DENTAL MATERIALS 22 (2006) 1081–1085

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In vitro biological response to a light-cured composite when used for cementation of composite inlays

Marco Annunziata[a], *Raffaella Aversa*[b], *Antonio Apicella*[b,*], *Antonio Annunziata*[a], *Davide Apicella*[a], *Curzio Buonaiuto*[a], *Luigi Guida*[a]

[a] *Department of Odontostomatological, Orthodontic and Surgical Disciplines, Second University of Naples "S.U.N.", Naples, Italy*
[b] *DISPAMA, Materials group, Second University of Naples "S.U.N.", via S. Lorenzo, Aversa (NA) Italy*

ARTICLE INFO

Article history:
Received 6 January 2005
Received in revised form 3 July 2005
Accepted 10 August 2005

Keywords:
Cytotoxicity
Primary culture
Indirect restoration
Composite
Luting cement

ABSTRACT

Objective. To define the cytotoxicity of a photo-cured composite when used as a bonding system under a composite inlay.
Methods. Composite specimens were photo-cured with or without a 2 mm composite inlay interposed between them and the light source. Samples were extracted in complete cell culture medium and the obtained eluates applied to primary cultures of human pulp and gingival fibroblasts. After 72 h of incubation, cell viability was evaluated by MTT assay. Survival rates were calculated with respect to negative controls.
Results. Both shielded and unshielded composite samples were cytotoxic to pulp and gingival cells. The inlay shielded composite samples reached a significantly higher level of cytotoxicity compared to the unshielded ones.
Significance. The results suggested that the cytotoxicity of a light-cured composite resin used as a bonding system for indirect composite restorations may be significantly increased as a result of an inlay light-shielding effect.

1. Introduction

Resin-based indirect restorations have been extensively used during the last decade as an esthetic alternative in posterior teeth, especially when large or multiple cavities are restored [1]. Indirect composite restorations offer some benefits with respect to the direct ones, such as a higher mechanical performance together with a significant reduction in polymerization shrinkage, limited to the dual-cured luting cement [2,3]. The main drawback remains the existence of a mechanical gradient between the cement layer and the inlay material. For instance, currently used luting cements are less wear resistant (due to their lower filler content), so that the formation of a marginal groove between tooth and restoration, the staining of the cement layer, and finally the failure, primarily for esthetic reasons, can occur [2–5].

The utilization, as a bonding system, of the same light-cured composite used for the fabrication of the inlay itself, has been recently proposed as an alternative to the conventional and more diffused dual-cured cements [6,7]. The rationale underlying this approach is that by using the same material for both the inlay and the bonding system, the mechanical gradient at the tooth-inlay interface can be reduced, improving the endurance of the cement layer over time. Furthermore, cement could be photo-cured at convenience after the inlay sitting, without the disadvantage of an early hardening which can occur using traditional dual-cured systems. The reduced mechanical properties of dual-cured cements with respect

* *Corresponding author.* Tel.: +39 081 19576396; fax: +39 081 2141001.
E-mail address: apicella@unina.it (A. Apicella).
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doi:10.1016/j.dental.2005.08.009

to composite resins has been shown in a recent paper [7], where Park et al. measured the microhardness of two types of restorative composites cured under a pre-polymerized resin composite overlay, compared to a conventional dual-cured luting cement. In that study, composite resins showed higher microhardness values than dual-cured cement, under any tested condition.

However, as known, the polymerization rate of a photo-cured material can be significantly affected by the intensity of the curing light. Several studies evidenced the dramatic effect of the thickness of composite increments on the their cure level and how poor polymerization can occur at depths greater than 2 mm [8–11]. A decreased degree of cure is known to consort not only with a decay of composite mechanical properties [11–14], but also with an alteration of the biological ones [15–18], with an increase of leached free monomers [19,20], that are demonstrated to be cytotoxic [21] and potentially harmful for the pulp [22] and the oral mucosa [23].

In this study, the authors wanted to investigate whether the biological properties of a photo-cured composite, in relation to primary cultures of human pulp and gingival fibroblasts could be affected when it is used as a bonding system under a pre-polymerized inlay.

2. Materials and methods

2.1. Materials and chemicals

The inlay and all the samples were fabricated using the composite Filtek Supreme (3M ESPE, St. Paul, MN, USA), whose characteristics are listed in Table 1.

All tissue culture biologics were purchased from Gibco Laboratories (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Inlay fabrication

To simulate an indirect restoration (inlay), a disc of composite (2 mm thickness × 7 mm diameter) was placed in a polyethylene mold and fully cured for 120 s on both sides by a conventional halogen light unit (Demetron Optilux, Kerr Company, USA; light intensity: 550 mW/cm^2). The light intensity was measured using a calibrated dental radiometer commonly available to clinicians. Light intensity was measured through the pre-polymerized inlay with the digital dental radiometer built into the Optilux curing light (Kerr, USA); the value was: 70 mW/cm^2.

Table 1 – Characteristics and composition (according with the manufacturer) of the material used to fabricate inlay and test samples

Product	Filtek Supreme
General description	Resin-based composite
Manufacturer	3M ESPE, St. Paul, MN, USA
Shade	A4
Composition	BIS-GMA, BIS-EMA, UDMA, TEGDMA, Nanosilica filler, zirconia/silica nanocluster and particles

2.3. Sample fabrication

Composite specimens (discs 1 mm thickness × 7 mm diameter) were placed on polyethylene strips and polymerized, applying the light tip close to the bottom of the strips. Some discs were cured with the inlay interposed between them and the light tip (shielded samples – SS) and some without the inlay (unshielded samples – US). All specimens were irradiated for 20 s with the above mentioned curing light unit and immediately processed for the eluate preparation.

2.4. Eluate preparation

The shielded and unshielded specimens were extracted in glass vials following standardized procedures [23]. High density polyethylene discs were used as negative control (Ctr) in parallel with the resin composite specimens. Eluates were prepared in cDMEM-F12, that is DMEM-F12 supplemented with 10% FBS, 1% L-glutamine, 1% pen-strep, and 0.25 μg/mL fungizone. The ratio between the specimen surface area and the extraction volume was 150 mm^2/mL.

After a 24 h incubation period at 37 °C under static conditions, the eluates were filtered through 0.22 μm cellulose acetate filters and immediately used for the cytotoxic assay.

2.5. Cell cultures

Primary human pulp cells (HPC) and gingival fibroblasts (HGF) were cultured by using an explant technique. Human deciduous molars were extracted just before their spontaneous exfoliation and immediately washed with sterile saline solution. The access to the pulp chamber was obtained by carefully removing the residual thin dentin floor with a sterile excavator and the pulp tissue aseptically removed. Gingival tissue was obtained from surgical operations (e.g. frenulectomies, flap operations); only the connective layer was dissected from the gingival samples by means of a surgical blade. Both pulp and gingival explants were washed two times with phosphate buffered saline (PBS) supplemented with antibiotics (1% pen-strep, 0.25 μg/mL fungizone) and cut into small pieces with a sterile surgical blade. Tissue fragments were digested in 1 mL of cDMEM-F12 containing 1 mg/mL collagenase at 37 °C for 3 h. After the incubation, released cells and tissue fragments were pelletted at 300 × *g* for 10 min, resuspended in cDMEM-F12, and plated in 35 mm Petri dishes at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. First cell islets were visible after 3–4 days, while confluence was reached in about 1–2 weeks. Cell cultures between the second and fifth passages were used in this study.

2.6. Cytotoxicity assay

Effect of control (Ctr) and test eluates (US and SS) on cell viability was measured by MTT assay. Both HGF and HPC were seeded in 24-well plates (4×10^4 cells/well) in cDMEM-F12. After overnight attachment, cells were exposed to the eluates (1 mL/well) for 72 h. Then, the cells were rinsed with PBS and

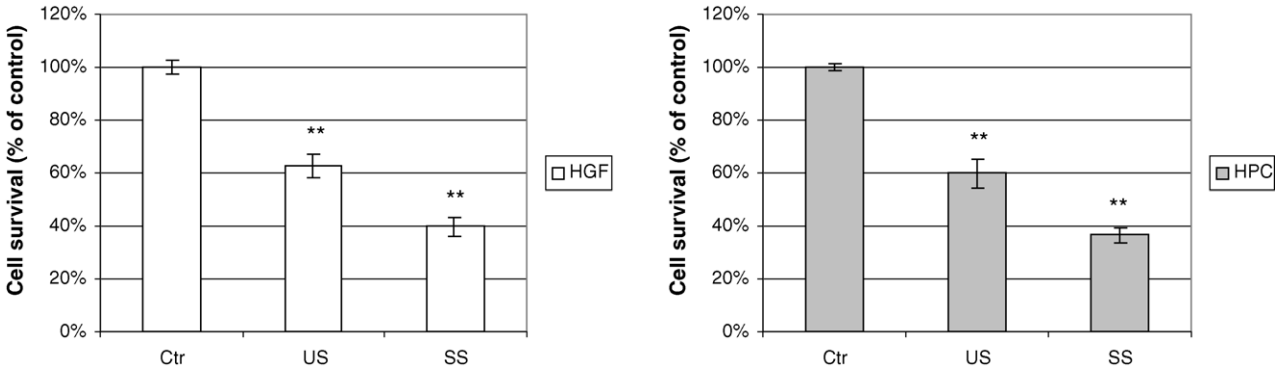

Fig. 1 – Cytotoxicity of 24 h eluates from a resin luting cement photo-cured with or without the interposition of a pre-polymerized composite inlay (shielded and unshielded samples). Survival of pulp and gingival cells is measured by MTT assay after 72 h exposure. Data are expressed as a percentage of negative control cultures (eluates from high density polyethylene specimens). Bars represents values (mean ± S.D.) from three independent experiments in triplicate. $^{**}P < 0.001$ compared to control; HGF = human gingival fibroblasts; HPC = human pulp cells; Ctr = control; US = unshielded samples; SS = shielded samples.

0.5 mL aliquots of freshly prepared MTT solution (0.5 mg/mL in DMEM without phenol red) were added to each well. After an incubation at 37 °C for 3 h, the MTT solution was removed, and the formazan crystals formed were dissolved in 1 mL/well of 0.1 N HCl in anhydrous isopropanol. The absorbance at 570 nm was spectrophotometrically measured.

2.7. *Statistical analysis*

Three independent experiments in triplicate were performed. Statistical analysis was performed by Student's *t*-test with the significance assigned at the $P < 0.05$ level.

3. Results

The effect of CTR, US and SS eluates on cell cultures is shown in Fig. 1. All eluates were cytotoxic to HGF and HPC. The presence of the inlay beyond the light tip during polymerization affected the degree of cytotoxicity of the samples tested.

Both US and SS eluates significantly reduced cell viability with respect to the control cells ($P < 0.001$). SS eluates were shown to be significantly more cytotoxic than the US ($P < 0.001$) both to HGF and HPC. HGF showed a rate of cell survival of $62.53 \pm 0.04\%$ of control when exposed to the US extract. This value decreased to $39.71 \pm 0.03\%$ for the SS extracts. Similar values were measured for the HPC, with a survival rate of $59.75 \pm 0.05\%$ and $36.52 \pm 0.03\%$ of control for US and SS eluates, respectively.

A comparison between HGF and HPC response to the eluates is shown in Fig. 2. Both cell cultures followed the same trend: although HPC seemed to be more sensitive with respect to HGF to the cytotoxicity of both US and SS extracts, no significant differences were found between the two cell populations ($P > 0.05$).

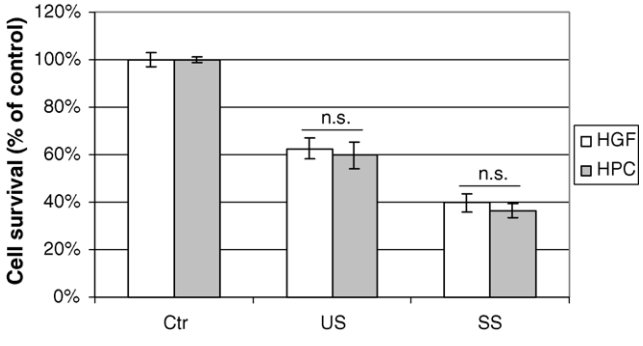

Fig. 2 – Comparison between the response of human pulp cells and gingival fibroblasts to the eluates. Cell survival is expressed as percentage of negative control cultures. Bars represents values (mean ± S.D.) from three independent experiments in triplicate. n.s. = not significant ($P > 0.05$); HGF = human gingival fibroblasts; HPC = human pulp cells; Ctr = control; US = unshielded samples; SS = shielded samples.

4. Discussion

For in vitro cytotoxicity testing either permanent cell lines or primary cultures are recommended. Although these are difficulties of isolation and maintenance, primary cells remain the gold standard for all biological assays, because of their homology to the original tissue [24]. In this study, primary cultures of human pulp cells and human gingival fibroblasts were used. The greatest care was taken during all phases, to prevent bacterial and, above all, external cell contamination (i.e. epithelial cells). Cell cultures were constantly examined during all steps of cell isolation, from the development of the first cell islets to reaching confluence: in any case epithelial colonies, or other cell typologies, were detected. Furthermore, only cultures at early passages were used, in order to maintain the cells as close as possible to their original phenotype. In order to evaluate cell viability after exposure to the resin composite samples, an indirect contact test was chosen. It was considered that such a test design was appropriate for the purpose of this study, because it was more similar to the in vivo situation,

where the luting cement covers the dentinal walls and only indirectly interacts with the pulp, by means of the tubular fluid, or with the oral mucosa, by means of the saliva. Cell viability was evaluated measuring cellular metabolic function by the MTT assay, a widely used test for in vitro biocompatibility evaluation, because of its reliability and sensitivity [25].

Both pulp cells and gingival fibroblasts produced a similar biological response and appeared to be adequate biological models for cytotoxicity testing under the experimental conditions. Once results were normalized to the respective controls, differences between HGF and HPC were present, but not significant. In particular, pulp cells, as previously described in the literature [15], tended to react more sensitively towards the test materials, with respect to HGF.

All composite samples, both shielded and unshielded, evoked a significant by lower cell survival when compared with the controls. The cytotoxicity of resin-based dental materials is widely evidenced in several studies using both cell lines and primary cultures [15,17,18,21,26]. This cytotoxic effect was also confirmed by the experience of the present study where both pulp and gingival fibroblasts showed a significantly lower viability (about 60% of the controls) when exposed to the eluates of fully polymerized composite samples (unshielded samples).

The presence of the inlay between the samples and the light tip during polymerization significantly affected their biological properties. Eluates derived from shielded samples showed a significantly higher cytotoxicity where compared with the unshielded ones. These results are in line with the current literature where, however, the importance of the shielding effect on composite cytotoxicity is only indirectly suggested. In a recent study [17], the authors reported that the cytotoxicity of all the investigated composites increased when polymerized in a 5 mm bulk increment rather than in more thin increments. The explanation to the observed phenomena can probably be found in a decreased polymerization rate reached by the shielded samples as a consequence of the attenuation of the curing light through the inlay, a phenomenon already known as decreased depth of cure [27,28]. In a recent paper, the inlay shielding effect on the polymerization levels and kinetics of light-cured composites was evidenced with a differential scanning calorimeter technique (DSC) [29]. In that study, the inlay shielded dental composite reached a significantly lower level of polymerization (63% of maximum attainable conversion) compared to the unshielded ones. It is widely evidenced in literature that the polymerization rate can significantly affect the cytotoxicity of a composite material, through the diffusion of a large number of unreacted resin monomers [16,19,20]. Several monomers contained in the composite material used in the current study (such as BIS-GMA, BIS-EMA, UDMA, TEGDMA as declared by the manufacturer) are known to diffuse from partially polymerized composite resins and to be cytotoxic in vitro [15,21]. Therefore, it is reasonable to assume that the biological response observed in our experiments to shielded samples, compared to the unshielded ones, can be related to an increased leaching of resin monomers from the composite, as a consequence of a reduced polymerization rate.

From a clinical point of view, when a photo-cured composite material is used as part of an inlay bonding system, the inlay can act as a shield. The degree of cure of those parts of the composite shielded by the inlay material can be significantly affected by light attenuation with a possible decay of both mechanical and biological properties. Uncured or incompletely cured resin composites can release a large amount of monomer into the surrounding environment, and this may occur just in the deeper part of the inlay cavity, where the dentinal wall is thinner and the composite is close to the pulp chamber.

5. Conclusions

This data suggested that the photo-polymerization of a light-cured composite bonding system through a pre-polymerized inlay could significantly affect its cytotoxicity towards the pulp and gingival cells, as a result of an inlay light-shielding effect. Practitioners should adequately ensure complete polymerization of the composite resin (e.g. using longer time of cure), when it is applied as a bonding system under composite inlays. Further studies are required to directly compare the polymerization kinetics of dual-curing cements and composite resins under inlay, as well as their in vitro biological properties.

REFERENCES

[1] Blank JT. Scientifically based rational and protocol for use of modern indirect resin inlays and onlays. J Esthet Dent 2000;12:195–208.
[2] Dietschi D, Scampa U, Campanile G, Holz J. Marginal adaptation and seal of direct and indirect class II composite resin restorations: an in vitro evaluation. Quintessence Int 1995:127–37.
[3] Dietschi D, De Siebenthal G, Neveu-Rosenstand L, Holz J. Influence of the restorative technique and new adhesive on the dentinal marginal seal and adaptation of resin composite class II restorations: an in vitro evaluation. Quintessence Int 1995;26:717–26.
[4] Van Meerbeek B, Inokoshi S, Willems G, Noack MJ, Braem M, Lambrechs P, et al. Marginal adaptation of four tooth-coloured inlay system in vivo. J Dent 1992;20:18–26.
[5] Gladys S, Van Meerbeek B, Inokoshi S, Willems G, Braem M, Lambrechts C, et al. Clinical and semiquantitative marginal analysis of four tooth coloured inlay system at 3 year. J Dent 1995;23:329–39.
[6] Campanella V. Restauri indiretti in composito nei settori posteriori: nuova tecnica costruttiva e cementazione sotto pressione. G IT Cons 2003;I-N 1(88):8–15.
[7] Park SH, Kim SS, Cho YS, Lee CK, Noh BD. Curing units' ability to cure restorative composites and dual-cured composite cements under composite overlay. Oper Dent 2004;29(6):627–35.
[8] Rueggeberg FA, Caughman WF, Curtis Jr JW. Effect of light intensity and exposure duration on cure of resin composite. Oper Dent 1994;19:26–32.
[9] Prati C, Chersoni S, Montebugnoli L, Montanari G. Effect of air, dentin and resin-based composite thickness on light intensity reduction. Am J Dent 1999;12:231–4.
[10] Silkas N, Eliades G, Watts DC. Light intensity effects on resin-composite degree of conversion and shrinkage strain. Dent Mater 2000;16:292–6.
[11] Price RB, Doyle G, Murphy D. Effects of composite thickness on the shear bond strength to dentin. J Can Dent Assoc 2000;66:35–9.

[12] Bream M, Davidson CL, Vanherle G, Van Doren V, Lambrecht P. The relationship between test methodology and elastic behavior of composites. J Dent Res 1987;66:1036–9.
[13] Ferracane JL. Correlation between hardness and degree of conversion during the setting reaction of unfilled dental restorative resin. Dent Mater 1987;1:11–4.
[14] Ferracane JL, Borge HX. Fracture toughness of experimental dental composite aged in ethanol. J Dent Res 1995;74:1418–23.
[15] Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. J Biomed Mater Res 1998;41(3):474–80.
[16] Geurtsen W, Spahl W, Leyhausen G. Residual monomer/additive release and variability in cytotoxicity of light-curing glass-ionomer cements and compomers. J Dent Res 1998;77(12):2012–9.
[17] Franz A, Konig F, Anglmayer M, Rausch-Fan X, Gille G, Rausch WD, et al. Cytotoxic effects of packable and nonpackable dental composites. Dent Mater 2003;19(5):382–92.
[18] Spagnuolo G, Annunziata M, Rengo S. Cytotoxicity and oxidative stress caused by dental adhesive systems cured with halogen and LED lights. Clin Oral Investig 2004;8(2):81–5.
[19] Ferracane JL, Condon JR. Rate of elution of leachable components from composite. Dent Mater 1990;6(4): 282–7.
[20] Ferracane JL. Elution of leachable components from composites. J Oral Rehab 1994;21:441–52.
[21] Hanks CT, Strawn SE, Wataha JC, Craig RG. Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res 1991;70(11):1450–5.
[22] Hebling J, Giro EM, Costa CA. Human pulp response after an adhesive system application in deep cavities. J Dent 1999;27(8):557–64.
[23] Hensten-Pettersen A. Skin and mucosal reactions associated with dental materials. Eur J Oral Sci 1998;106:707–12.
[24] International Standard Organization. Biological evaluation of medical devices – Part 5. Tests for cytotoxicity: in vitro methods, 10993-5, 1st ed. Geneva, Switzerland; 1992.
[25] Wataha JC, Craig RG, Hanks CT. Precision of and new methods for testing in vitro alloy cytotoxicity. Dent Mater 1992;8(1):65–70.
[26] Schedle A, Franz A, Rausch-Fan XH, Spittler A, Lucas T, Samorapoompichit P, et al. Cytotoxic effects of dental composites, adhesive substances, compomers and cements. Dent Mater 1998;14:429–40.
[27] Bennec AW, Watts DC. Performance of two blue light-emitting diode dental light curing units with distance and irradiation-time. Dent Mater 2004;20:72–9.
[28] Ruytes IE. Conversion in different depths of ultraviolet and visible light activated composite materials. Acta Odont Scand 1982;40:179–92.
[29] Simeone M, Lanza A, Rengo S, Aversa R, Apicella D. Inlay shading effect on the photopolymerisation kinetic of a dental composite material used as bonding system in an indirect restoration technique. Dent Mater 2005 [Epub ahead of print].