B,C). Accordingly, there was a decrease in the number of retracted round cells; most cells remained spread out and spindle shaped when 1:10 diluted extracts of BBR and BBF were applied. Furthermore, non-PRG bulk-fill RBCs, including SDR, EXP and TNC, had little effects on the morphology of L929 cells; approximately 80% of cells remained spindle shaped when treated with the undiluted extracts. When the specimen thickness of these materials was increased to 4 mm, no significant effect on cell morphology was also observed.

Cell Viability Analysis

Varying levels of cell viability detected with extracts of the RBCs were normalized against the untreated cells set as the negative control at 100%. Differential cytotoxic effects were observed with the various bulk-fill RBCs (PRG and non-PRG) and the standard composites. Results were again dependent on chemical composition, specimen thickness, and extract concentrations (Table 2 and Figure 2). Undiluted (1:1) extracts of ZFR and ZFF at 2-mm thickness significantly reduced the cell viability to 66% ($p<0.0001$) and 79% ($p<0.05$), respectively (Figure 2a). Cell viability was further reduced to 36% and 28%, respectively, when specimen thickness of ZFR and ZFF was increased to 4 mm ($p<0.0001$).

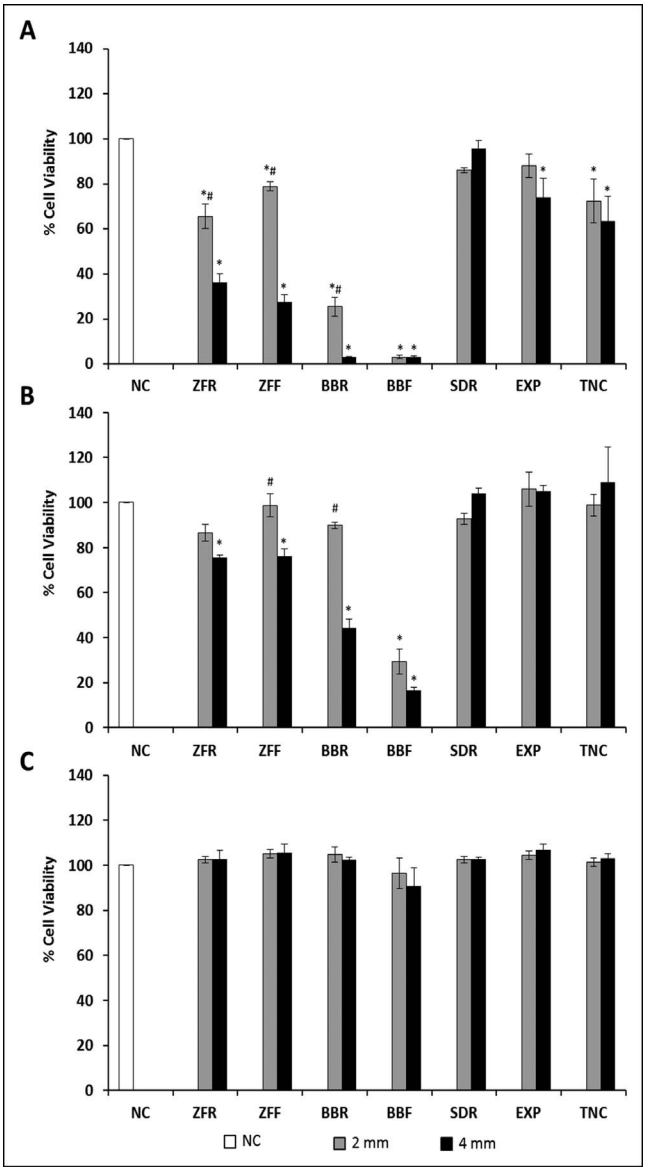

Figure 2. *Cellular viability of L929 mouse fibroblasts after treatment with extracts of various resin composites. The cell cultures were exposed to (A): original extracts (1:1), (B):1:2 diluted extracts, and (C): 1:10 diluted extracts at 37 °C for 24 hours, and cellular viability of treated and untreated cell cultures (negative control, NC) were determined by MTS assay in four replicate cultures (n=4). Results are normalized to the mean values of the negative control and presented as mean (± SD) percentage cell viability. * indicates significant decrease compared with the negative control; # indicates statistically significant differences between cell viability values of 2-mm and 4-mm composite extracts (analysis of variance, Scheffe's* post hoc *test, n=4).*

For the bulk-fill RBCs, undiluted extracts of bulk-fill PRG RBCs (BBR and BBF) at 2-mm thickness resulted in drastic decline of cell viability to below 30% ($p<0.0001$). Cell viability was further reduced to less than 5% when the cells were treated with BBR and BBF at 4-mm thickness ($p<0.0001$; Figure 2A). At 2-mm and 4-mm increments, BBR and BBF were significantly more cytotoxic than the non-PRG bulk-fill RBCs ($p<0.0001$) and standard RBCs ($p<0.01$; Figure 2A). Undiluted extracts of non-PRG bulk-fills, including SDR, EXP, and TNC, at 2-mm thickness reduced cell viability to 86% ($p\geq0.54$), 88% ($p\geq0.79$), and 72% ($p<0.001$), respectively. When the specimen thickness of these materials was increased from 2 mm to 4 mm, no significant difference in cell viability was observed ($p>0.05$). Ranking of material cytotoxicity (from most to least cytotoxic) based on the cell viability of L929 after 24-hour exposures to undiluted extracts were as follows: BBF, BBR, ZFR, TNC, ZFF, SDR, EXP (2-mm thickness) and BBR, BBF, ZFF, ZFR, TNC, EXP, SDR (4-mm thickness).

DISCUSSION

We compared the cytotoxicity of five bulk-fill RBCs and two standard composites using L929 mouse fibroblast cell line as specified in the ISO guidelines.[19-21] The use of immortalized cell lines, in this case L929, offers the greatest advantage of low interbatch variability and accuracy of response to toxic challenge.[22] A number of cell types, including human gingival fibroblasts,[23] periodontal ligament fibroblasts,[23,24] and dental pulp stem cells,[25] have been proposed for dental materials testing as these cell types are present in the oral tissues. The L929 mouse fibroblast cell line, however, remains to date one of the most common cell lines for cytotoxicity evaluation of dental materials.[22,26,27]

With the exception of bulk-fill PRG RBCs (BBR and BBF), bulk-fill RBCs generally have comparable or higher cell viability than the standard composites. Significantly lower cell viability was observed for RBCs cured at 4-mm thickness compared with 2-mm thickness, with the exception of SDR, EXP, and TNC at 1:1 and 1:2 dilutions. The differential cytotoxic effects observed in ZFR and ZFF at 2-mm and 4-mm thickness implied inadequate curing of these RBCs at 4 mm, resulting in the release of leachable toxic monomers. For the bulk-fill PRG RBCs (BBR and BBF), the differential cytotoxic effects between 2-mm and 4-mm specimens may be attributed in part to greater fluoride and other ion release in addition to the degree of conversion.[12,13] The degree of conversion for 4-mm-thick BBR and BBF specimens might also be compromised by the intensity of the LED curing light used. The manufacturer recommends an irradiance of 1000 mW/cm^2 for 10 seconds for both materials. Although the total LED (intensity×time) used in the study was greater than that

endorsed by the manufacturer, the intensity may not be sufficient to penetrate to 4-mm depths. The effects of RBCs on cell viability were paralleled by alterations of cell morphology. This was most evident in cells treated with BBR and BBF. In this instance, the cells demonstrated a transition from spindle shaped to a retracted and condensed morphology in a dose-dependent manner. The condensed nuclei morphology observed here is likely indicative of apoptosis, although further confirmative assay would be required.

BBF was found to be more cytotoxic than BBR. This was not unexpected as flowable materials are made less viscous by reducing filler content and adding diluents that have been shown to result in a more persistent mass leaching at toxic levels.[28] When the non-PRG materials were compared, the difference between regular (TNC and EXP) and low viscosity (SDR) products was also evident for 4-mm-thick specimens. Extracts of BBR and BBF induced morphologic alterations and cytotoxicity in a dose-dependent manner, linking cell morphologic changes with the cell viability. The cytotoxic effects of bulk-fill PRG RBCs (BBR and BBF) can be attributed to the release of fluoride and other ions, in addition to such monomers as triethylene glycol dimethacrylate (TEGDMA) and bisphenol A diglycidyl ether dimethacrylate (Bis-GMA), which are also present in other RBCs. Supplementary ions released from PRG fillers include aluminum, boron, sodium, silicon, strontium, and zinc[29,30] and are constituents of the fluoroaluminosilicate glass utilized in the PRG technology. Inhibition of enzyme activity, generation of ROS, impairment of the antioxidant defense system, induction of inflammation, and apoptosis by low concentrations of fluoride in L929 mouse fibroblasts,[31] human dental pulp stem cells,[18] and human gingival fibroblasts[32] have been reported. The correlation between concentration of fluoride ion released and cytotoxic response is shown to be high, positive, and significant.[18,33] The other bulk-fill RBCs (SDR, EXP, and TNC) also incorporate fluoride-containing glasses into their formulations (Table 1). Fluoride release with PRG-filled materials is, however, higher than for materials with fluoroaluminosilicate glass in the unreacted form.[34] The fluoride-containing glass must be reacted with polyacids for effective fluoride release. This explains in part the better biocompatibility of PRG RBCs compared with conventional and ceramic-reinforced glass ionomer cements.[27]

Undiluted extracts of SDR, EXP, and TNC at 4-mm thickness resulted in significantly higher cell viability (>60%) than that of the standard composites (ZFR and ZFF). The slight reduction in cell viability is most likely due to release of TEGDMA or Bis-GMA inducing cytotoxicity by generation of ROS, as previously described.[35-37] Among the bulk-fill RBCs, only SDR and EXP demonstrated acceptable cell viability (>70%) at 4-mm thickness based on the ISO cutoff of 70% cell viability.[20] Findings supported manufacturers' claims of curing at 4-mm thickness without causing cytotoxicity. It is important to note that not all bulk-fill RBCs can be adequately cured at 4-mm thickness.[38] Cell viability increases with extraction dilution. At 1:10 dilution, all RBCs, with the exception of BBR, showed almost 100% cell viability. Clinically, physical and mechanical properties, including polymerization shrinkage, must be considered in addition to biocompatibility. Flowable bulk-fill RBCs have been shown to shrink more than nonflowable ones.[38] The shrinkage stress can result in cuspal movement and microleakage, leading to postoperative sensitivity and possible pulpal inflammation.

Our study has several limitations. First, only a single cell line and test model were used for the assessment of cytotoxicity. It is important to note that cytotoxicity testing using an established cell line, in this case L929 mouse fibroblasts, would serve only as a general and preliminary assessment. Besides the differences in cell lines and primary cells, different test models including direct and indirect contact tests would also result in variability in cytotoxic responses.[26,39] Careful selection of more clinically relevant cell types as well as appropriate testing models and methods would be the next step to confirm the cytotoxic properties of biomaterials. We are currently investigating the use of neural crest stem cells and fibroblasts derived from human embryonic stem cells as human cell–based models and employing transcriptomic and proteomic analysis for cytotoxicity and genotoxicity testing of dental composites.[31,40,41] Comprehensive chemical analyses of culture medium extracts for organic and inorganic compounds are also necessary to identify plausible cytotoxic agents. In our study, the curing light distance was standardized at 1 mm away from the composite specimens. Clinically, this may be difficult to achieve because of curing light access and the presence of a matrix band and holder. Curing light-related parameters, including light type, distance, intensity, curing modes, and penetration may also influence the cytotoxicity of bulk-fill RBCs and warrants further in-depth investigations.

CONCLUSION

We evaluated the biocompatibility of contemporary bulk-fill RBCs based on the ISO 10993. Within the limitations of our study, we conclude that chemical composition, specimen thickness, and extract concentrations from RBCs have significant effects on cell viability and morphology. The cytotoxic effects of RBCs on cell viability paralleled changes in cell morphology. Among the bulk-fill RBCs, PRG materials BBR and BBF have significantly higher cytotoxicity. This may be attributed in part to the release of fluoride and other ions associated with the use of PRG technology. Only SDR and EXP demonstrated acceptable biocompatibility at 4-mm thickness based on the ISO cutoff of 70% cell viability.

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Regulatory Statement

This study was conducted in accordance with all the regulatory provisions, guidelines and policies of the National University of Singapore.

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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