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Effect of heat treatment on cytotoxicity and polymerization of universal adhesives

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To assess, *in vitro*, the influence of heat air treatment on cytotoxicity and degree of conversion (DC) of universal self-etch adhesives (Ambar Universal APS, Scotchbond Universal Adhesive, and Tetric N-Bond Universal) in an NIH/3T3 fibroblast cell culture. Samples were divided into three groups: 1) no heat treatment (control), 2) 37°C and 3) 60°C heat treatment before photopolymerization. Cytotoxicity was analyzed by MTT assay and the DC by FTIR. All adhesives heated at 60°C showed reduced cytotoxicity levels when compared with those heated at 37°C. In general, DC of Ambar Universal APS presented the highest DC than Scotchbond Universal Adhesive and Tetric N-Bond Universal, and the hot air treatment do not influence the conversion. Heat treatment at 60°C was able to reduce the cytotoxicity of universal self-etch adhesives, even, the heat treatment does not enhances the DC.

Keywords: Cytotoxicity, Degree of conversion, FTIR, MTT, Universal adhesives

INTRODUCTION

Self-etching adhesives have been increasingly used in daily practice, as they present shorter working time, easier handling, low postoperative sensitivity than total etch systems[1]. The formula of these systems comprises both hydrophilic and hydrophobic bifunctional monomers, acidic monomers containing carboxylic acid-derived radicals, additives, solvents, aromatic amines, photoinitiators, and other components[2]. However, many of these components can change the biological behavior of the dentin-pulp complex when used in deep cavities, due to their cytotoxic effects[3]. This explains why self-etching adhesives have long been studied by several investigators[4-6].

Both acidic and non-acidic components of adhesive systems have cytotoxic effects on cells[7]. Uncured residual monomers may affect mitochondrial activity, causing morphological alterations and membrane rupture[8]. Therefore, adequate photopolymerization is paramount to maximize the physical properties, clinical performance, and biocompatibility of self-etching adhesives[9].

Cytotoxicity is directly related to the conversion of monomers into polymers, meaning that it depends on the effectiveness of the polymerization technique employed[10]. Obtaining optimized polymers depends on parameters such as light intensity, duration and mode of light application, wavelength, and irradiation time[11,12].

All these factors have a direct influence on polymerization rates and thus on the cytotoxicity of adhesive systems, potentially leading to cell damage and even cell death[13]. There are variations in cytotoxicity values reported in the literature according to the methodology and materials employed. Also, some studies have demonstrated differences in cytotoxicity between *in vitro*[14,15] and *in vivo*[16] samples, as well as when comparing different adhesive systems (total-etch *vs.* self-etch) in different dentin thicknesses[17]. Resin components, for instance, are highly aggressive when in contact with cells: The literature reports a decrease in their aggressive behavior as soon as they are removed from the environment containing cells[14].

With the goal of improving polymer cross-linking and consequently increasing the rates of resinous polymer conversion, thus decreasing the number of residual monomers, some authors have proposed to increase photopolymerization time of resinous components[18,19], whereas other authors have tested the use of external sources of heating before photopolymerization, within biologically acceptable limits[20-25], resulting in immediate conversion gains, and also decreasing the concentration of final solvent in the adhesive system[26]. Temperatures of 23, 37, and 60ºC have been used to assess the mechanical properties of resinous compounds, and 60ºC has been reported to be the optimal temperature to improve polymer properties and system stability[20,22,26].

The objective of this study was to assess, *in vitro*, the influence of heat air treatment on cytotoxicity and degree of conversion of universal self-etch adhesives.

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MATERIALS AND METHODS

Cytotoxicity assay

Three different adhesives (Table 1) were used in this *in vitro* study (two different batches from each manufacturer): Ambar Universal APS (FGM, Joinville, Santa Catarina, Brazil), Scotchbond Universal Adhesive (3M ESPE, St. Paul, MN, USA), and Tetric N-Bond Universal (Ivoclar Vivadent, Schaan, Liechtenstein). Vials were sterilized with ethylene oxide (Esteriliplus, Porto Alegre, Rio Grande do Sul, Brazil), and the amounts necessary to produce specimens (9 mm diameter×1 mm thickness) were dispensed onto a sterilized glass slide. Specimens were immediately prepared in three different forms: 1) no heat treatment (air jet of dental chair (23°C) distant 10 cm from the slide for 10 s before photopolymerization); 2) jet of warm air (37°C) distant 10 cm from the slide for 10 s before photopolymerization; 3) jet of hot air (60°C) distant 10 cm from the slide for 10 s before photopolymerization. The hot air was provided by a heater device coupled to an air blow, presenting an output similar to the air jet of dental chair. Temperature was checked previous of each application.

All specimens were subsequently light-cured for 10 s using a VALO Cordless light-emitting diode (LED) curing unit (Ultradent, Salt Lake City, UT, USA), with a light guide diameter of 15 mm, irradiation intensity of 1,000 mW/cm^2 +/−10%, wavelength between 395 and 480 nm (manufacturer-provided information), and a distance of 5.0 mm.

The cells used in this study were NIH/3T3 mouse fibroblasts (ATCC® —American Type Culture Collection— TCC, Old Town, MD, USA) cultured in Dulbecco's modified Eagle media (DMEM; Invitrogen®, CA, California, USA). This medium was supplemented with 10% of fetal bovine serum, 100 U/mL of penicillin (Gibco, Grand Island, NY, USA), 100 U/mL of streptomycin (Gibco), and 100 μg/mL of gentamycin (Gibco). Cells were kept in a humidified incubator at a temperature of 37ºC and 5% of CO_2.

Immediately after the light-curing process, specimens from the three groups were immersed in the DMEM medium. The specimen surface area to medium volume ratio was 3 cm²/mL, according to ISO 10993-12. Surface area was calculated based on the total dimensions of the specimen, disregarding porosity. Extracts were tested for cell viability after remaining 24 h and 7 days in the incubator.

The MTT method was used to assess cytotoxicity. This assay measures the ability of live cells to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma Aldrich, St. Louis, MO, USA) to insoluble blue-to-purple formazan crystals. At each treatment time (24 h and 7 days), the culture medium was removed and 10% of an MTT solution (5 mg/mL) in phosphate buffer solution was added to each well. Subsequently, cultures were incubated at 37ºC, protected from light, until the presence of blue-to-purple formazan crystals was observed. For the solubilization of formazan crystals, 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and absorbance was measured at 570 nm wavelength using a spectrophotometer and an ELISA microplate reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA). The percentage of viable cells was calculated and compared to the results obtained with the negative control (cells cultured in DMEM). The assay was validated using a positive toxicity control (cells treated with 2% sodium hypochlorite).

Table 1 Materials tested, composition as informed by manufacturer, light-curing time, batch, and manufacturers

Adhesive	Composition	Light-curing time (s)	Batch number	Manufacturer
Ambar Universal APS	Active components: MDP (10-methacryloyloxydecyl dihydrogen phosphate), methacrylate monomers, photoinitiators, coinitiators, stabilizer. Inactive components: inert matter (silica nanoparticles) and vehicle (ethanol).	10	200416 050716	FGM
Scotchbond Universal Adhesive	Bisphenol A diglycidyl ether dimethacrylate (Bis-GMA), 2-hydroxyethyl methacrylate, silanated silica, ethyl alcohol, decamethylene dimethacrylate, water, 1,10-decanediol phosphate methacrylate, acrylic and itaconic acid copolymer, camphorquinone, N,N-dimethylbenzocaine, 2-dimethylaminoethyl methacrylate, methyl ethyl ketone	10	475261 654110	3MESPE
Tetric N-Bond Universal	Silane, water, initiators, ethanol, Vitrebond copolymer, 2-hydroxyethyl methacrylate (HEMA), bisphenol A diglycidyl ether dimethacrylate (Bis-GMA), decamethylene dimethacrylate, 10-methacryloyloxydecyl dihydrogen phosphate (MDP)	10	U18895 T34374	Ivoclar Vivadent

Degree of conversion (DC)

The DC was evaluated by a FTIR coupled to a horizontal attenuated total reflectance (Vertex 70, Bruker Optics, Ettlingen, Germany), according to previous study[27]. A 10μL drop of each sample (n=3) was directly dispensed onto the ATR crystal, received one of the air treatments, covered by a acetate strip and photoactivated for 10 s. The percentage of unreacted carbon–carbon double bonds (% C=C) was determined from the ratio of absorbance intensities of aliphatic C=C (peak height at 1,636 cm^{-1}) against internal standard before and after curing of the specimen. The aromatic carbon–carbon bond (peak height at 1,608 cm^{-1}) absorbance was used as an internal standard. The DC was determined by subtracting the % C=C from 100%.

Statistical analysis

Means cytotoxicity (expressed by cell viability), expressed in percentual in relation to the negative control, of adhesive systems was compared in terms of cell viability rates in NIH/3T3 mouse fibroblast cultures using a two-way ANOVA followed by Tukey *post-hoc*, for 24 h and seven days data. DC results were evaluated using ANOVA and Tukey *post-hoc* test for the comparisons between treatments and between adhesives. All analysis was performed at 5% of significance.

RESULTS

All materials testes exhibit cytotoxicity (Table 2). The heat treatment at 60°C enhance the cell viability (p<0.05) in comparison to control or 37°C air. On 7 days analysis, Ambar Universal APS shown more viable cells (p<0.05) than Scotchbond Universal Adhesive and Tetric N-Bond Universal. At the 24-h cell viability analysis, no differences were detected among the samples not subjected to heat treatment (control) in terms of cell viability. In the samples treated with warm air (37°C), cell viability results were similar for Scotchbond Universal Adhesive and Ambar Universal APS, but lower for Tetric N-Bond Universal. In the samples treated with hot air (60°C), all three adhesive systems showed increased cell viability when compared to the control group and to the warm air-stream group (37ºC). No differences were observed among the three self-etching adhesives subjected to 60°C heat treatment. In general, at the 7-day analysis, cell viability results were lower than those observed at 24 h. Seven-day analyses of adhesives treated by 60°C hot air reveals that the cell viability decreases in the following order Ambar Universal APS, Scotchbond Universal Adhesive and Tetric N-Bond Universal.

The DC (Table 3) ranged from 43.1±5.3% for Tetric N-Bond Universal without treatment to 69.7±4.6 for Ambar Universal APS heated at 37°C. There is

Table 2 Mean±standard deviation cell viability (%) obtained using the MTT assay at 24 h and 7 days in Universal adhesive systems subjected to heat treatment before polymerization

Adhesive	24 h			7 days		
	no treatment (control)	warm air (37°C)	hot air (60°C)	no treatment (control)	warm air (37°C)	hot air (60°C)
Ambar Universal APS	14.64±1.08^{Ac}	15.96±0.37^{Ab}	30.12±0.33^{Aa}	6.34±0.26^{Ac}	7.04±0.38^{Ab}	15.32±0.37^{Aa}
Scotchbond Universal Adhesive	14.93±1.18^{Ac}	16.27±0.65^{Ab}	29.83±0.11^{Aa}	5.92±0.16^{Bb}	6.24±0.17^{Bb}	14.76±0.30^{Ba}
Tetric N-Bond Universal	14.13±0.33^{Ab}	11.45±0.33^{Bc}	28.51±0.27^{Ba}	5.78±0.35^{Bc}	6.87±0.17^{Ab}	13.88±0.07^{Ca}

Distinct capital letters indicate statistical significance (p<0.05) in the same column; distinct lowercase letters indicate statistical significance (p<0.05) in the same row, according time.

Table 3 Mean±standard deviation of DC (%) of universal adhesives in function of heat treatment

Adhesive	Heat treatment		
	no treatment (control)	warm air (37°C)	hot air (60°C)
Ambar Universal APS	57.9±2.6^{Ab}	69.7±4.6^{Aa}	66.8±2.1^{Aa}
Scotchbond Universal Adhesive	48.2±4.2^{ABa}	64.3±5.0^{ABa}	52.4±6.7^{Ba}
Tetric N-Bond Universal	43.1±5.3^{Ba}	51.2±8.2^{Ba}	46.7±2.3^{Ba}

Distinct capital letters indicate statistical significance (p<0.05) in the same column; distinct lowercase letters indicate statistical significance (p<0.05) in the same row.

no statistical interaction (p=0.397) between factors (adhesive/treatment) in study. Ambar Universal APS presented higher DC than other Tetric N-Bond Universal for all treatment groups (p<0.05) and higher DC than Scotchbond Universal Adhesive at 60°C treatment (p<0.05). Heat treatment did not influence (p>0.05) the DC of Scotchbond Universal Adhesive and Tetric N-Bond Universal, however the hot air treatment at 37 and 60°C enhances (p<0.050) the DC of Ambar Universal APS.

DISCUSSION

The cytotoxicity of dental materials and its relationship with cell viability have been investigated by several authors using different methods, materials[28], polymerization modes[20], and material concentrations[29-31], as these parameters often have irreversible deleterious effects on pulp tissues. The present study was conducted to investigate cytotoxicity through cell viability results observed after 24 h and 7 days of incubation based on the extraction of uncured products. The results confirmed the presence of high levels of cytotoxicity and showed that toxicity increases with time. The adhesive thickness used in the present study represents a strong challenge to materials, stronger than in clinical scenario. This thickness was used in accordance to ISO 10993 protocol for cytotoxicity and could overestimate the cell aggression. However, the use of heat treatment by 60°C air reduces the cytotoxicity.

Several cell functions are used in biocompatibility tests to assess the cytotoxicity of dental materials. Some studies[32,33] have investigated cell adhesion, proliferation, and metabolism in 3T3, L929, and W138 fibroblast and osteoblast cell lines. In the present study, we assessed the behavior of mouse fibroblasts according to modified parameters of Stanford. These cell lines show a type of cell that is also present in human pulp and gingival tissues, and they were chosen due to their reproducible growth rates, easy handling, easy availability when compared with primary cells, in addition to being an immortal cell line[34]. Cell inviability as determined by the MTT test does not necessarily mean a higher occurrence of apoptosis and tissue necrosis; rather, it means that, in addition to these events, there may also be a higher number of cells showing reduced metabolic activity. The MTT assay is a well-established method of determining cell viability and has been used in previous studies to test the cytotoxicity of dental adhesives and other dental materials, especially because it is simple, fast, and objective[7,35].

Souza Costa *et al.*[7] emphasized that uncured adhesives are highly toxic when compared with cured ones, and underscored the importance of effective polymerization, considering that both acid and non-acid components of adhesive systems, when uncured, lead to cytotoxicity. Therefore, effective polymerization of both composite resins and resinous cements stands out as a key factor to ensure good cell viability rates[28]. In the present study, an attempt was made to enhance adhesive polymerization through the previous application of warm and hot air stream, so as to reduce cytotoxicity.

Polymerization at high temperatures (60 a 70ºC) has been associated with extra cross-linking in the formation of composite resin polymer chains, as a result of the excessive energy available (heat). In mechanical tests, this effect is demonstrated by increased resin homogenization and consequently lower standard deviation values in specific parameters, *e.g.*, hardness and diametral tensile strength[29]. Moreover, the frequency of collision of active groups and non-reacted radicals increases when curing temperature is below the glass transition temperature, resulting in additional polymerization and a higher rate of conversion[31,34]. In addition, previous studies[18,20] also showed that heat treatment before resin polymerization had a significant influence on resinous material properties. In the present study, samples not treated with a jet of hot air (60°C) showed high cytotoxicity levels, at both 24 h and 7 days. Conversely, in the group treated with hot air (60°C) prior to polymerization, cytotoxicity results decreased, again at both 24 h and 7 days. However, in this study the DC did not increase by hot air treatment, and could not explain the cytotoxicity reduction. What can contribute to these results is the higher solvent evaporation on hot air group than warm air or control groups. All tested adhesives have ethanol in its composition, and thinking in an ethanol boiling temperature of 78°C and vapor pressure 44.6°C mmHg at 20°C[36], it could be expected that the hottest air tested in this study is capable of evaporate a large amount of solvent.

In sum, cell viability of universal adhesive systems reduces with time between 24 h and 7 days, and the use of heat treatment was paramount to avoid this decrease. Samples treated with a jet of hot air (60°C) showed 7-day cell viability rates similar to those observed in non-heated samples at 24 h. This finding is extremely relevant, as it suggests that heat treatment helps maintain better cell viability rates when compared with non-heated samples.

Future studies should assess other tests combined with heat treatment and investigate the effects of heating on cytotoxicity, conversion rates, solvent evaporation rates, and genotoxicity. It is important to highlight that research on the use of heat treatment to enhance polymerization has been conducted by different universities and dental specialties[23], always pointing to benefits associated with heating before polymerization.

From a clinical standpoint, the authors of the present study are aware that it would be difficult to standardize the clinical use of heat treatment on adhesive systems, as the manufacturers would have to include this option in their equipment, so that the pressure and temperature of the air stream could be adjusted. Even though this scenario may not be so far from our current reality, a great deal of research needs to be conducted to find the optimal air stream temperature, pressure, and flow. The results here obtained can be used as preliminary findings in that regard, not only by investigators and academics, but also in clinical practice.

In the present study, all universal adhesives

analyzed showed high levels of cytotoxicity, which increased with time of exposure to the extraction medium (24 h and 7 days). Heat treatment at 60°C, prior to photopolymerization, reduced cytotoxicity in all adhesives, as evidenced by the results observed both at 24 h and 7 days of analyses.

CONCLUSION

Heat treatment at 60°C was able to reduce the cytotoxicity of universal self-etch adhesives, even, the heat treatment do not enhances the DC.

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