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Relation between conversion degree and cytotoxicity of a flowable bulk-fill and three conventional flowable resin-composites

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Abstract. – OBJECTIVE: The aim of this study is to evaluate if the cytotoxic effects of the Surefil SDR flow, bulk fill flowable composite resin and three conventional flowable materials (Venus Diamond Flow, Filtex Supreme XTE Flowable and Enamel plus HRi Flow) correlated with the conversion degree (DC); hardness and depth of cure are also assessed.

MATERIALS AND METHODS: Disks of each materials – cured using LED lamp - are utilized to evaluate DC (by FT-IR technique), amount of leached monomers (by HPLC technique), hardness (by Vickers hardness tester) and cytotoxicity (by MTT test).

RESULTS: All tested materials show light cytotoxic effects, independently from DC values. Both the latter parameter and the hardness, in fact, change in function of thickness and type of material. HPLC results show that the monomers amount leached from each specimen is influenced by thickness but it is always very low which justifies the absence of any cytotoxic effect.

CONCLUSIONS: Our findings suggest that there are not statistically significant differences in cytotoxicity in all experimental conditions, notwithstanding the differences in hardness and in degree of conversion.

Key Words:
Cytotoxicity, Degree of conversion, Flowable bulk fill composite resins, Monomers leachability, Vickers hardness.

Introduction

Resin-based composites (RBCs) should possess several key mechanical and biological properties like hardness, low degradation, good biocompatibility, low polymerization shrinkage, elastic modulus, etc.[1,2]. The flowable composite resins - characterized by reduced filler content[3] - were introduced in the second half of the nineties and applied in clinical situations with difficult access or requiring good penetration because of their low elastic modulus permitting a low viscosity as well as a great bond-strength[4]. The shrinking stress, which occurs using the RBCs in clinical practice, influences the marginal sealing quality and effectiveness which can evolve to a secondary carious process leading to restoration failure. In order to avoid the shrinking stress, many studies have been carried out focusing on the dental curing light, the polymerization period, the composite resin features and, finally, on the material application technique[5,6]. The flow composites have been designed and manufactured to overcome the above reported problem, taking into account that such materials, with a low amount of filling, are less rigid and show an elastic modulus lower than the micro-hybrid composite resins. Moreover, the low viscosity allows the material to perform a better linkage both with dentin and enamel so adapting to the microstructural defects of the floor and walls of the cavity preparation[7]. For all these reasons, many clinicians in performing the "Open Sandwich" technique to treat both II and V cavity classes prefer to use the flow resins instead of the glass ionomer cement[8,9]. Recently, a new technique has been proposed[10] where a thin layer of flowable composite is applied to the cavity floor and then co-cured with a following layer of a packable

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composite improving the marginal sealing of the restoration with decreased microleakage. Some producers and researchers alike have proposed to use flowable bulk-filling composites at higher thickness[11], in order to maintain the features to reduce irregularities of the cavity base and, consequently, the C-factor[12]. Flow resins are also employed as pit-and-fissure sealants[13] and as fiber-reinforced composite retainers for orthodontic or periodontal use[14].

The mechanical and biological properties of RBCs are related (in different ways) to the double bonds degree of conversion (DC) involved in the polymerization reaction[15]. In particular, DC decreases proportionally to the material depth in the light-curing materials; as a matter of fact, the energy associated to the light-curing declines during the passage through resin composites because of the light scattering phenomenon, due to the size and to the refractive index of the filler particles[15].

A DC decrease provokes the enhancement of monomers leachability (with possible toxic effects on pulp cells) and reduces the hardness. Although the polymerization reaction of self-curing resins is more homogeneous than the photo-polymerization, their DC is lower because the autocatalytic process is less efficient. DC is most commonly evaluated by Fourier Transform Infrared Spectroscopy (FT-IR), an analytical technique capable of determining the signal of the unreacted aliphatic C = C double bonds present in a polymerized sample[16].

During the restoration of cavity with light-curing resin composites, 2 mm depth represents - for the above reported reasons - the maximum depth increment[17]. In an attempt to solve this problem, various manufacturers have recently introduced on the market the bulk-fill resin composites, applicable in a single layer up to 4 mm[18-21], and characterized by a decrease of polymerization shrinkage and a lack of negative effects on DC[22]. Dentsply Caulk firm claimed that "Surefil SDR *flow* (Smart Dentin Replacement, stress decreased resin) is based on a urethane dimethacrylic structure responsible for the polymerization shrinkage and stress reduction"[19]. Many researchers have already investigated some physical properties of Surefil SDR *flow*[12,15,22,23], although hardly a report has yet dealt with its biological and mechanical properties compared to those of traditional materials.

The definition of the hardness of a material is not unique, but this conventional property depends on the method used for its determination and is generally defined as the resistance that a material opposes to its penetration[24].

For the depth of cure (DoC) - commonly indicated as the thickness of a RBC adequately cured – a more complete definition has been recently proposed: "the maximum thickness that should be used for each successive RBC increment"[25].

DoC can be determined by employing different methods, like the ISO 4049 "scraping test", or by calculating ratio (hardness or DC values) of the bottom and top surfaces of resin composite samples[17,26,27]. Some authors[28,29] suggested that the hardness ratio values should be 0.8-0.9 for an acceptable cure of resin composite. Subsequently, Bouschlicher et al[27] studied the relationship between hardness ratio and DC ratio, and found that when the former has a 0.8 value, the latter usually is 0.9.

The objective of the present study was to assess the cytotoxicity of a bulk-fill material: Surefil SDR *flow* (SDR) and three conventional flowable materials: Venus Diamond Flow (VDF), Filtex Supreme XTE Flowable (XTE) and Enamel plus HRi Flow (HRI) and to correlate it to the degree of conversion. Moreover, physical properties in term of hardness, hardness ratio DC ratio were evaluated.

Materials and Methods

Sample Preparation

VDF, XTE, SDR and HRI (Table I) samples – constituted by disks – were prepared using two overlapped stainless steel molds (Figure 1). The disks, covered and separated by transparent polyester strips (Hawe-Neos Dental CH-6934 Bioggio, Switzerland) to avoid the mutual bond, were subjected – at the same time – to photo-polymerization using a LED lamp (ART-L3 Curing LightPro with 1,000 mW/cm^2 light intensity) at a distance of 2 mm from the material surface for 40", according to producer's instructions.

The obtained samples were so composed by:

1) Disk 1 (6 mm Ø × 2 mm) with a top surface directly irradiated
2) Disk 2 (6 mm Ø × 2 mm) with a top surface irradiated by the light filtered from disk 1 for a total thickness of 4 mm.

Subsequently, the cured disks, easily removed from molds by extrusion, were available for the evaluation of DC, amount of released monomers, Vickers hardness and cytotoxicity.

Table I. Manufacturers' information about materials tested in the study.

Material name	Company	Material type	Matrix type (wt %)	Filler type	Filler Ø	Filler loading	Lot. N.
Venus Diamond Flow (VDF)	Heraeus Kulzer	Flowable, nano-hybrid, thixotropic, multifunctional	UDMA, EBPADMA Bis-GMA (10-15% wt), [a] TEGDMA (10-15% wt),[a]	Ba-F-Al glass	50 nm-20 μm	63.5-65.1	6604358
Filtex Supreme XT Flow (XTE)	3M espe	Flowable, thixotropic, low wear nano-hybrid,	BIS-EMA (1-5% wt),[a] functionalized dimethacrylate (1-5% wt).[a]	Ytterbium trifluoride	0.1-5.0 μm	65	N420784
				non-agglomerated/non-aggregated silica nanofiller	75 nm		
				loosely bound agglomerated zirconia/silica	5-20 nm		
Surefill SDR Flow (SDR)	Dentsply Caulk			nanocluster non-agglomerated/non-aggregated zirconia nanofiller	5-10 nm 0.6-10 μm		
				aggregate particles			
		Flowable, fluoride ion release, up to 4 mm thickness, low shrinkage stress Bulk-fill	Polymerization modulator, EBPADMA, TEGDMA, Modified	Ba-B-F-Al silicate glass, SiO_2 amorphous, Sr-Al silicate glass, TiO2, SiO_2 highly dispersed, KF	4.2 μm	68	1208214
Enamel plus HRI flow (HRI)	Micerium s.p.a.	Flowable, micro-hybrid, light-curing composite	UDMA resin UDMA, Bis-GMA, BDDMA.	glass filler	0.7 μm	55	2012009382
				highly dispersed SiO_2	0.012 μm		

UDMA: Diurethane dimethacrylate, EBPADMA: Ethoxylated Bisphenol A dimethacrylate, Bis-GMA: Bisphenol A glycerolate dimethacrylate, TEGDMA: Triethylene glycol dimethacrylate, Bis-EMA: (2,2-Bis[4-methacryloxypolyethoxyphenyl]propane), BDDMA: 1,4-Butanediol dimethacrylate, Ba: Barium, F: Fluoride, Al: Aluminum,

Cell Cultures

All chemicals and reagents used in this study (cell culture grade) were obtained from Sigma-Aldrich, Milan, Italy, unless otherwise specified

Human pulpar cells (HPCs) were obtained (with informed consent) from a healthy patient subjected to third molars extraction for orthodontic reasons. Tooth surfaces were cut to reveal the pulp chamber, pulp tissues were harvested, cut into small pieces, digested in a solution of type I collagenase (3 mg/mL) and dispase (4 mg/mL) for 1 h at 37°C and then cultured in Dulbecco's

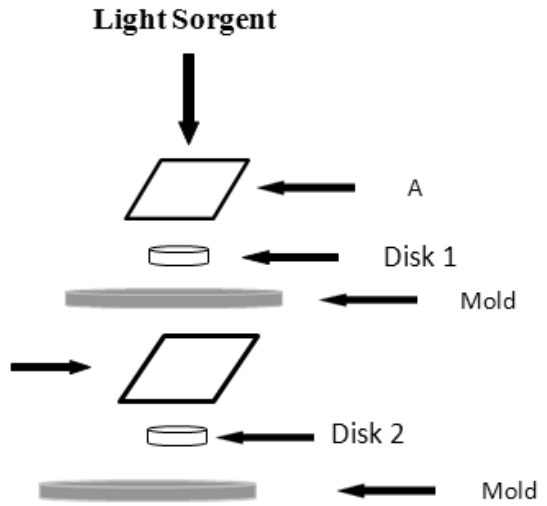

Figure 1. Samples preparation by stainless steel molds: Composite disks (6 mm Ø, 2 mm) 1 and 2. A: transparent polyester strip. Stainless steel molds (2 mm h)

Modified Eagles Medium (DMEM), supplemented with 10% Fetal Calf Serum (FCS), L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), penicillin (50 UI/mL) and streptomycin (50 μg/mL), at 37°C in a 5% humidified CO_2 atmosphere. HPCs between the second and fifth passages were used in this study[30,31].

Toxicity Studies

In order to evaluate the cytotoxic effects of the methacrylic monomers released by materials, each specimen was immersed in DMEM (2.8 mL) and left on site for 24 h at 37°C. HPCs (1 x 10^4) in DMEM (0.20 mL) were seeded in individual wells of a 96-well tissue culture plate (Costar, Cambridge, MA, USA) and cultured to sub-confluent monolayer for 24 hours; DMEM extracts of the dishes (0.20 mL) were then added to cell monolayers (undiluted and diluted from 50% to 10%) by medium change and similar volumes of DMEM were added to the control wells. After 24 h of incubation, the cell viability was evaluated by MTT test, according to Wataha et al[32]: 20 μl of a solution of MTT in PBS (phosphate buffer, 5 mg/mL) were added to the medium (0.20 mL) and, after incubation for 4 h at 37°C, the produced intracellular formazan crystals were solubilized with a solution of HCl in isopropanol (4×10^{-2} N, 0.20 mL). The absorbance of the solution contained in each well was determined using an automatic microplate photometer (PackardSpectracount™, Packard BioScience Company, Meriden, CT, USA) at a wavelength of 570 nm. Each experiment was performed in sextuplicate, repeated four times (n=4) and the cell cytotoxicity was calculated according to the following equation[33]:

$$\% \text{ cell mortality} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Materials were rated as slightly, moderately or severely cytotoxic when the toxic effects, relative to controls, were less than 30%, between 30% and 60%, or greater than 60%, respectively[34].

Conversion Degree

The top and bottom surfaces of the disks, prepared as previously described, were analyzed by a Spectrum One FTIR spectrophotometer (Perkin Elmer, Norwalk, CT, USA) equipped with an ATR (attenuated total reflection) outfit. All FT-IR spectra were recorded in the following conditions: 1000-3000 cm^{-1} wavenumber range, 16 scans were averaged at a resolution of 4 cm^{-1}. Monomers DC values were determined through the following equation:

$$\% \text{ DC} = 1 - \frac{\text{Am (c)} \times \text{Aar (u)}}{\text{Am (c)} \times \text{Aar (u)}} \times 100$$

where Am is the absorbance area of the signal related to the C = C bond of the methacrylic moiety (1637 cm^{-1}) in the cured (c) or uncured (u) material; Aar is the signal of the aromatic ring of Bis-GMA (1609 cm^{-1}) in cured (c) and uncured (u) material[35]. Three specimens (n = 3) were used in the determination of each ratio for each material.

In HRI, the absorption peak (A ester) of the C = O ester groups (1716 cm^{-1})[36] was used as reference because no aromatic peak was identified in the specimens and DC was thus calculated as follows:

$$\% \text{ DC} = 1 - \frac{\text{Am (c)} \times \text{A ester (u)}}{\text{Am (c)} \times \text{A ester (u)}} \times 100$$

The DoC of the specimens calculated as DC ratio was determined as follows:

$$\text{CD ratio 2 mm} = \frac{\text{CD2mm}}{\text{CD0mm}}$$

$$\text{CD ratio 4 mm} = \frac{\text{CD4mm}}{\text{CD4mm}}$$

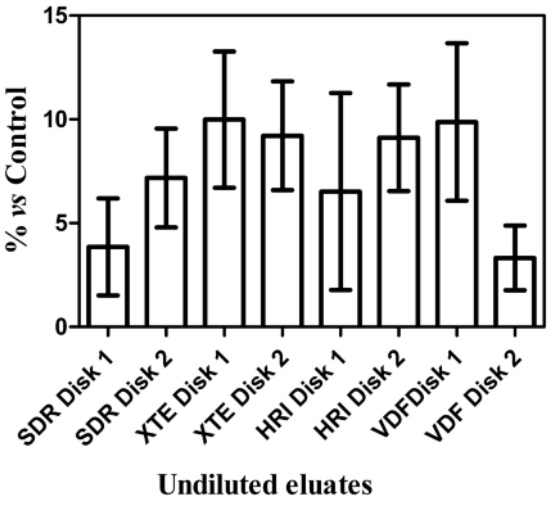

Figure 2. Cytotoxic effect in HPCs after 24 h incubation with eluates obtained from specimens; exposed HPCs did not show differences in mitochondrial dehydrogenase activity compared to control cell cultures. Each value represents the mean ± standard deviation (SD) for 4 independent experiments.

Monomers Leaching Evaluation

High Performance Liquid Chromatography (HPLC) was used to determine the amount of monomers (1,4-Butanediol dimethacrylate – BDDMA – and Triethylene glycol dimethacrylate – TEGDMA –) leached from cured samples. Three specimens (n = 3) were used for each material. Each specimen was prepared as previously reported and immersed in DMEM (2.8 mL) and left on site for 24 h at 37°C. The media were then centrifuged (13000 g, 15 min) and filtered (0.45 μm syringe filter, Whatman, Maidstone Kent, UK). Finally, samples were diluted in acetonitrile (1:10) and analyzed using a JASCO HPLC system (2 PU-980 pumps, UV-970 UV/VIS detector and AS-1555 autosampler). The assays (50 μL injected volume) were performed at a wavelength of 214 nm with a C-18 (5 μm) Supelco reversed phase column (250 × 4.6 mm) using as mobile phase (0.7 mL/min) a mixture of water (A) and methanol (B) gradient from 40% to 20% of A (30 min).

TEGDMA and BDDMA concentration in each sample was quantified using a calibration line performed with standard solutions (Sigma Aldrich, Milan, Italy) before and after each analysis.

Hardness Evaluation

Surface Vickers hardness (VH) was determined using a Vickers hardness tester (Microhardness Tester MHT4, Zeiss, Jana, Germany) and a 100 g load (0.981 N) applied for 15 seconds, slope: 10 gf/s. Three specimens (n = 3) were used for each material, and three indentations were recorded for each sample at different points of the irradiated top and non-irradiated bottom surfaces. For each surface, the mean value was then evaluated and converted into a Vickers hardness number (VHN) according to Erdemir et al[37] and Lombardini et al[38]. VHN values were expressed as N/mm^2 (MPa).

The DoC of the specimens calculated as VHN ratio was determined as follows[39]:

$$\text{VHN ratio 2 mm} = \frac{\text{VHN 2mm}}{\text{VHN 0mm}}$$

$$\text{VHN ratio 4 mm} = \frac{\text{VHN 4mm}}{\text{VHN 4mm}}$$

Statistical Analysis

Each value represents the mean of three experiments in sextuplicate. All results are expressed as the mean ± Standard Deviation (SD). The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls. If necessary, comparison of means by Student *t*-test was used: $p < 0.05$ was considered significant.

Results

Cytotoxicity

In our experimental conditions, the toxic effect induced by all tested materials was slight (5-10%), either when the eluates were added to the cells undiluted (Figure 2) or diluted (from 50% to 90%) (data not shown). No statistical differences were observed analyzing the cytotoxic effects induced by the different depth of disks (Figure 2).

Monomers Leaching Evaluation

Specimens were analyzed by HPLC to evaluate BDDMA and TEGDMA leaching. BDDMA presence was detected in the eluate from HRI samples, while TEGDMA was found in the eluate from XTE and SDR disks (Figure 3). The results showed that the monomers concentration detected is very low which justifies the absence of any cytotoxic effect. As expected, in all experimental conditions (except in the case of SDR specimens) the quantity of released monomers increases with the enhancement of the specimen

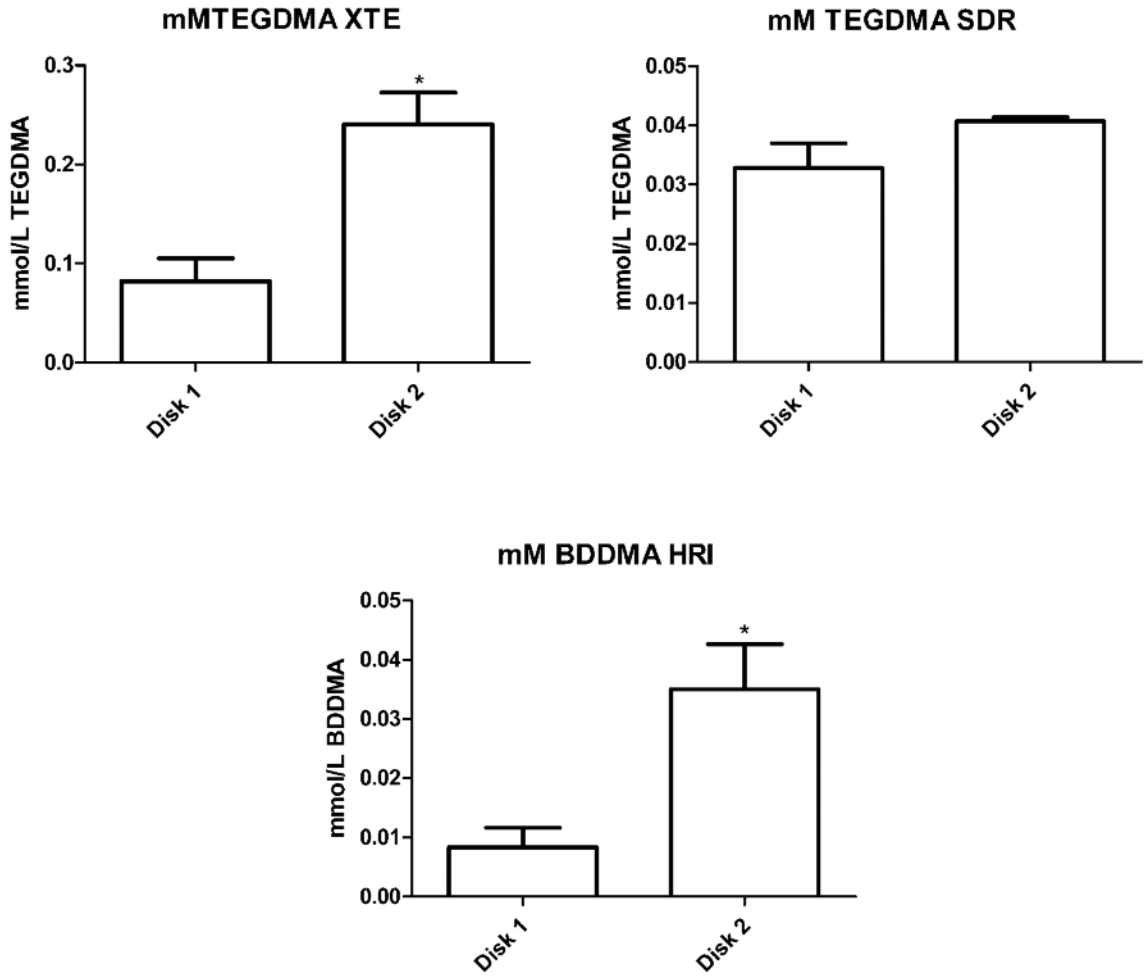

Figure 3. HPLC analysis: TEGDMA presence was detected in eluates derived from XTE and SDR disks (Panel A). BDDMA was found in eluates from HRI samples (Panel B). Monomers concentration was quantified using calibration curves using standard solutions before each analysis. Bars represent mean values ± SD from 3 repeated experiments (n = 3). $^{*}p < 0.05$.

thickness (Figure 3): although the difference of monomers release between 0-2 and 2-4 mm are statistically significant ($p < 0.05$) monomers concentrations still exerted slight cytotoxic effects in both cases. Regarding VDF, no HPLC signals were recovered.

Conversion Degree

When both surfaces of disk 1 and upper surface of disk 2 (depth 2 mm) were considered, all materials reached statistically similar DC values (Table IIa), with the only exception of XTE that statically diminished its DC also at depth of 2 mm (Table IIa). Comparing the behaviour of the materials, results showed that:

- Upper surfaces of disks 1: the analysis of results showed a conversion degree of SDR not significantly different in comparison to the other materials; while that of XTE was higher than VDF ($p < 0.05$) and HRI ($p < 0.001$) (Table IIb).
- Bottom surfaces of disks 1: the conversion degree of all materials did not show significant differences.
- Upper surfaces of disks 2: the conversion degree value of SDR was not statistically higher in respect to XTE and HRI, whereas that of VDF was lower than that of XTE ($p < 0.05$), SDR ($p < 0.001$) and HRI ($p < 0.05$) (Table IIb).
- Bottom surfaces of disks 2: the conversion degree of SDR was significantly higher than that of XTE ($p < 0.001$), VDF ($p < 0.001$) and HRI ($p < 0.001$) (Table IIb).

Table IIa. Conversion degree values. Conversion degree of samples. The top and bottom surfaces of the disks were analyzed by a Spectrum One FTIR spectrophotometer equipped with an ATR.

	SDR	VDF	XTE	HRI
Disk 1: 0 mm	70.30 ± 4.95 ^{A}	68.19 ± 2.47^{A}	76.73 ± 5.58^{A}	63.16 ± 5.31^{A}
Disk 1: 2 mm	70.08 ± 4.02^{A}	59.84 ± 4.86^{A}	67.14 ± 3.35^{B}	62.89 ± 3.61^{A}
Disk 2: 2 mm	71.70 ± 3.28^{A}	57.51 ± 2.24^{A}	65.49 ± 5.00^{B}	64.07 ± 1.96^{A}
Disk 2: 4 mm	64.67 ± 3.19^{B}	32.39 ± 2.72^{B}	40.74 ± 6.43^{C}	47.79 ± 4.58^{B}
	Disk 1; 0 mm vs. Disk 2; 4 mm**	Disk 1; 0 mm vs. Disk 2; 4 mm***	Disk 1; 0 mm vs. Disk 2; 4 mm***	Disk 1; 0 mm vs. Disk 2; 4 mm***
	Disk 1; 2 mm vs. Disk 2; 4 mm*	Disk 1; 2 mm vs. Disk 2; 4 mm***	Disk 1; 2 mm vs. Disk 2; 4 mm***	Disk 1; 2 mm vs. Disk 2; 4 mm***
	Disk 2; 2 mm vs. Disk 2; 4 mm**	Disk 2; 2 mm vs. Disk 2; 4 mm***	Disk 2; 2 mm vs. Disk 2; 4 mm***	Disk 2; 2 mm vs. Disk 2; 4 mm***
			Disk 1; 0 mm vs. Disk 2; 2 mm***	
			Disk 1; 0 mm vs. Disk 1; 2 mm***	

Values represent the means ± SD of three independent experiments (n = 3). Similar letter in each line indicate statistically similar means. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls, the values of each material were compared at different depth. (See also text in Materials and Methods).

Table IIb. Conversion degree of samples. Values represent the means ± SD of three independent experiments (n = 3).

	Disk 1: 0 mm	Disk 1: 2 mm	Disk 2: 2 mm	Disk 2: 4 mm
SDR	70.30 ± 4.95 A	70.08 ± 4,02A	71.70 ± 3.28A	64.67 ± 3.19E
VDF	68.19 ± 2.47 A	59.84 ± 4.86A	57.51 ± 2.24 B	32.39 ± 2.72 F
XTE	76.73 ± 5.58B	67,14 ± 3.35A	65.49 ± 5.00H	40.74 ± 6.43C
HRI	63.16 ± 5.31A	62.89 ± 3.61A	64.07 ± 1.96G	47.79 ± 4.58D
	XTE vs. VDF*		VDF vs. SDR***	VDF vs. SDR***
	XTE vs. HRI***		VDF vs. XTE*	VDF vs. XTE*
			VDF vs. HRI*	VDF vs. HRI***
			SDR vs. HRI*	XTE vs. SDR***
			SDR vs. XTE*	HRI vs. SDR***

Similar letter in each column indicate statistically similar means. *$p < 0.05$; ***$p < 0.001$, the different materials were compared at the same depth. (See also text in Materials and Methods).

The above results were confirmed by the analysis of depth of cure (calculated as conversion degree ratios); as a matter of fact $DC_{2\ mm}$ / $DC_{0\ mm}$ of all materials did not show statistically significant differences; on the contrary $DC_{4\ mm}$ / $DC_{0\ mm}$ of SDR was significantly higher in comparison to the other materials (Table IIc).

Table IIc. Conversion degree ratios of the samples. Values represent the means ± SD of three independent experiments (n = 3). (See also text in Materials and Methods).

Depth of cure	SDR	VDF	XTE	HRI
DC_{2mm}/DC_{0mm}	0.99 ± 0.06	0.88 ± 0.06	0.87 ± 0.04	0.99 ± 0.03
DC_{4mm}/DC_{0mm}	0.92 ± 0.05	0.47 ± 0.03	0.53 ± 0.03	0.76 ± 0.02

Table IIIa. VHN mean values. Values represent the means (MPA) ± SD of the three independent experiments (n = 3). Three indentations were recorded at different points for the irradiated top and non-irradiated bottom surfaces.

Vickers hardness values	SDR	VDF	XTE	HRI
Disk 1: 0 mm	248.0 (11.36)^{A}	205.3 (12.01)^{A}	457.8 (81. 48)^{A}	462.7 (11.75)^{A}
Disk 1: 2 mm	263.2 (30.26)^{A}	184.7 (6.65)^{B}	286.6 (11.92)^{B}	364.5 (16.60)^{B}
Disk 2: 2 mm	261.2 (11.40)^{A}	180.7 (6.89)^{B}	274.8 (25.96)^{B}	364.5 (8.80)^{B}
Disk 2: 4 mm	203.0 (12.21)^{B}	129.3 (9.06)^{C}	210.2 (7.99)^{C}	273.1 (13.88)^{C}
	Disk 1; 0 mm vs. Disk 2; 4 mm***	Disk 1; 0 mm vs. Disk 2; 4 mm***	Disk 1; 0 mm vs. Disk 2; 4 mm***	Disk 1; 0 mm vs. Disk 2; 4 mm***
	Disk 1; 2 mm vs. Disk 2; 4 mm***	Disk 1; 2 mm vs. Disk 2; 4 mm***	Disk 1; 2 mm vs. Disk 2; 4 mm*	Disk 1; 2 mm vs. Disk 2; 4 mm***
	Disk 2; 2 mm vs. Disk 2; 4 mm***	Disk 2; 2 mm vs. Disk 2; 4 mm***	Disk 2; 2 mm vs. Disk 2; 4 mm*	Disk 2; 2 mm vs. Disk 2; 4 mm***
		Disk 1; 0 mm vs. Disk 2; 2 mm**	Disk 1; 0 mm vs. Disk 2; 2 mm***	Disk 1; 0 mm vs. Disk 2; 2 mm***
		Disk 1; 0 mm vs. Disk 1; 2 mm***	Disk 1; 0 mm vs. Disk 1; 2 mm***	Disk 1; 0 mm vs. Disk 1; 2 mm***

Similar letter in each line indicate statistically similar means. $^{*}p<0.05$; $^{***}p<0.001$. The group means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls, the values of each material were compared at different depth. (See also text in Materials and Methods).

Hardness

The VHN mean values for the top and bottom surfaces measured for each group are shown in Table IIIa. The statistical analysis indicated significant differences among the examined specimens. In particular: SDR showed results congruent with DC, in fact both surfaces of disk 1 and upper surface of disk 2 (depth 2 mm) reached statistically similar VHN values (Table IIIa) and differences are present only when depth of 4 mm was considered. While all other materials showed a significant reduction of VHN values at a depth of 2 mm (Table IIIa).

Comparing the behaviour of the materials, the results showed that the VHN of the top surfaces (thickness = 0 mm) of the materials decreased in the following order: HRI > XTE > SDR > VDF. Moreover, for thickness = 2 mm and 4 mm, the VHN values of VDF were significantly lower in comparison to the other three composites (Table IIIb), whereas HRI values were significantly higher than those of the other three ones (Table IIIb). The VHN ratios of each material analysed are reported in Table IIIc; as expected, top surfaces showed higher mean values than the bottom ones.

Table IIIb. VHN mean values. Values represent the means (MPA) ± SD of the three independent experiments (n = 3). Three indentations were recorded at different points for the irradiated top and non-irradiated bottom surfaces.

	Disk 1: 0 mm	Disk 1: 2 mm	Disk 2: 2 mm	Disk 2: 4 mm
SDR	248.0 (11.36)^{A}	263.2 (30.26)^{A}	261.2 (11.40)^{A}	203.0 (12.21)^{H}
VDF	205.3 (12.01)^{A}	184.7 (6.65)^{C}	180.7 (6.89)^{D}	129.3 (9.06)^{E}
XTE	457.8 (81. 48)^{B}	286.6 (11.92)^{F}	274.8 (25.96)^{G}	210.2 (7.99)^{H}
HRI	462.7 (11.75)^{B}	364.5 (16.60)^{I}	364.5 (8.80)^{L}	273.1 (13.88)^{M}
	SDR vs. HRI***	SDR vs. HRI***	SDR vs. HRI***	SDR vs. HRI***
	SDR vs. XTE***	SDR vs. XTE*	SDR vs. VDF*	SDR vs. VDF***
	VDF vs. HRI***	SDR vs. VDF***	VDF vs. HRI***	VDF vs. HRI***
	VDF vs. XTE***	VDF vs. HRI***	VDF vs. XTE***	VDF vs. XTE***
	VDF vs. XTE***	XTE vs. HRI***	XTE vs. HRI***	XTE vs. HRI***

Similar letter in each column indicate statistically similar means. $^{*}p<0.05$; $^{***}p<0.001$. The different materials were compared at the same depth. (See also text in Materials and Methods).

Table IIIc. VHN ratios of the samples. Values represent the means ± SD of three independent experiments (n = 3).

Depth of cure	SDR	VDF	XTE	HRI
VHN_{2mm}/VHN_{0mm}	1.06 (0.12)	0.90 (0.05)	0.64 (0.13)	0.79 (0.03)
VHN_{4mm}/VHN_{0mm}	0.82 (0.06)	0.63 (0.03)	0.47 (0.09)	0.59 (0.03)

Moreover, the hardness ratio at 4 mm depth for SDR was significantly higher than the ratios from the other materials, consistently with the data from DC determinations.

Discussion

In this study, we evaluated if the cytotoxicity of a bulk-fill material and of three conventional flowable materials varies with degree of conversion. This hypothesis was formulated since both cytotoxicity and the other chemical-physical features are – more or less directly – related to conversion degree[15]. According to the obtained results, this hypothesis was rejected since there were not statistically significant differences in cytotoxicity in all experimental conditions.

Significant differences in DC values were observed either among different depths of each material and among different materials at the same depth. In particular, as far the latter aspect is regarded we found that irradiated top surface of XTE showed the better performance in respect to the other materials while, when the thickness reached 4 mm, SDR showed the best performance. Thus, although XTE is characterized by the best conversion degree at a thickness = 0 mm (76.73 ± 5.58), SDR showed a more linear behavior from the upmost to the deepest layer and its conversion degree is by far the best compared to the DC values of the other materials ([40]). Such behavior is stressed by depth of cure obtained from the conversion degree ratio: at a thickness = 4 mm, only SDR exceeds the 0.9 value whereas the other materials range between 0.47 and 0.76 (just as indicated by the producers). At a thickness = 2 mm, all the tested materials show a conversion degree value between 0.88 and 1.0; therefore, the polymerization depth (determined through depth of cure) settles – as expected – to 2 mm for HRI, XTE and VDF and to 4 mm for SDR.

The different conversion degree observed between thickness = 2 mm and 4 mm affects the uncured monomers release: as a matter of fact, disks 2 of XTE and HRI leached respectively TEGDMA and BDDMA in significantly higher amount than disks 1 and similar results were obtained by the comparison of VDF HPLC signals. It is noteworthy that the same analysis, performed on SDR, revealed no differences in TEGDMA release from disk 1 and 2. Despite

the increment of monomer leaching in every tested material, the concentrations of the released substances remain at sub-cytotoxic levels, demonstrating that the toxicity, in our experimental conditions, is not affected by DC. The release of bioactive molecules and the cytotoxicity assays are very important for the evaluation of biomaterials[41]. As a matter of fact, different harmful components can leak from the composite resins, in particular uncured methacrylic monomers or oligomers, that may cause, or at least contribute to, adverse biological effects (*i.e.* damage to the oral soft tissues, as already observed *in vivo*[41] and to a remarkable *in vitro* cytotoxicity in primary and immortalized cell cultures[42]. Research on biocompatibility of dental materials revealed that different methacrylates were able to induce glutathione depletion[43] and mitochondrial damages with consequent increase of reactive oxygen species production[44].

Regarding VHN results, it is interesting to note that only SDR showed results totally congruent with DC values, while VDF, XTE and HRI showed significant differences – in hardness values – between 0 and 2 mm.

Comparing the behavior of the materials tested, the results regarding the hardness showed that HRI and XTE were characterized by values significantly higher in respect to SDR and VDF (at all the considered depths), not only due to the Bis-GMA presence but also to the amount and dimension of inorganic filler[27]. Since micro-hardness values depend on applied weight force (most commonly 100-500 grf)[39], it was necessary to convert all the obtained results to the same units for a suitable correlation with the data reported in literature[15,22,38]. The comparison is also complicated by the different polymerization conditions utilized in the works reported and a standardization of all the variables should be warranted to even out the results obtained by the different research groups[26,40]. At a thickness = 2 mm, the depth of cure values (obtained as the ratio of hardness of bottom and top layers) of HRI and VDF samples range between about 1 and 0.8 whereas they drop markedly for XTE and VHN. At a thickness = 4 mm, only SDR (as can be observed from conversion degree values) keeps a satisfactory depth of cure (0.82) whereas the values of the other materials range between 0.47 and 0.63 confirming that SDR polymerizes adequately also with a thickness = 4 mm, consistently with what reported by producer[19].

It has been recently demonstrated[17] that the ISO 4049 "scraping test" overestimates depth of cure of bulk-filled materials. It was moreover evidenced[45] that also depth of cure, determined by hardness ratio and conversion degree ratio, overestimates the polymerization thickness of the resin based materials supporting the appropriateness of the use of calorimetry, electron paramagnetic resonance imaging or atomic force microscopy in order to measure more precisely such parameter[45].

Conclusions

Our findings demonstrated that all tested materials cause slight cytotoxic effect independently from their DC values. Comparing these parameters among the evaluated materials it is possible to note that VDF, HRI and XTE are characterized - compared to SDR - by a less satisfying polymerization at depth = 4 mm which determines a higher release of uncured substances, without a significant change in cytotoxicity.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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