Cytotoxicity of Composite Materials Polymerized with LED Curing Units

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Laboratory Research

Cytotoxicity of Composite Materials Polymerized with LED Curing Units

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Clinical Relevance

Curing light intensity is one of the main parameters for proper resin composite cure. Inadequate polymerization of a composite can be harmful to vital pulp tissue and can compromise the durability and quality of the final restoration.

SUMMARY

The proper intensity and illumination time of a curing light is of great importance for the complete polymerization of resin composites and long-lasting resin composite restorations. Inadequately cured resin composites can have a cytotoxic effect on pulp tissue by releasing unreacted monomers. This study determined whether there is any difference in cytotoxicity between composite materials illuminated with different curing modes of LED curing units. Thin layers of two composite materials were polymerized using three different modes of the Bluephase C8 LED curing unit: a high intensity mode (HIP–800 mW/cm², 20 seconds), a soft-start mode (SOF–650 mW/cm² first 5 seconds, 800 mW/cm² next 25 seconds) and a low intensity mode (LOP–650 mW/cm², 30 seconds). Lymphocyte cultures were treated with both polymerized and unpolymerized composites using one of the modes stated above. Cells were analyzed using the trypan blue exclusion test, the acridine orange/ethidium bromide dying technique and an alkaline comet assay. Significant cytotoxicity was observed for 120 mg of unpolymerized composites and those polymerized with the HIP polymerization mode. A significant level of DNA damage was detected for 120 mg of unpolymerized composites. However, curing via the LOP program exhibited the lowest genotoxicity. Longer curing time with lower intensity results in less cytotoxicity than shorter curing exposure using a higher intensity of light emitted from the curing light source.

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INTRODUCTION

The use of light-cured composite materials has recently increased due to improvements in formulation, simplification of bonding procedures and increased aesthetic demands from patients. Adequate polymerization is a crucial factor in maximizing the physical properties and clinical performance of resin composites.[1] Halogen light-curing units remain the most widely used light source for resin composite curing. To overcome the problems inherent with halogen curing units, new devices, based on solid-state light-emitting diode (LED) technology, have been developed for polymerizing resin composites.[2]

Regardless of the light source used, the illumination of 500-800 mW/cm^2 lasting 30-40 seconds (15-24 Jcm^{-2}) is necessary to polymerize an increment of composite, which must be sufficiently thin to receive the full power density of the curing light. Despite using thick increments of 1 to 3 mm, it is important to note that complete polymerization is never achieved. Composite materials create highly cross-linked networks during polymerization and, under normal curing conditions, they achieve approximately 45%-70% conversion.[3-4] The composition of composite materials, filler particle size and the type and kinds of interactions between monomers and filler, affect the rate of polymerization. The light-curing unit can also have a significant impact on the ability to polymerize the composite material.[5] The intensity of light, polymerization mode and bandwidth of light output can also modify the response of the material. Theoretically, a 100% conversion of monomer to polymer is possible, but as much as 25% to 50% of the methacrylate monomer double-bonds actually remain inactive in the polymer.[6] Even in fully set restorative materials, substantial amounts of short-chain polymers remain unbound, resulting in a potential release of unreacted toxic components around pulp tissue.[7-9] There is also a correlation between the amount of uncured resin monomers in a composite and the magnitude of the cytotoxicity effect. To overcome this problem of inadequate polymerization, new curing methods have been introduced (the soft-start and exponential programs, pulse program and high and low intensity program).

The cytotoxicity of dental composites and their components have been studied using different methods.[10-13] The toxicity of a dental material can be evaluated by an *in vitro* test and through clinical studies in humans. *In vitro* studies are primarily performed to evaluate the cytotoxicity (cell damage) or genotoxicity (specific DNA damage or chromosomal aberration) of a dental material. Contrary to other cytogenetic techniques, comet assay, which was used in this experiment, does not require cell cultivation; thus, it detects primary DNA damage *in situ* at the level of each individual cell. Comet assay is used to detect single and double DNA strand breaks and other alkali labile sites (apurinic and apyrimidinic sites, DNA adducts, DNA cross-linking, DNA-protein links and incomplete excision repair). Due to its simplicity, high sensitivity and reliability, comet assay is even used in the evaluation of genotoxic potential of various chemical and physical agents.[14-15]

Unfortunately, little is known about the effect of the different curing modes on the cytotoxicity of composites. In this study, the authors evaluated possible toxicity and/or genotoxicity of two different composite materials and three light curing procedures, both separately and in their mutual interaction. The composites were tested both as polymerized and unpolymerized. To evaluate genome damage, comet assay on peripheral blood lymphocytes, as a recommended sensitive biomarker, were used.[16-17] When evaluating the cytotoxic effect, apoptotic cells were distinguished from necrotic ones by using a combination of fluorescent dyes.

METHODS AND MATERIALS

Reagents

Histopaque-1077 (1.077 g/mL at 25°C), NaCl (≥98% CAS No 7647-14-5), penicillin-streptomycin (50×, liquid, stabilized), trypan blue (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate CAS No 72-57-1), acridine orange (≥98.0% CAS No 65-61-2), ethidium bromide (≥95.0% CAS No 1239-45-8), low melting agarose (grade for Molecular Biology, DNase, RNase, none detected CAS No 39346-81-1), normal melting agarose (grade for Molecular Biology, DNase, RNase, none detected CAS No 9012-36-6), N-Lauroylsarcosine sodium salt (≥94% CAS No 137-16-6), Na_2EDTA (≥99% CAS No 6381-92-6), Tris-HCl (≥99.9% CAS No 77-86-1), Triton X-100 (grade for Molecular Biology CAS No 9002-93-1), Dimethyl sulfoxide (DMSO) solution (75 wt % in H_2O CAS No 67-68-5) and NaOH (≥98% CAS No 1310-73-2) were purchased from Sigma-Aldrich, St Louis, MO, USA.

Gibco RPMI medium (with D-glucose at 4.5 g/L, HEPES buffer at 2.383 g/L, L-glutamine with sodium bicarbonate 1.5 g/L, sodium pyruvate 0.1100 g/L) was purchased from Invitrogen, Paisley, UK.

Cell Cultures

To evaluate genome damage, primary human lymphocyte cultures were used. The advantage of using primary lymphocyte cultures lies in their closer resemblance to cells found *in vivo*. The lymphocytes were freshly isolated from three young, healthy, non-smoking donors with no record of exposure to any chemical or physical agent that might interfere with the results of the testing. The volunteers were acquainted with the purpose of the study, and they gave an informed consent for participation in the study. The lymphocytes were isolated using a standard procedure.[18]

Anticoagulant-treated blood was mixed 1:1 (v/v) with balanced salts solution (0.9% NaCl), layered on the Histopaque-1077 solution and centrifuged at 600 rpm for 40 minutes at room temperature. The layer containing lymphocytes was carefully removed and the cells were re-suspended in balanced salts solution. They were washed twice by centrifugation at 600 rpm for 10 minutes. The final pellet was gently re-suspended in culture Gibco RPMI medium. Viability of the cells was checked by supravital staining with 0.1% trypan blue, and it was always above 95%. Lymphocyte cultures were set up by introducing the cells to 5 ml of Gibco RPMI medium with penicillin and streptomycin added to obtain a cell density of 10^6/ml. No newborn calf serum or mitogen was added. The cultures were cultivated at 37°C in a 5% CO_2 atmosphere.

Preparation of Composites

This study comprised the cyto/genotoxicity testing of two composites: Tetric EvoCeram (Vivadent, Schaan, Liechtenstein) (Lot H3269, exp 2009/10) and Tetric Ceram (Vivadent) (Lot G06835, exp 2008/03). Two different weights (Sartorius BLG10S, Goettingen, Germany) of each composite were tested: 60 mg and 120 mg. After weighing, the unpolymerized composites were directly introduced into the lymphocytes cultures. Other composite samples were mechanically pressed between two Mylar sheets to obtain a thin layer and proper cure of the resin composite samples and were polymerized by the LED curing unit, Bluephase C8 (Vivadent). The light curing tip was applied directly onto the Mylar sheet and the composites were cured using three different modes: a high intensity mode (HIP, 800 mW/cm^2, 20 seconds illumination for each tested composite material), a soft-start mode (SOF, 650 mW/cm^2 first 5 seconds, 800 mW/cm^2 next 25 seconds) and a low intensity mode (LOP, 650 mW/cm^2, 30 seconds illumination). Thereafter, the polymerized composites were separated from the Mylar sheets and introduced into the lymphocyte cultures. The cultures were treated for 72 hours at 37°C in a 5% CO_2 atmosphere. The negative control cultures were treated with 1 ml of saline solution (NaCl 0.9%) for 72 hours. The positive control cultures were treated with 1 mM H_2O_2 on ice for five minutes, which is the standard procedure for comet assay.[14-15] The same culture was used for both cytotoxicity and genotoxicity testing.

Cytotoxicity Testing

For composite testing, after 72 hours of treatment, the cultures were centrifuged at 600 rpm for 10 minutes and 4.5 ml of supernatant was removed. The pellet was gently re-suspended. On the microscopic slide, 20 µl of suspension was mixed with 20 µl of 0.4% trypan blue. The suspension mixed with dye was covered by a cover slip and analyzed under a CX 40 light microscope (Olympus, Tokyo, Japan) using 1000x magnification. For each lymphocyte culture (for each sample of composite cured using a specific curing program), 1000 lymphocytes were analyzed, counting the unstained (viable) cells. The blue cells were considered to be nonviable.[19]

To detect early apoptosis and necrosis, a method described by Duke and Cohen[20] was used. Fifty microlitres of suspension obtained in the same way as the trypan blue exclusion test were mixed with 50 µl of a solution of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml). The suspension mixed with dye was covered with a cover slip and analyzed under the epifluorescence microscope, AX 70 (Olympus, Tokyo, Japan), at 600x magnification. For each lymphocyte culture (for each sample of composite cured using specific curing program), 500 lymphocytes were analyzed, counting the unstained (viable) cells. The nuclei of vital cells emitted a green fluorescence; apoptotic lymphocytes emitted a green fluorescence surrounded by a red echo and necrotic red fluorescence.

Comet Assay

The comet assay was performed according to the standard protocol identified by Singh and others.[21] Five µl of the same cell cultures that were used for cytotoxicity testing were suspended in 100 µl of 0.5% low melting agarose to obtain 10,000 lymphocytes per slide. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerization of each gel-layer. After solidification of the 0.5% agarose layer, the slides were immersed in a lysis solution (1% N-lauroylsarcosine sodium salt, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4°C. After one hour, the slides were placed in an electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) at 0˚C for 20 minutes to allow the DNA to unwind. The electrophoresis was performed at 300 mA and 1.0 V/cm in a horizontal electrophoresis platform for 20 minutes. The slides were neutralized with a Tris-HCl buffer (pH 7.5) and stained with ethidium bromide (20 µg/ml) for 10 minutes. Each slide was analyzed using a Leitz Orthoplan epifluorescence microscope (Leitz, Wetzlar, Germany). One hundred comets per slide were analyzed by the Comet assay II automatic digital analysis system (Perceptive Instruments Ltd, Suffolk, Halstead, UK) measuring tail length and tail intensity (% DNA). During analysis, the edges and eventually the damaged parts of the gel and debris, superimposed comets, comets of uniform intensity and comets without a distinct head ("clouds," "hedgehogs" or "ghost cells") were avoided. For each lymphocyte culture (for each sample of composite cured using specific curing program), 100 comets were analyzed.

Statistical Analysis

To test differences between the results of the cytotoxicity testing obtained by the trypan blue exclusion technique and acridine orange/ethidium bromide dying, a χ^2 test was used. For the comet assay endpoints, the Mann-Whitney *U*-test was carried out. All calculations were performed using a commercial program, Statistica 5.0 (StatSoft, Tulsa, OK, USA). *P*-values less than 0.05 were considered significant.

RESULTS

The trypan blue exclusion test showed a slight and statistically insignificant decrease in the number of viable lymphocytes treated with 120 mg of Tetric Ceram polymerized using the HIP mode and an unpolymerized one (Table 1). The latter method significantly increased the number of apoptotic cells detected by acridine orange/ethidium bromide dying. The same method revealed a significant increase in the number of necrotic lymphocytes in cultures treated with both doses of unpolymerized Tetric Ceram, 120 mg of unpolymerized Tetric EvoCeram, 120 mg of Tetric Ceram cured using each of three tested modes and 120 mg of Tetric EvoCeram cured using HIP (Table 1).

Significantly higher tail length values were detected only in lymphocytes treated with 120 mg of unpolymerized Tetric Ceram and Tetric EvoCeram (Table 2). The same dose of unpolymerized Tetric EvoCeram also exhibited a significantly higher percentage of DNA contained in the tail. Although comet assay parameters for cultures treated with polymerized composites did not differ significantly from the control, the lowest level of DNA damage was detected after the LOP curing mode (Table 2).

DISCUSSION

Effective composite cure is a critical parameter, not only to ensure optimum physical properties of the cured restoration, but to also ensure that clinical problems do not arise as a result of the cytotoxicity of inadequately polymerized materials. HEMA and TEGDMA are the main (co)monomers released from resin-based materials after the hardening of resin composite.[22] Within the last decade, the cytotoxicity and genotoxicity of some methacrylates have been identified in a number of investigations. It has been well established that the comonomer TEGDMA causes gene mutations *in vitro*. Gerzina and Hume[23] have found that TEGDMA and HEMA can diffuse through dentin into the pulp space and, therefore, can also irritate the pulp and gingiva. Although details of the mechanisms leading to cell death, genotoxicity and cell-cycle delay are not completely understood, resin monomers may be able to alter the function of cells in the oral cavity.

Table 1: *Results of the cytotoxicity testing of two different composites using the trypan blue exclusion technique and combined acridine orange/ethidium bromide dying.*

Composite	Amount/mg	Light Curing Procedure	Viability/% ± SE	Apoptotic/% ± SE	Necrotic/% ± SE
Tetric EvoCeram	60	Non-polymerized	0.2 ± 0.1	0.6 ± 0.2	0.8 ± 0.1
	120		0.3 ± 0.1	1.0 ± 0.2	1.8 ± 0.6[1]
	60	SOF[2]	0.2 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
	120		0.1 ± 0.0	0.8 ± 0.4	1.0 ± 0.3
	60	HIP[3]	0.2 ± 0.1	1.0 ± 0.3	1.0 ± 0.2
	120		0.0 ± 0.0	1.1 ± 0.6	1.6 ± 0.4[1]
	60	LOP[4]	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.1
	120		0.0 ± 0.0	0.7 ± 0.1	0.5 ± 0.1
Tetric Ceram	60	Non-polymerized	0.2 ± 0.1	0.8 ± 0.2	1.7 ± 0.5[1]
	120		0.8 ± 0.3	2.5 ± 0.6[1]	4.2 ± 1.0[1]
	60	SOF[2]	0.2 ± 0.1	0.6 ± 0.1	1.1 ± 0.7
	120		0.1 ± 0.1	1.0 ± 0.7	2.3 ± 0.9[1]
	60	HIP[3]	0.3 ± 0.1	1.3 ± 0.4	1.6 ± 0.4[1]
	120		0.7 ± 0.2	1.3 ± 0.3	2.9 ± 0.9[1]
	60	LOP[4]	0.2 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
	120		0.0 ± 0.0	0.9 ± 0.3	1.7 ± 0.4[1]
Negative control (0.9% NaCl)			0.2 ± 0.1	0.8 ± 0.1	0.3 ± 0.0
Positive control (0.1 M H_2O_2)			25.3 ± 6.1[1]	9.1 ± 1.4	21.7 ± 4.5

Statistical significance was tested by Mann-Whitney U-test. Significantly different results (p<0.05) were obtained:
1 compared to negative control
2 compared to SOF curing mode
3 compared to HIP curing mode
4 compared to LOP curing mode

Table 2: *Comet Assay Endpoints for the Testing of Different Curing Procedures*

Composite	Amount/mg	Light Curing Procedure	Tail Length/ µm ± SE	Tail Intensity/ % DNA ± SE
Tetric EvoCeram	60	Non-polymerized	17.2 ± 0.65	1.23 ± 0.24
	120		21.1 ± 0.42[1]	1.27 ± 0.31
	60	SOF[2]	18.1 ± 0.60	1.31 ± 0.18
	120		19.6 ± 0.56	1.91 ± 0.35
	60	HIP[3]	18.9 ± 0.60	1.62 ± 0.33
	120		19.3 ± 0.87	1.47 ± 0.26
	60	LOP[4]	17.6 ± 0.58	1.08 ± 0.33
	120		17.3 ± 0.49	1.92 ± 0.38
Tetric Ceram	60	Non-polymerized	16.9 ± 0.58	1.04 ± 0.35
	120		22.5 ± 0.59[1]	2.41 ± 0.36[1]
	60	SOF[2]	18.0 ± 0.51	1.85 ± 0.37
	120		20.1 ± 0.95	2.14 ± 0.55
	60	HIP[3]	17.9 ± 0.93	1.94 ± 0.58
	120		20.6 ± 0.86	1.38 ± 0.41
	60	LOP[4]	17.2 ± 0.53	1.09 ± 0.47
	120		19.0 ± 1.09	1.93 ± 0.36
Negative control (0.9% NaCl)			17.7 ± 0.80	1.37 ± 0.36
Positive control (0.1 M H_2O_2)			37.2 ± 5.02	10.3 ± 2.07

[1] *compared to negative control*
[2] *compared to SOF curing mode*
[3] *compared to HIP curing mode*
[4] *compared to LOP curing mode*

The goal of this study focused on the impact of the polymerization program used for the hardening of composite material on the cytotoxicity and genotoxicity of the tested composite materials. If a light-activated resin restoration does not receive sufficient energy at the correct wavelengths from the curing unit, the effects of wear may be increased;[24] this could result in greater breakdown of the restoration at the margins, decreased bond strength between the tooth and the restoration, reduced hardness and greater cytotoxicity.[25-27] It is well documented that energy density (power density x exposure duration) of the light curing unit influences the degree of cure, depth of cure and mechanical properties of a resin composite. A given energy density can be delivered with different combinations of power density and exposure duration. That means that, on the same exposure duration, an increase in power density leads to improved cure, as does an increase in exposure duration at the same power density.[28-29] Some studies, based on the degree of cure measurements, concluded that a reciprocal relationship exists between power density and exposure duration. Furthermore, different curing modes may result in similar degrees of conversion but in a different polymer network structure, which leads to different mechanical properties.[28-29]

Some studies previously conducted with the same curing units show the influence of energy density on the degree of conversion. The higher the energy density, the higher the degree of conversion that was achieved.[30-31] Other studies revealed temperature measurements and concluded that higher energy density leads to higher temperature rise.[4,32] In the current study, the highest cytotoxicity was found in the case of polymerization with a high intensity polymerization program. Based on what was mentioned earlier, higher intensity leads to a higher degree of conversion, but also to higher temperature rise. This leads to the conclusion that temperature rise has probably a greater influence on cell culture cytotoxicity than the effect of the unpolymerized monomer.

Resin composites have been shown to be cytotoxic in several tissue cell systems.[10-13] However, it is difficult or even impossible to compare the results of different cell culture experiments, because of the many variations in experimental conditions, such as cell type, cell material contact method and exposure time. Although both techniques were used in this experiment, trypan blue exclusion assay and combined acridine orange/ethidium bromide dying, which were used in the cytotoxicity evaluation results, indicated that the number of cells undergoing cell death by necrosis and apoptosis was always higher compared to those incorporating trypan blue. The cells with an intact plasma membrane do not take up trypan blue. Thus, using trypan blue reveals only cells that have substantially altered membrane

permeability. However, cell membrane integrity is only affected at a very late stage in apoptosis. Therefore, trypan blue visibly detects only a part of the necrotic cells that are permeable and could only highlight apoptotic cells at a very late stage, when they potentially display membrane lesions. It was therefore logically observed that trypan blue staining substantially underestimates the number of non-viable apoptotic cells.[33]

Yap and others[2] showed that composite cure and cytotoxicity associated with LED lights is device dependent. They concluded that composite materials cured with LED lights were more cytotoxic than composites cured with conventional halogen curing lights.

Hofmann and others[34] compared the release of unreacted components from resin composites after curing with standard, soft-start and fast cure modes. They concluded that samples cured using a fast cure method showed the greatest solubility and sorption. However, in a study by Nalasci and others,[35] it was revealed that curing methods did not have a significant effect on the cytotoxicity of composite materials. The results of the current study somewhat agree with those of Nalasci and others. In the current study, the most cytotoxic effect was obtained by using the HIP polymerization mode. The reason for those results may be the shorter polymerization time. However, previous studies by the current authors show that the degree of conversion of the HIP mode is better than the LOP mode of the same curing unit.[31] It is questionable as to why the LOP program, with its lower degree of conversion, shows less cytotoxicity. One answer may be in the temperature reached with the HIP mode. While higher light intensity brings a higher temperature rise,[4,32,36-37] another answer may be the difference in the polymer network of composite materials formed after polymerization with the LOW and HIP polymerization mode.[28-29] Based on the limitations of this study, further studies that enhance the degree of conversion measurements and temperature rise with their influence on cell culture cytotoxcity need to be done.

It can also be concluded from this study that both tested composites, in their unpolymerized form, exhibited higher cytotoxicity and genotoxicity than polymerized materials, although the pH value of treated cell cultures did not change, even after 72 hours of cultivation. This could indicate the presence of cyto/genotoxic monomers, which are inactivated by their conversion during the light curing process. Both composites that were tested when cured using the HIP mode exhibited higher, although not statistically significant, cytotoxicity compared to the other two modes. These observations might suggest that using a higher power of the light source within a shorter period of time is less efficient in monomer conversion than using a lower source of power during longer periods of time. Thus, applying the HIP mode increased results in the higher amount of unconverted residual monomers that could exhibit a cyto/genotoxic effect that is released from the polymerized composite, initiating cell death by apoptosis or necrosis.[38-39] The authors deduced that considerable amounts of those monomers are released during the first 24 hours after polymerization. Thus, the highest toxicity could be expected within the first few days.

Further studies on the elution time and release of components from composite materials polymerized with different curing modes and methods would be helpful to better understand the biological risks of these modern restorative materials and curing lights for their photo-polymerization.

CONCLUSIONS

The results of this study show a dependence between the curing light intensity, curing time and cytotoxic/genotoxic effect of cell culture. The highest cytotoxic effect was obtained in the case of polymerization of composite materials with the HIP mode, while the lowest effect was obtained using the LOP polymerization program.

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