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ORIGINAL ARTICLE

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Cytotoxicity and oxidative stress caused by dental adhesive systems cured with halogen and LED lights

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Abstract The aim of the present study was to investigate the cytotoxicity of two "one bottle" adhesive systems after polymerization with a conventional halogen or a light emitting diode (LED) lamp. We hypothesized that different polymerization sources might enhance the intracellular production of reactive oxygen species (ROS), leading to reduced cell survival. Two "one bottle" adhesive systems (Optibond Solo and Scotchbond One) were cured with a commercial halogen (Optilux 500) and an LED source (Elipar Freelight, 3 M). The specimens were extracted for 24 h in complete cell culture medium or in phosphate-buffered saline (PBS). Endothelial cells (ECV 304) were exposed to the extracts for 24 h and survival rates were evaluated by the MTT assay. Then, ROS generation was monitored by the oxidation-sensitive fluorescent probe 2'7'-dichlorofluorescin diacetate (DCFH-DA). Extracts from all materials except for Optibond Solo polymerized with the halogen lamp were rated significantly cytotoxic. Scotchbond One cured with LED was the most toxic material, which reduced cell survival to about 23% compared with control cultures. Significantly higher amounts of ROS were produced in cell cultures treated with adhesives polymerized with the LED lamp compared with the materials cured with the commercial halogen light source. We demonstrated that the production of intracellular ROS by extracts of the adhesive systems depended on the light sources used for curing of the materials. These results suggested a possible link between ROS production and cytotoxic activity.

Keywords Dental adhesives · Photopolymerization · Cytotoxicity · Reactive oxygen species · Light-curing units

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Introduction

Modern dentine bonding systems (DBSs) have been widely used by clinicians without relevant incidence of unfavorable effects. However, cytotoxic effects of current dentin adhesive systems have been reported from in vivo and in vitro studies [1, 2, 3, 4, 5, 6]. Resin monomers present in adhesive systems are cytotoxic in vitro [7, 8, 9] and their release in vivo into the pulp has been implicated as the possible cause of the reported adverse phenomena [2, 3, 10]. A number of factors contribute to monomer release, including the degree of conversion of the system, which is never complete [11, 12, 13, 14, 15]. The degree of the light-induced conversion of monomers to polymers is influenced by parameters as different as the intensity of the light around the wavelength triggering the photoinitiator system, the duration of irradiation, the concentrations, types and mixtures of photoinitiators, co-initiators, stabilizers, and inhibitors as well as the types and proportions of monomers and fillers [11, 16, 17].

Among the different light curing units (LCU) available in dental practice, halogen lamps are the most frequently used, although recently the light emitting diode (LED) technology has been successfully proposed [18, 19, 20]. The differences among the LCUs reflect the differences among materials in terms of depth and degree of polymerization. Consequently, the amounts of leachable residual monomers may vary with the light source used for curing [18, 19, 20].

In the biological evaluation of adhesive systems, an interesting possibility would be to detect the production of intracellular reactive oxidative species (ROS) induced by leachable monomers. Intracellular ROS are generated both in healthy and diseased tissues. In healthy cells, ROS can be produced by incomplete reduction of oxygen during catabolism. Disruption of important macromolecules through free radical reactions within host cells may hamper cellular functions or even lead to early cell death. ROS have been shown to cause disruption at multiple cellular sites, resulting in lipid peroxidation, protein oxidation, and nucleic acid damage. ROS may

induce cell damage directly, or act as an intracellular messenger during cell death induced by various other kinds of stimuli [21]. Recently, ROS production has been described as an early expression of cellular stress in dental monomers cytotoxicity [22, 23, 24].

Here, we assessed the influence of different light sources on the cytotoxicity of two dental adhesives. We investigated the survival rates in cell cultures exposed to extracts of specimens of two "one bottle" adhesive systems cured with halogen or LED lamps. In particular, we studied ROS production as a possible mechanism of early oxidative cellular damage induced by resinous adhesives.

Materials and methods

Preparation of test specimens and extracts

Two "one bottle" adhesive systems, Optibond Solo (Kerr, West Collins Orange, CA, USA) and Scotchbond One (3 M, St. Paul, MN, USA), were tested (Table 1). Two commercially available dental curing lamps were used to polymerize the adhesives: a halogen curing unit (Optilux 500, Demetron/Kerr, Danbury, CT, USA) and an LED curing unit (Elipar Freelight, 3 M). The adhesives were shaken and then poured into sterile circular Teflon molds (6 mm diameter × 1 mm thickness). Each adhesive was cured with either the halogen or the LED curing lamp. The light tip (cleaned with ethanol) was applied directly on the mold edge and the adhesives were light-cured for 40 s at room temperature.

The test specimens were extracted in glass vials following standardized procedures [25]. Extracts were prepared in phosphate-buffered saline (PBS) completed with glucose for the analysis of ROS production or in cell culture medium (RPMI 1640) supplemented with 10% FBS, 1% L-glutamine, and 1% pen-strep for cytotoxicity testing (MTT assay). The ratio of the surface area of the test specimens to the extraction volume was 150.8 mm^2/ml, which is in line with ISO 10993-12 (1996) [25]. After a 24-h incubation period at 37°C without agitation, the extracts were filtered through 0.22-μm cellulose acetate filters (Millipore) and stored at −25°C until use as described [26]. Then, the original extracts were diluted sixfold immediately before testing because higher concentrated extracts induced acute cytotoxic effects (MTT test) in preliminary range finding experiments (data not shown).

Endothelial cell cultures

ECV 304 is an immortal human endothelial cell line spontaneously transformed with HUVEC (ATCC—American Type Culture Collection, Rockville, MD, USA). These cells have been used in previous cytotoxicity studies [27]. The cells were grown and maintained in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 1% pen-strep at 37°C in a humidified air atmosphere containing 5% CO_2.

Cytotoxicity testing (MTT assay)

ECV 304 cells seeded in 96-wells plates (2×10^4 cell/well) were grown in complete medium for 24 h. The cell cultures were exposed to diluted extracts of the adhesive systems for 24 h. Then, the cells were rinsed with PBS and 200-μL aliquots of MTT (Sigma, 1 mg/ml in PBS containing 1 g/L w/v glucose) were added into each well. After a 3-h incubation period at 37°C, the MTT solution was removed, and the insoluble formazan crystals formed were dissolved in 200 μL of dimethyl sulfoxide (Sigma). The absorbance was measured at 540 nm using a 96-well plate spectrophotometer (LP400 Microplate Spectrophotometer, Bio-Rad).

Measurement of reactive oxygen species (ROS)

Generation of reactive oxygen species was measured using an oxidation-sensitive fluorescent probe, 2'7'-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probe). The oxidized form (2'7'-dichlofluorescein, DCF) is highly fluorescent [28]. Twenty-four hours before the experiments, ECV 304 cells were incubated in RPMI 1640 with 0.2% FBS in order to induce cell quiescence. Then, cells were detached by 250 mM trypsin/4 mM EDTA, and re-suspended in PBS at a density of 2×10^6 cells/mL. DCFH-DA (10 μM) was added for 20 min at 37°C in the dark. The cells were washed with PBS, and re-suspended in 3 ml of each extract of the cured test specimen. The cells re-suspended in PBS served as a negative control. DCFH-DA was further added to all samples. Immediately after re-suspension, the formation of DCF was monitored fluorimetrically (Perkin-Elmer LS 50 B) for 120 min at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. Viability of cells was evaluated with Trypan Blue separately after each experiment. The cell viability always exceeded 95% (data not shown).

Statistical analysis

Experimental results are given as mean values (and SEM) of the numbers of experiments, as indicated in the figure legends. The results of the MTT assays are expressed as the percentages of the corresponding negative controls (untreated cell cultures). The significance of the differences between sample values was determined by analysis of variance (ANOVA) with Bonferroni approximation for multiple comparisons. $P<0.05$ was considered necessary for statistical significance.

Results

Extracts of Scotbond One specimens cured with LED (ScbO-LED) were the most toxic media tested after a 24-h exposure period because cell survival was reduced to 23% compared with control cultures (Fig. 1). The cytotoxic effect of ScbO-LED was followed by Scotch-

Table 1 Test materials and their composition according to manufacturers

Material (manufacturer)	Resin monomers	Solvent	Inorganic fillers	Other substances
Scotchbond One (3 M, St. Paul, MN, USA)	BisGMA, HEMA, MMPAS, UDMA, GDMA	Water, ethanol	Not determined	-
Optibond Solo (Kerr, West Collins Orange, CA, USA)	BisGMA, HEMA, GPDMA	Ethanol	Silicon dioxide, Ba-Al-boro-silicate, Cycloeptadione, Dimethylamine benzoate	Na_2SiF_6, BHT

BHT 2,6-di-tert-butyl-para-cresol or butylated hydroxytoluene, *BisGMA* bisphenol A-diglycidyl ether dimethacrylate, *GDMA* glycerol dimethacrylate, *GPDMA* glycerol phosphate dimethacrylate, *HEMA* 2-hydroxyethyl methacrylate, *MMPAS* methacrylate-modified polyacrylic acids, *Na_2 SiF_6* disodium hexafluoro-silicate, *UDMA* urethane dimethacrylate, *n.d.* not declared, or data not available from manufacturer

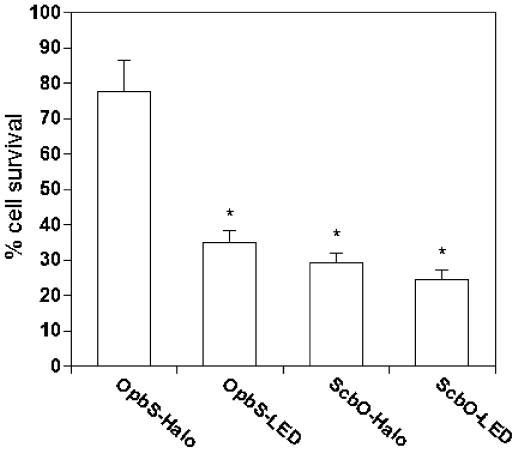

Fig. 1 Viability of ECV 304 cells assessed by MTT assay after exposure to polymerized adhesives for 24 h. Data are expressed as percentage of the negative-control cultures. Values (means ± SEM) from three independent experiments in triplicate are presented (n=3); *indicates significant ($p \leq 0.05$) differences from untreated control cultures (one-way ANOVA)

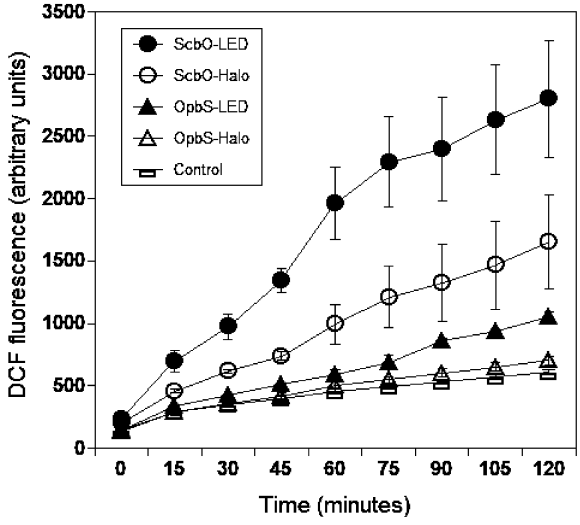

Fig. 2 Generation of ROS in ECV 304 cells in contact with the extracts of the polymerized adhesives monitored for 120 min. Control (cells without extracts) represents the basal level of intracellular ROS production in time. Data are expressed as arbitrary units of DCF fluorescence. Results represent the means ± SEM of three independent experiments in duplicate (n=3)

bond One cured with a halogen Lamp (ScbO-Halo) (29.3%), Optibond Solo cured with LED lamp (OpbS-LED) (35%), and Optibond Solo cured with halogen lamp (OpbS-Halo) (77.61%) (Fig. 1). The reduction of cell survival was significant in cell cultures treated with OpbS-LED, ScbO-Halo and ScbO-LED compared with control cells. Moreover, significant differences were detected between OpbS-LED and OpbS-Halo, while no significant differences were rated between ScbO-LED and ScbO-Halo.

In order to investigate if the cytotoxicity of the adhesive systems might be correlated with the generation of ROS, the intracellular ROS production was monitored for 120 min with extracts exposure. We found that intracellular amounts of ROS increased in a time-dependent pattern in all cell cultures (Fig. 2). Extracts of Scotbond One specimens cured with LED (ScbO-LED) induced the highest increase of ROS among all extracts tested during an initial 15-min incubation period and after 120 min. After an incubation period of 120 min, statistically significant differences of ROS production were observed between untreated cell cultures and cultures treated with ScbO-Halo or ScbO-LED. In contrast, there were no significant differences between untreated control cultures and cell cultures treated with Optibond Solo (OpbS -LED and OpbS-Halo). After 120 min, the increase in ROS production of ScbO-LED was 4.5-fold higher than controls, followed by ScbO-Halo (2.75-fold), OpbS-LED (1.75-fold), and OpbS-Halo (1-fold). Furthermore, ROS production between cell cultures treated with extracts of ScbO-LED and ScbO-Halo was significantly different, while no significant differences were shown between OpbS-LED and OpbS-Halo.

Discussion

Usually DBSs are polymerized by photo activation, and free monomers may be released from resinous materials before and after polymerization. These monomers may pass through the dentinal tubules and reach the pulp tissue, causing pulpal irritation. An insufficient photo activation contributes to an increase of the unreacted monomers level through a lower degree of polymerization and cross-linking [12, 14, 15]. For this reason, the biological properties of light polymerized dental materials may be influenced by the quality of the LCU used. Currently, halogen lamps are the most frequently used light sources for the polymerization of resin-based dental materials. To overcome the problems inherent to halogen LCUs, solid-state LED technology has been proposed.

In the present study, cytotoxic and cellular stress-promoting effects (ROS production) on endothelial cells by dentin adhesives which were cured with halogen or LED LCUs were quantified using two assays. The MTT test measures cellular metabolic function and is widely used for in vitro biocompatibility evaluation because of its reliability and sensitivity [29]. DCFH-DA is used in order to measure the production of intracellular reactive oxidative species induced by the adhesive extracts. DCFH-DA is a non-fluorescent probe that readily diffuses through the cell membrane and, reacting with peroxides or hydroperoxide, is oxidized to its fluorescent form (DCF).

In healthy cells only small amounts of ROS, including hydrogen peroxide, superoxide anion and the hydroxyl radical, are produced during catabolism and cellular respiration, and they are efficiently eliminated by an elaborated system of antioxidant enzymes. A massive

production of intracellular ROS may be responsible for cytotoxic effects causing cellular damage by lipid, protein, and nucleic acid oxidation [30, 31, 32]. ROS may induce cell death directly or act as an intracellular messenger induced by various other kinds of stimuli [21].

Recently, some components of resin-based dental materials such as monomers and photoinitiators have been described to increase ROS production [22, 23, 24]. Here, all the extracts, excluding OpbS-Halo, were rated significantly cytotoxic by the MTT assay. In ROS detection, we obtained similar results after 120 min, although only ScbO cured either with halogen or LED lamp showed statistically significant differences from controls. This increase of ROS in exposed cell cultures may be caused directly by reactive compounds released from incompletely cured adhesives. On the other hand, it cannot be ruled out that the amounts of ROS increased indirectly because of an inactivation of cellular systems which protect from oxidative damage. For instance, it has been reported that resin monomers like TEGDMA may lead to a depletion of glutathione and a decrease of cell viability [24].

The cytotoxicity of dental adhesives has been widely investigated and evidenced by a number of researchers [2, 3, 4, 5, 6, 9]. In accordance with other reports, the two tested adhesives in our study proved to be cytotoxic on cell cultures. OpbS was rated less toxic than ScbO in the MTT assay, either cured with a halogen or an LED lamp. Furthermore, OpbS proved to produce fewer free radicals than ScbO in all cases.

A number of studies investigated the relationship between the properties of light-cured dental materials and the type of LCU used for their polymerization [11, 18, 19, 20, 32]. It has been shown that different LCUs can affect the release of resin monomers [33]. A high amount of leachable monomers in resin-based materials may indicate poor conversion and poor mechanical properties [16]. In addition, the release of monomers has a potential impact on the biocompatibility of dental materials [12, 13]. In most cases these results confirmed that the mechanical properties (such as compressive and flexural strength) and depth of cure were higher in materials irradiated with halogen lamps compared with LED lamps [18, 20].

Our results are in line with these findings; however, we focused on the biological rather than mechanical properties of dental adhesives. We demonstrated that the production of intracellular ROS by the adhesive extracts was both light source-dependent and time-dependent and that cytotoxicity is light source-dependent. OpbS produced only a small amount of ROS and also showed only low cytotoxicity when cured with halogen light. Furthermore, ScbO produced high amounts of ROS and also showed high cytotoxicity when cured either with a LED or a halogen lamp. These results suggested a possible link between ROS production and cytotoxic activity.

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