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Influence of Preheating Self-Adhesive Cements on the Degree of Conversion, Cell Migration, and Cell Viability

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Abstract: Enhancing the degree of polymerization can mitigate the cytotoxic effects of resinous materials, as residual monomers have been identified as a significant contributor to cytotoxicity. Hence, the aim of the current research was to evaluate the influence of preheating self-adhesive cements at 39 °C on cell migration, cytotoxicity, and degree of conversion. RelyX U200, Set PP, and MaxCem Elite were subjected to Fourier Transform Infrared Spectroscopy–Attenuated Total Reflection (FTIR–ATR). Self-adhesive resin cements were applied onto an ATR device, with samples subjected to either heated or room temperature conditions, followed by photoactivation. For the cytotoxicity analysis, extracts (24 h and 7 days) were placed in contact with NIH/3T3 cells. For cell migration, images were captured of each sample until the possible closure of the cleft occurred. A two-way analysis of variance (ANOVA) was conducted to assess the effect of preheating on the degree of conversion and cell viability within the self-adhesive cements tested. A significance level of 5% was set for statistical purposes. In the results of the degree of conversion, preheating did not improve the conversion of cements ($p > 0.05$). For the 3-(4-5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), preheating did not improve the results within 24 h, however, it generated positive results within 7 days for the Set PP resin cement ($p < 0.05$). For cell migration, high rates of cell death were found in all groups. It is concluded that preheating at 39 °C causes a positive effect only in increasing the cell viability of the Set PP resin cement and that both materials analyzed are highly cytotoxic.

Keywords: cell migration assays; cytotoxicity; degree of conversion; dental cements; resin cements

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1. Introduction

In the realm of dental materials, adhesive cements are pivotal for the successful placement and longevity of restorations. Broadly, these cements are classified into two categories: conventional cements and self-etch cements. Conventional cements necessitate the application of a separate adhesive system prior to their use, ensuring a strong bond between the restoration and the tooth structure. In contrast, self-etch cements are formulated to combine both adhesive and cementation properties, eliminating the need for a prior adhesive system. This integration not only simplifies the cementation process but also reduces the

potential for technique-sensitive errors associated with multiple application steps [1–3]. Self-adhesive cements were introduced in dentistry with the promise of combining the high clinical performance of resin cements with simplification of operative steps since they are materials composed of polymers capable of adhering to the dental structure without the need for additional use of an adhesive [1,2]. They are currently the first choice for esthetic restorations with the advantages of being able to adhere to various substrates, high resistance, and low solubility in the oral environment, in addition to several color options [1–3].

This material contains resin monomers, such as 2-hydroxyethyl methacrylate (HEMA), bisphenol A-glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), and camphorquinone, which have a degree of cytotoxicity to be always considered, and are still capable of reaching the pulp cells through the dentinal tubules after solubilization of the hybrid layer [4–7]. In addition to methacrylates and low-molecular-charge resin components, self-etching monomers were added to its composition to significantly reduce the pH and promote demineralization of the tooth structure [8].

The cytotoxicity levels of these materials are significant since residual monomers are left in the curing process [9–11]. The conversion of monomers into polymers is crucial for the full development of the physical properties and clinical performance of resin cements, as well as for minimizing their toxicity. Dual-curing self-adhesive cements exhibit lower cytotoxicity levels compared to conventionally self-cured cements, highlighting the benefits of enhanced polymerization in reducing potential adverse effects [11]. In general, the literature is unanimous in highlighting that resinous materials have a high cytotoxic potential, generating a chronic inflammatory process that may be irreversible [7–13]. The interaction at a molecular level of these materials with cells can cause tissue reactions such as inflammation, immunological changes, necrosis, and cellular apoptosis [14–16]. Cytotoxicity is also due to the change in pH that occurs with the materials during the polymerization process. Therefore, neutralizing the pH of these cements is of great importance to avoid the impact on the final polymerization, mainly considering the effect of the formulation of methacrylate monomers in this process [17].

An increase in the degree of polymerization can contribute to reducing the cytotoxicity of resinous materials, since, as previously reported, residual monomers are one of those responsible for this [18,19]. Therefore, several authors have proposed preheating materials with the aim of providing greater mobility to photoinitiators and monomers present in the resin matrix [7,20–26]. The increase in temperature increases the kinetic energy of the molecules, changing the way they bond [27], favoring the conversion of monomers into polymers in order to reduce the cytotoxicity of the materials [20–26]. Thus, the objective of the present study was to analyze the effect of preheating at 39 °C on the degree of conversion, cytotoxicity, and migration of cells exposed to self-adhesive resin cements. Accordingly, the null hypothesis for this study is that preheating at 39 °C has no effect on the degree of conversion and cytotoxicity to self-adhesive resin cements.

2. Materials and Methods

2.1. Degree of Conversion (DC)

Three different self-adhesive resin cements (Table 1) were used.

Samples of each material ($n = 8$) were made from a standardized volume of cement that was dispensed onto the crystal of the attenuated total reflectance (ATR) device, being retained within a cylindrical matrix measuring 5 mm in diameter by 1 mm in depth. In the groups where the materials were heated, the HotSet device (Technolife, São Paulo, Brazil) was preheated to 39 °C and positioned on the matrix with the material already dispensed for 20 s. After removing the heating device, the sample was immediately light-activated for 40 s using a light-emitting diode (LED) device with a power setting of 1000 mW/cm^2 (VALO, Ultradent, Salt Lake City, UT, USA). In the other groups, the specimens were manufactured at a temperature of 23 ± 2 °C and followed the same photoactivation pattern.

Table 1. Materials tested, composition as informed by manufacturer, batch, and manufacturers.

Cement	Composition	Lot	Manufacturer
RelyX U200	Base paste: silane-treated glass powder, 2-propenoic acid, 2-methyl,1,10-[1-(hydroxymethyl)-1,2-ethanediyl] ester, triethylene glycol dimethacrylate (TEGDMA), silane-treated silica, fiber glass, sodium persulfate, and tert-butyl peroxy-3,5,5-trimethylhexanoate. Catalyst paste: silane-treated glass powder, dimethacrylate substitute, silane-treated silica, sodium p-toluenesulfonate, 1-Benzyl-5-phenylbarbituric acid, calcium salts, 1,12-Dodecanediol dimethacrylate, calcium hydroxide, and carbon dioxide titanium.	8077839	3M/ESPE, St. Paul, MN, USA
Set PP	Fluoro-aluminosilicate glass, urethane dimethacrylate, camphorquinone, acid monomer.	S21101231	SDI, Bayswater, Victoria, Australia
Maxcem Elite	Hydroxyethyl methacrylate (HEMA), methoxyphenol (MEHQ), comene hydroperoxide (CHPO), unpolymerized acrylic monomers, titanium dioxide (TiO_2) and pigments.	8399866	Kerr Corporation, Orange, CA, USA

The degree of conversion was assessed using Fourier transform infrared spectroscopy (FTIR). The spectrophotometer (VERTEX 70, Bruker Optics, Ettlingen, Germany) was connected to an ATR device featuring a 2 mm horizontal diamond crystal (Platinum ATR-QL, Bruker Optics, Ettlingen, Germany) positioned at a 45° angle with the mirror [28]. The percentage of unreacted carbon–carbon double bonds was calculated by comparing the absorbance intensities of the aliphatic (peak at 1640 cm^{-1}) and aromatic carbon–carbon (peak at 1715 cm^{-1}) double bonds, with measurements taken at a resolution of 4 cm^{-1} over 32 scans.

2.2. Cytotoxicity Assay

2.2.1. Preparation of Test Specimens

The test specimens of each material (Table 1) were produced at a temperature of 23 ± 2 °C or pre-heated with the HotSet device (Technolife, Joinville, SC, Brazil) at 39 °C for 1 min before being photopolymerized, totaling 8 samples per group. A sterile metallic matrix was used to produce the test specimens. Each specimen was 1.95 cm in diameter and 2 mm thick, totalizing a final volume of 3 cm^3 for each sample [29]. For the unheated specimens, the matrix was positioned on a sterilized glass plate and filled with cement. Afterwards, the specimen was photopolymerized for 40 s using a high-power photopolymerizer (VALO; Ultradent, Salt Lake City, UT, USA) with a light emitter with a diameter of 15 mm and an irradiation intensity of 1000 mW/cm^2 at a 5.0 mm distance. For the heated specimens, the matrix was placed on the HotSet preheated to 39° and the cement was dispensed into the matrix and after 1 min; the polymerization process was the same for all the specimens. The equipment was previously decontaminated with 70% alcohol. All procedures were performed in a laminar flow cabinet. After obtaining samples from the aforementioned groups, cytotoxicity tests were performed using 3-(4-5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays and cell migration assays.

2.2.2. Means of Cell Extraction

After obtaining the specimens from the six experimental groups (3 at room temperature and 3 pre-heated), each sample of 3 cm^3 in volume was immersed in Dulbecco's modified Eagle medium (DMEM; Invitrogen®, Carlsbad, CA, USA) without supplementation in accordance with ISO 10993:12.29 [29]. The surface area was calculated based on the total dimensions of the specimen. The extracts were kept at 37 °C in an incubator containing 95% O_2 and 5% CO_2 for periods of 24 h and 7 days to later be exposed to cell culture. Due to the intense cytotoxicity of the materials, part of the extraction solution was also diluted in unsupplemented DMEM at a ratio of 1:10 to allow an adequate analysis of cell viability [30].

All cell culture assays were performed with the NIH/3T3 line (ATCC®—American Type Culture Collection—TCC, Old Town, MD, USA), fibroblasts originating from mouse embryos. The strain was thawed, expanded, and maintained in cell culture flasks in an incubator at 37 °C containing 95% O_2 and 5% CO_2. The cells were maintained in the flasks in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Gibco, Grand Island, NY, USA), 100 U/mL streptomycin (Gibco, Grand Island, NY, USA), and 100 µg/mL gentamicin (Gibco, Grand Island, NY, USA) until the start of the trials.

2.3. Cell Viability Testing

To evaluate the cell viability, the MTT method was used, which has as its principle the determination of the ability of viable cells to reduce the MTT, forming insoluble violet-colored formazan crystals. The cultured cells were transferred to a 96-well plate and maintained under standard culture conditions for 24 h to allow them to adhere to the bottom of the plate. After this period, they were washed with Dulbecco's phosphate-buffer saline (DPBS) to remove the culture medium and then exposed to the extraction media (24 h and 7 days), in triplicates and incubated for 24 h. The negative control group consisted of cells exposed to unsupplemented DMEM medium and not exposed to the materials, while cells selected for the positive control were also maintained under the same conditions as the negative control during this incubation. After 24 h of exposure, the extraction medium was removed, and the cells were washed with DPBS. At this time, cells selected for the positive control were exposed to a 2% NaClO solution for 5 min and washed with DPBS. Next, 200 µL of a solution containing 10% MTT (5 mg/mL) in DMEM was added to each well, and the cells were returned to the incubator under the same culture conditions for 4 h for the formation of violet formazan crystals. To solubilize these crystals, 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the spectrophotometric reading of the absorbance, at a wavelength of 570 nm, was performed on a plate reader (Bio-Rad Microplate Reader Benchmark, Inc., Hercules, CA, USA). The percentage of viable cells was calculated in relation to the negative control and assay validation was performed using a positive control.

2.4. Cell Migration Assay

A total of 8×10^4 NIH/3T3 cells were transferred into 3.85 cm^2 K12-012 (Kasvi) 12-well plates. Before transferring the cells to the plates, two parallel lines 2 cm apart were drawn on the bottom of the plate from the outside using a permanent marker, passing horizontally through all the wells. The entire procedure was carried out under aseptic conditions inside a laminar flow cabinet. The cells were maintained under normal culture conditions in DMEM medium with 10% FBS for 24 h to allow them to adhere to the plate. Afterwards, the culture medium was replaced with DMEM supplemented with 5% FBS for 24 h, and the following day it was replaced again with DMEM with 0.5% FBS for another 24 h. After 24 h, the medium was removed and replaced with DPBS buffer. The cells were observed under the microscope and formed a complete cell monolayer in all wells. With the aid of a 200 µL tip, a wound was made in the monolayer perpendicular to the drawn lines using the tip to scrape the bottom of the well from top to bottom in a single movement. The DPBS was removed and replaced with DMEM exposed to the materials and diluted 1:10 in duplicates. A pair of wells were filled with unsupplemented DMEM to serve as a control group. An image was captured of each well immediately after this procedure, this being time 0. Successive images were captured until the wound closed in the control group. The times were 0 h, 24 h, and 48 h. Images were analyzed using the Image-Pro Plus 7.0 software. The cleft space was determined by quantifying the pixels present in the empty space without living cells. The cell migration workflow used in this study is detailed in Figure 1.

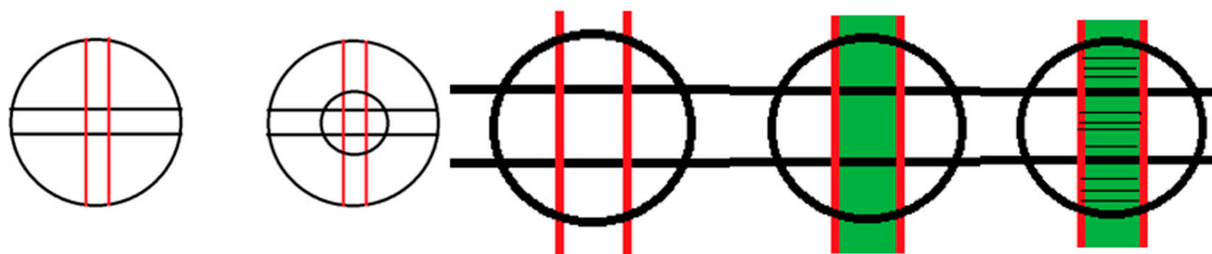

Figure 1. Cell migration images for resin cements.

2.5. Statistical Analysis

A two-way analysis of variance (ANOVA) was conducted to assess the effect of preheating on the degree of conversion and cell viability within the self-adhesive cements tested. The sample size for cytotoxicity assay was defined according to ISO 1099312 [29]. Statistical significance of 5% was adopted. The Sigma Plot (Version 12, Systat, San Jose, CA, USA) was employed for the statistical analyses.

3. Results

3.1. Degree of Conversion

The means and standard deviations of the degree of conversion for all self-adhesive cements, both polymerized at room temperature and preheated, are shown in Table 2. The degree of conversion for the three cements analyzed was not significantly affected by preheating the material ($p > 0.05$) according to the two-way ANOVA statistical analysis with a significance level of 5%.

Table 2. Average conversion degree values (%) according to the temperature of self-adhesive resin cements.

Material	Room Temperature (23 °C)	Preheating (39 °C)
RelyX U200	34.13 ± 1.65	31.27 ± 1.45
Set PP	35.59 ± 4.55	39.02 ± 1.47
MaxCem Elite	32.42 ± 6.22	34.55 ± 2.31

There was no statistical difference between groups.

3.2. Cell Viability

When the three materials, both at room temperature and preheated, were analyzed without dilution, cell death occurred for all, often preventing obtainment of results from living cells. However, when dilution was carried out, it was possible to perform the analysis since cell viability could be checked and measured. In Table 3, in the analysis with a dilution of 1:10, it was possible to observe that preheating had a positive effect on cell viability over a period of 7 days ($p < 0.05$) for the Set PP self-adhesive resin cement. In contrast, the other materials did not show any influence of heat on cell viability in either analysis period (24 h and 7 days).

Table 3. Means ± standard deviations of cell viability (%) according to the material and treatment.

Material	Dilution 1:10 24 h		Dilution 1:10 7 Days	
	Control	Preheated	Control	Preheated
RelyX U200	58.2 ± 32.6 Aa	78.8 ± 11.8 Aa	88.6 ± 14.3 Aa	94.4 ± 14.2 Aa
Set PP	77.5 ± 6.1 Aa	74.2 ± 13.5 Aa	74.5 ± 8.0 Ab	95.1 ± 8.4 Aa
MaxCem Elite	67.0 ± 0.6 Aa	69.3 ± 13.9 Aa	76.8 ± 6.5 Aa	71.6 ± 3.5 Ba

Different capital letters indicate statistical difference in the same column. Different lowercase letters indicate statistical differences between groups in the same analysis period.

3.3. Cell Migration

According to the visual results obtained, when compared to the control group, it was possible to observe that all experimental groups did not have any type of cell migration within 24 h (Figure 2), confirming the high cytotoxicity of the material.

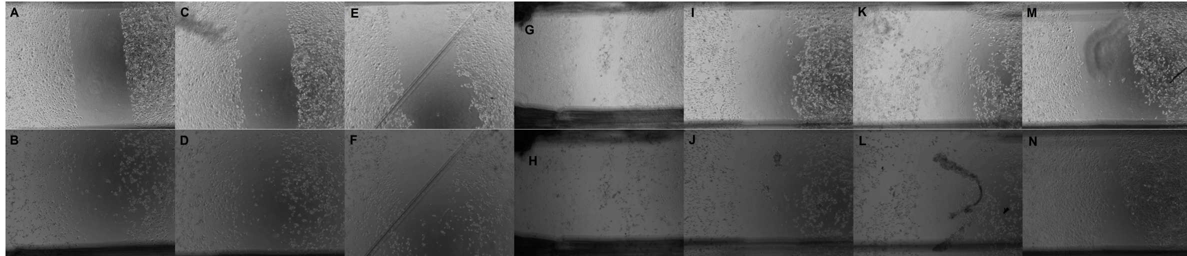

Figure 2. Cell migration images for resin cements in the 24 h analysis. Groups: (**A**,**B**)—U200 control time 0 and 1, respectively; (**C**,**D**)—U200 preheated time 0 and 1, respectively; (**E**,**F**)—Set PP control time 0 and 1, respectively; (**G**,**H**)—Set PP preheated time 0 and 1, respectively; (**I**,**J**)—MaxCem Elite control time 0 and 1, respectively; (**K**,**L**)—MaxCem Elite preheated time 0 and 1, respectively; (**M**,**N**)—negative control (no treatment).

In the 7-day analysis (Figure 3), it can be observed that cell migration in the control U200, pre-heated U200, and pre-heated Set PP samples was much lower than the cell growth observed in the control group (DMEM not supplemented).

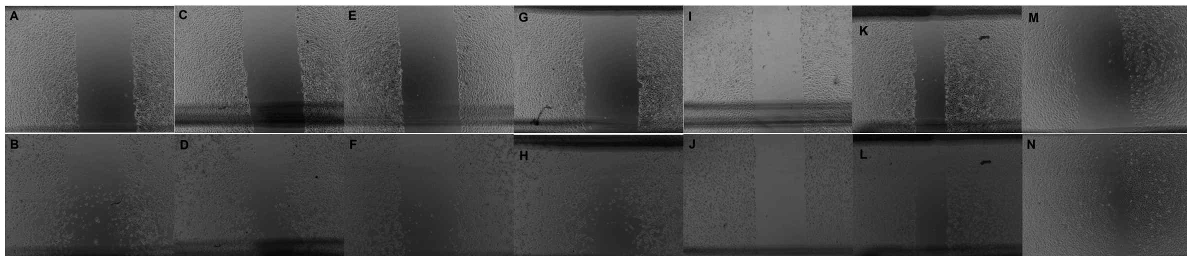

Figure 3. Cell migration images for resin cements in the 7-day analysis. Groups: (**A**,**B**)—U200 control time 0 and 1, respectively; (**C**,**D**)—U200 preheated time 0 and 1, respectively; (**E**,**F**)—Set PP control time 0 and 1, respectively; (**G**,**H**)—Set PP preheated time 0 and 1, respectively; (**I**,**J**)—MaxCem Elite control time 0 and 1, respectively; (**K**,**L**)—MaxCem Elite preheated time 0 and 1, respectively; (**M**,**N**)—negative control (no treatment).

It is worth noting that in these experimental groups, it was observed that the cells were unable to survive for the 48-h duration of the experiment and were all dead after 24 h. Furthermore, the Set PP control, MaxCem Elite control, and pre-heated MaxCem Elite samples showed a small regression in the area, indicating that there was also cell death even though they were pre-heated (Table 4).

Table 4. Cell migration (%) into resin cements at 24 h and 7 days.

Material	Scar Area (24 h)			Scar Area (7 Days)		
	T0	T1	% Migration	T0	T1	% Migration
U200 control	396935	0	0	419929	383524	8.67
U200 preheated	393159	0	0	395202	309167	21.77
SetPP control	476130	0	0	412537	417019	−1.09
SetPP preheated	378987	0	0	393932	364406	7.33
MaxCel Elite control	454822	0	0	358327	370650	−3.44
MaxCem Elite preheated	555235	0	0	221451	237891	−7.42
Negative control	340301	225141	33.84	401688	273951	66.66

4. Discussion

The increase in the degree of monomer conversion of resinous materials is directly related to the reduction in cytotoxicity caused by these materials [7,31]. The result of the present investigation led to the partial rejection of the null hypothesis since the preheating at 39 °C of self-adhesive resin cements tested was affected by the cell viability (%) according to the material and treatment.

Several studies demonstrate an increase in the degree of conversion when materials are exposed to increased temperature [22,32–34]. The results obtained in the present study partially diverge from part of the literature mentioned, where some studies used temperatures of 54 °C, 60 °C, and even 68 °C, while in the present study, the materials were preheated to 39 °C. Additionally, there is a reduction in temperature in the present study by up to 50% when the material is removed from the device's heating base [32]. It may be thought that the temperature used is not sufficient to provide greater conversion of monomers into polymers, since increasing temperature provides an increase in kinetic energy and greater mobility of the photoinitiators and monomers present in the resin matrix [35].

It is worth taking into account here whether increasing the temperature of the material could cause repercussions on the pulp organ. Even though the present study did not work with pulp, the repercussions that may occur with pre-heating or with applications of heat on materials that will come into direct contact with the dentin and sometimes close to the pupil organ are always taken into account. A previous study demonstrated that the application of an air jet at 69 °C on dentin with a thickness of 0.5 mm for 10, 20, 30, and 40 s promoted an increase in intrapulpal temperature by 5.8 °C, 10.1 °C, 13.6 °C, and 16.6 °C, respectively. A classic 1965 study reports that an increase of 5.5–11 °C in intrapulpal temperature results in different levels of pulp necrosis in primates [36]. However, Baldissara et al., [37] demonstrated that an increase of 9–15 °C in intrapulpal temperature was not enough to cause pulp necrosis after 3 months. Following the same idea, but under laboratory conditions, Zimmer et al., [7] applied a jet of air at 50 °C to a 0.4 mm thickness of dentin for 5, 10, and 20 s and observed that increasing the temperature did not promote a reduction in viability of MDPC-23 cells.

Because increasing the temperature of the resinous material can increase the degree of conversion of monomers into polymers, there will be a reduced number of free monomers that can diffuse through the dentin to the pulp and cause cytotoxic effects [5–8,38]. However, in the present research, a high rate of cytotoxicity was found in all tests carried out, even after preheating the material. The results of high cytotoxicity were remarkable in the first 24 h of the cell migration test where no cell growth was observed. In the 7-day analysis, some groups had a small cell growth and others showed a regression in the area, corroborating with da Silva et al., [13], who demonstrated that resin cements have high cytotoxicity. Toxicity was high even when the sample extracts were diluted in a ratio of 1:10, making it in a certain way closer to a clinical situation [30,39].

The MTT test performed in the present study is widely found in the literature and recommended by international standards [29]. However, it should be noted that more tests are needed to determine the cytotoxic effect of materials used in routine dental clinics. In the current manuscript, the cell migration test is carried out, which has complementary results to the MTT and demonstrates by optical microscopy the existence or non-existence of cell migration. The results prove, for both preheated and unheated materials, a low rate of cell migration, which is the effect of intense aggression to adhered cells, characterized by the process of apoptosis and cellular disorganization. Therefore, more in vitro studies of self-adhesive resin cements are necessary to guide in vivo tests, especially since these materials are used in close proximity to the dental pulp, particularly in cases of rehabilitation of vital teeth that require significant removal of tooth structure.

In addition to evaluating the degree of conversion and cell viability, it is essential to carry out more studies that evaluate the physical and mechanical properties of these materials since the literature already demonstrates that heating can reduce the viscosity

of the material and promote a satisfactory adaptation to cavity preparation walls [17,40]. Researchers working with heated resins and cements utilize various temperatures for the heating process. However, many argue that 39 °C strikes an optimal balance, combining effective polymerization potential with minimal risk of pulp irritation. In the present study, the temperature of 39 °C did not promote an improvement in the properties evaluated, such as the degree of conversion. The device also had the possibility of working at a temperature of 69 °C. However, the temperature of 69 °C promoted by the heating device immediately started polymerization of the cements, making its evaluation unfeasible. Therefore, it is essential to establish the ideal temperature to achieve the optimal properties of self-adhesive resin cements, along with their best possible biological characteristics. Further, microscopic live/dead images in the cell viability assay could provide better visual illustration and clarity. The high toxicity observed in the study is a significant concern, but it is crucial to consider the limitations of the research. As this study was conducted in vitro, the results may not fully translate to clinical scenarios. In vitro studies often use controlled environments that do not completely mimic the complex conditions found in the human oral cavity, such as variations in temperature, pH, and the presence of saliva and other biological factors. Additionally, the exposure times and concentrations of the materials tested in vitro may differ from those encountered in clinical practice, potentially leading to an overestimation or underestimation of cytotoxicity. Therefore, while the findings provide valuable insights, further in vivo studies are necessary to confirm the relevance of these results to real-world clinical applications and to ensure patient safety.

5. Conclusions

Within the limitations of this current study, conclusions were made as follows:

- All tested materials were strongly cytotoxic and exhibited a low degree of conversion.
- The Set PP resin cement showed an increase in cell viability when preheated to 39 °C, without any gains in the degree of conversion.
- As a clinical conclusion, preheating self-adhesive resin cement can be discarded, as it does not enhance the material's properties and introduces an unnecessary additional step in the clinical workflow. Furthermore, due to the observed cytotoxicity, extra caution is advised when using these materials in cavities close to the pulp complex, to minimize potential adverse effects on patient health.

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