

# Effect of heat treatment on cytotoxicity of self-adhesive resin cements: Cell viability analysis

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## ABSTRACT

**Objective:** The aim of the study was to assess, *in vitro*, the influence on cytotoxicity of heat treatment applied before photopolymerization, while mixing three self-adhesive resin cements, in an NIH/3T3 fibroblast cell culture, based on cell viability measures. **Methods:** Samples were divided into three groups: (1) no heat treatment while mixing (control), (2) 37°C, and (3) 60°C heat treatment while mixing. Cements were light-cured immediately after mixing and immersed in Dulbecco's Modified Eagle Media for the extraction of possibly uncured products after 24 h and 7 days. Cultures contained 0.5 mL of NIH/3T3 fibroblasts per well at a concentration of  $0.4 \times 10^5$  cells/mL and specific extracts for each sample. **Statistical Analysis Used:** Data were statistically analyzed with ANOVA and *post hoc* Student–Newman–Keuls (significance of 5%). **Results:** Cement cytotoxicity increased with time, as shown by the higher values observed at 7 days. There was a slight difference in intragroup cytotoxicity levels between 24 h and 7 days. Heat treatment at 60°C was associated with a major decrease in cytotoxicity levels in all three groups, both at 24 h and at 7 days, with no differences among the cements. **Conclusions:** Heat treatment at 60°C should be considered as a strategy to reduce cytotoxicity of self-adhesive resin cements, as evidenced by the results observed at 24 h and 7 days of analysis.

**Key words:** Biocompatibility, dental materials, toxic substances

## INTRODUCTION

With the evolution of esthetic dentistry, self-adhesive resin cements have become indispensable in clinical practice. These materials are used in several procedures, for example, cementation of indirect restorations, porcelain laminate veneers, and fixed prostheses, especially because of their low solubility in water and the strength of their bond to enamel and dentin.<sup>[1,2]</sup> Because of these properties, the use of

self-adhesive resin cements is associated with a lower degree of infiltration and marginal staining, lower postoperative sensitivity, in addition to reinforcement of the bond between the restoration and the tooth.<sup>[3,4]</sup> The large number of resin cements available in the market and the introduction of self-adhesive systems have increased their use in clinical practice, especially

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because they simplify the cementation technique, eliminating the need for previous treatment of the tooth substrate and decreasing sensitivity. In addition, these cements present a strong bond to dentin, similar to that of conventional adhesive cements.<sup>[5-7]</sup>

Adequate conversion of monomers into polymers is essential to maximize the physical properties and clinical performance of resin cements, as well as to reduce their cytotoxicity.<sup>[8]</sup> Polymerization may be influenced by several factors, for example, ceramic translucency,<sup>[9,10]</sup> thickness,<sup>[9,11,12]</sup> curing time,<sup>[13]</sup> type of curing unit,<sup>[14,15]</sup> battery level,<sup>[16]</sup> light intensity, wavelength, and type of initiator.<sup>[17]</sup> Different types of curing light units have been proposed and assessed for the photopolymerization of restorative materials, always with the goal of enhancing physical properties and clinical performance and consequently reducing cytotoxicity.<sup>[18-20]</sup>

Several attempts have been made over recent years to enhance polymerization rates, and it is currently known that the molecular cross-linking density of methacrylate-based resin materials can be improved with the use of high temperatures either before or during polymerization.<sup>[21,22]</sup> Several authors have demonstrated superior physical properties of resin materials as a result of a higher degree of conversion of monomers into polymers obtained through different light-curing methods employing heat.<sup>[23-26]</sup>

Cytotoxicity measurement based on cellular behavior and viability is the first step in assessing the biocompatibility of dental materials for subsequent use in clinical practice.<sup>[27]</sup> Cytotoxicity depends on the quality and amount of monomers and derivatives released, which may irritate the pulp and oral soft tissues and eventually lead to a toxic reaction.<sup>[28,29]</sup> Methacrylate-based dental materials are known to present a high level of cytotoxicity and are therefore likely to penetrate the pulp and induce cytotoxic effects.<sup>[30]</sup>

Several protocols have been used to assess cellular behavior, viability, and cytotoxicity, including the trypan blue exclusion assay, chromium release assay, DNA synthesis, and cellular metabolism (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]) assay. In particular, the MTT is considered a relatively simple assay, yet as thorough and reliable as the others, and therefore it is widely used to determine cytotoxicity of different materials in cell cultures.<sup>[31-33]</sup>

The objective of this study was to assess, *in vitro*, the influence on cytotoxicity of heat treatment applied before photopolymerization, while mixing three self-adhesive resin cements, in an NIH/3T3 fibroblast cell culture, based on cell viability measures.

## METHODS

### Sample preparation

Three self-adhesive resin cements were used in this *in vitro* study: RelyX U200 (3M ESPE, Saint Paul, Minnesota, USA), Multilink N (Ivoclar Vivadent, Schaan, Liechtenstein), and BisCem (Bisco Inc., Schaumburg, Illinois, USA) [Table 1]. Resin cement dispensers were sterilized with ethylene oxide (Esterilipus, Porto Alegre, Rio Grande do Sul, Brazil), and the necessary amounts of base and catalyst paste 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 while mixing the pastes (control); (2) jet of warm air (37°C) distant 10 cm from the slide for 10 s while mixing; and (3) jet of hot air (60°C) distant 10 cm from the slide for 10 s while mixing. All specimens were subsequently light cured for 20 s using a VALO Cordless light-emitting diode curing unit (Ultradent, Salt Lake City, Utah, USA).

### Cell culture

The cells used in this study were NIH/3T3 mouse fibroblasts (ATCC®-American Type Culture Collection-TCC, Old Town, Maryland, USA) cultured in Dulbecco's Modified Eagle Media (DMEM; Invitrogen®, Carlsbad, California, USA). This medium was supplemented with 10% of fetal bovine serum, 100 U/mL of penicillin (Gibco), 100 U/mL of streptomycin (Gibco), and 100 µg/mL of gentamycin (Gibco). Cells were kept in a humidified incubator at a temperature of 37°C and 5% of CO<sub>2</sub>.

### Extraction medium

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 cm<sup>2</sup>/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.

### Cytotoxicity assay

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) 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-buffered solution was added to each well. Subsequently, cultures were incubated at 37°C, 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 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 Inc., Hercules, California, 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).

### Statistical analysis

The cytotoxicity of light-cured self-adhesive resin cements without previous heat treatment and with warm and hot air treatment was compared in terms of cell viability rates in NIH/3T3 mouse fibroblast cultures using three-way ANOVA followed by *post hoc* Student–Newman–Keuls. Examiners were blinded to group allocation. Results were expressed as mean and standard deviation. Significance was set at 5%.

## RESULTS

Table 2 shows the cell viability results obtained for the three self-adhesive resin cements. Groups treated with both temperatures (37°C and 60°C) showed cytotoxicity, with a reduced number of viable cells, regardless of the cement used. Resin cement cytotoxicity increased with time, with the highest values observed at 7 days for all cements.

At the 24h cell viability analysis, no differences were detected among the samples not subjected to heat treatment in terms of cell viability. In the samples treated with warm air (37°C), 24 h cell viability results were similar to those obtained without heat treatment in both RelyX and BisCem samples. Multilink N, in turn, showed a significantly lower cell viability rate. Finally, in the samples treated with hot air (60°C), all three cements showed higher cell viability rates when compared to either the warm air group (37°C) or the group with no heat treatment. However, no differences were observed among the three self-adhesive resin cements subjected to 60°C heat treatment.

After 7 days of incubation, in turn, cell viability rates were lower than those obtained at 24 h in both the group treated with warm air (37°C) and in the one not subjected to heating. Conversely, in the group treated with hot air (60°C), cell viability results showed a marked increase when compared with the nonheated group, in all self-adhesive resin cements.

**Table 1: Self-adhesive resin cements used, composition, light-curing time, batch, and manufacturer**

Cement	Composition	Light-curing time (s)	Batch	Manufacturer
RelyX U200	Silanated filler (glass powder), dimethacrylate monomers, 1-benzyl-5-phenylbarbituric acid, calcium salt, 1,12-dodecanediol dimethacrylate, sodium p-toluenesulfonate, silanated silica, calcium hydroxide, methacrylated aliphatic amine, titanium dioxide	20	622725	3M ESPE
Multilink N	Dimethacrylate, HEMA, barium glass, ytterbium trifluoride, spherical mixed oxides	20	U44037	Ivoclar
BisCem	Bis-GMA, dimethacrylate monomer, glass particles, and acid monomer	20	1500003825	Bisco

HEMA: Hydroxyethylmethacrylate, Bis-GMA: Bisphenol A glycidyl methacrylate

**Table 2: Cell viability rates obtained using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 24 h and 7 days in light-cured self-adhesive resin cements without and with previous heat treatment**

Cement	24 h			7 days		
	No heat	37°C	60°C	No heat	37°C	60°C
RelyX	14.93±1.77 <sup>A</sup>	14.56±0.71 <sup>A,C</sup>	23.25±0.45 <sup>B</sup>	5.41±0.68 <sup>D</sup>	6.24±1.73 <sup>D</sup>	14.70±0.65 <sup>A</sup>
Multilink N	13.93±1.35 <sup>A</sup>	12.24±1.15 <sup>C</sup>	24.05±0.40 <sup>B</sup>	6.33±1.06 <sup>F</sup>	8.43±0.61 <sup>F</sup>	15.31±1.15 <sup>A</sup>
BisCem	15.52±1.37 <sup>A</sup>	15.89±0.30 <sup>A</sup>	23.88±1.05 <sup>B</sup>	5.78±0.69 <sup>D</sup>	6.77±0.77 <sup>D</sup>	13.87±0.68 <sup>A,C</sup>

Data presented as mean±SD. Different letters indicate statistical differences (ANOVA or Student–Newman–Keuls test):  $P<0.05$ . SD: Standard deviation, ANOVA: Analysis of variance

Furthermore, samples treated with hot air (60°C) were able to maintain high rates of cell viability, showing similar results to those found at 24 h in the group not subjected to heat treatment. Again, the three groups treated with hot air showed similar cell viability results among themselves.

## DISCUSSION

Several studies have been conducted over the past few years to analyze the biocompatibility of methacrylate-based resin materials, which are known to have severe cytotoxic effects on pulp tissues. In the present study, we measured cell viability rates in the extraction medium in contact with cells after 24 h and 7 days of incubation. Not only did we confirm the presence of toxicity in resin cements but also we found that toxicity increases significantly with time.

Cell characteristics and functions have been used to analyze and investigate cytotoxicity of methacrylate-based resin materials. Cell adhesion, proliferation, and metabolism in 3T3, L929, and W138 fibroblast and osteoblast cell lines are among the parameters that have been investigated.<sup>[31,33,34]</sup> In the present study, we assessed the behavior of mouse fibroblasts according to modified parameters of Stanford. Although these cells are more sensitive to cytotoxicity than human cells, they are indicated for this type of study by the American National Standard ISO 10993-5 due to their reproducible growth rates, easy handling, and easy availability when compared with primary cells and in addition being an immortal cell line.<sup>[31]</sup> 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.

In this study, resin dispensers were sterilized and placed onto sterilized glass slides. Then, the base and catalyst pastes were mixed (9 mm diameter × 1 mm thickness), light cured, and immediately immersed in the monomer extraction medium. It is important that materials are tested immediately after photopolymerization, to avoid the loss of toxic substances that may be released by the material after light curing. The longer the time elapsed between photopolymerization and cell viability analysis, the less faithful and consequently less reliable the results will be. Studies have demonstrated<sup>[28]</sup> the relevance of immediate versus late cytotoxicity analysis of methacrylate-based resin materials and its

effects on cell vitality,<sup>[28-30]</sup> as well as the importance of effective photopolymerization in an attempt to minimize cytotoxicity.<sup>[8]</sup>

Dioguardi *et al.*<sup>[35]</sup> tested five different resin cements for cytotoxicity and found differences between the brands assessed. Still, all cements presented low cytotoxicity rates, which remained low even after 1 week of contact with cells. We did not confirm these findings of low cytotoxicity in the present study. On the contrary, all toxicity values were high, in all cements analyzed, both at the 24 h and at the 7-day analyses. In addition, in our sample, cell viability continued to reduce with time, as observed on the 7-day analysis.

Residual uncured monomers released during the light-curing process are one of the factors responsible for the cytotoxicity of resin materials. However, according to Goldberg,<sup>[28]</sup> there are other mechanisms that contribute to cytotoxicity, for example, leachable components created by erosion or degradation over time, ion release, and bacteria located at the interface between the tooth and the adhesive.

Uncured resin cement debris, such as monomers, degradation products, initiators, activators, or stabilizers, produces cellular cytotoxicity. These products can be reduced through enhancement of the polymerization process, as better cross-linking will result in a better polymer. Heat treatment applied during the mixing of base and catalyst pastes before polymerization has enabled a good reduction rate, according to some studies,<sup>[21,22]</sup> producing a resin with superior properties. In the present study, heat treatment at 60°C probably resulted in a higher rate of polymer cross-linking (enhanced polymerization) and thus fewer residual monomers.

Ergun *et al.*<sup>[8]</sup> reports that effective polymerization is one of the most important factors when dealing with methacrylate-based dental materials so as to improve their physical properties, clinical performance, and biocompatibility. Klein-Júnior *et al.*<sup>[25]</sup> and Ferracane and Condon<sup>[23]</sup> also showed that heat treatment before resin polymerization had a significant influence on 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) before polymerization, cytotoxicity results decreased, again at both 24 h and 7 days.

Resin cement cytotoxicity is known to increase with time.<sup>[35]</sup> In the present study, we compared cytotoxicity



results after 24 h and 7 days of incubation. The results showed that heat treatment had a significant effect in preventing the increase of cytotoxicity. Samples treated with a jet of hot air (60°C) showed 7-day cell viability rates similar to those observed in nonheated samples at 24 h. This finding is extremely relevant, as it suggests that heat treatment helps maintain better cell viability rates when compared with nonheated samples.

There is a great concern that cytotoxicity mechanisms may be related to the release of residual monomers during the conversion of monomers into polymers, i.e., to the early stages of polymerization.<sup>[28]</sup> In this sense, the present study obtained markedly satisfactory results during early polymerization, as heat treatment probably allowed for a higher degree of polymer cross-linking and consequently resulted in a lower number of residual monomers.

Heat treatment before photopolymerization significantly increases monomer conversion rates to above the levels observed with traditional methods.<sup>[36]</sup> This can be explained by the lower viscosity and increased mobility of radicals as a result of heating. Moreover, the frequency of collision of active groups and nonreacted radicals increases when curing temperature is below the glass transition temperature, resulting in additional polymerization and a higher rate of conversion.<sup>[37-40]</sup>

According to the present results, small physical modifications to the environment where cement mixing and homogenization take place, for example, using a jet of hot air at 60°C to heat the cement and glass slide, can play major roles in reducing material cytotoxicity. Further studies are warranted to evaluate how this cytotoxicity can be further reduced and thus cause less damage to patients.

## CONCLUSIONS

The results obtained in the present study showed that heat treatment at 60°C, before photopolymerization, while mixing self-adhesive resin cements, should be considered as a strategy to reduce cytotoxicity of self-adhesive resin cements, as evidenced by the results observed both at 24 h and 7 days of analysis.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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