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Effect of various polymerization protocols on the cytotoxicity of conventional and self-adhesive resin-based luting cements

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Abstract: OBJECTIVES This study evaluated the cytotoxicity of resin-based luting cements on fibroblast cells using different polymerization protocols. MATERIALS AND METHODS Two conventional dual-polymerized (RelyX ARC, VariolinkN) and two self-adhesive resin cements (RelyX Unicem, Multilink Speed) specimens were polymerized using four different polymerization protocols: (a) photo-polymerization with direct light application, (b) photo-polymerization over ceramic and (c) resin nano-ceramic discs and (d) auto-polymerization. The specimens were then assigned to four groups to test cytotoxicity at 0, 1, 2 and 7 preincubation days (n = 5). MTT test was performed using NIH/3T3 fibroblast cells. Data were analysed using three- and one-way ANOVA. Multiple comparisons were made using Bonferroni post hoc test ($p < 0.05$). RESULTS The highest cytotoxic values were recorded at day 2 for conventional resin cements and at day 0 for self-adhesive resin cements. Self-adhesive resin cements showed the most cytotoxic effect at the second day, while conventional resin cements presented immediate cytotoxicity. Auto-polymerized resin specimens and especially Multilink Speed demonstrated the most cytotoxic effect regardless of the preincubation time. Cytotoxicity of cements tested reached the lowest level at day 7. Interposition of ceramic or nano-ceramic restorative material did not significantly affect the cytotoxicity of tested luting cements ($p > 0.05$). CONCLUSIONS Cytotoxicity of dual-polymerized resin cements was material-dependent and decreased gradually up to 7 days. Photo-polymerization plays an important role in reducing the cytotoxic effects. CLINICAL RELEVANCE When luting ceramic or resin nano-ceramic restorations of which thickness does not exceed 2 mm, the level of cytotoxicity with the tested materials is not significant. Luting of restorative materials that do not allow for light transmission such as metal-fused porcelain, clinicians should be cautious in the use of dual-polymerized conventional resin cements as only auto-polymerization of resin cements takes place under such materials.

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Effect of various polymerization protocols on the cytotoxicity of conventional and self-adhesive resin-based luting cements

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Short title: P*olymerization protocol effect on cytotoxicity of resin luting cements*

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Abstract

Objectives: This study evaluated the cytotoxicity of resin-based luting cements on fibroblast cells using different polymerization protocols.

Materials and Methods: Two conventional dual-polymerized (RelyX ARC, VariolinkN) and two self-adhesive resin cements (RelyX Unicem, Multilink Speed) specimens were polymerized using four different polymerization protocols: a) photo-polymerization with direct light application, b) photo-polymerization over ceramic and c) resin nano-ceramic discs and d) auto-polymerization. The specimens were then assigned to four groups to test cytotoxicity at 0, 1, 2 and 7 preincubation days (n=5). MTT test was performed using NIH/3T3 fibroblast cells. Data were analyzed using 3- and 1-way ANOVA. Multiple comparisons were made using Bonferroni post-hoc test ($p<0.05$).

Results: The highest cytotoxic values were recorded at day-2 for conventional resin cements and at day-0 for self-adhesive resin cements. Self-adhesive resin cements showed the most cytotoxic effect at 2nd day, while conventional resin cements presented immediate cytotoxicity. Auto-polymerized resin specimens and especially Multilink Speed, demonstrated the most cytotoxic effect regardless of the preincubation time. Cytotoxicity of cements tested reached the lowest level at day-7. Interposition of ceramic or nano-ceramic restorative material did not significantly affect the cytotoxicity of tested luting cements ($p>0.05$).

Conclusions: Cytotoxicity of dual-polymerized resin cements was material-dependent and decreased gradually up to 7 days. Photo-polymerization plays an important role in reducing the cytotoxic effects.

Clinical Relevance: When luting ceramic or resin nano-ceramic restorations of which thickness does not exceed 2 mm, level of cytotoxicity with the tested materials is not significant. Luting of restorative materials that do not allow for light transmission such as metal-fused porcelain, clinicians should be cautious in the use of dual-polymerized conventional resin cements as only auto-polymerization of resin cements takes place under such materials.

Keywords: Adhesion ● Cytotoxicity ● Dual-polymerized resin cements ● Resin nano-ceramic ● Auto-polymerized resin cements ● Lithium disilicate

Introduction

Resin-based dual-polymerized luting cements are widely used for cementation of indirect restorations. Biocompatibility of such materials is an important consideration since the cement is directly in contact with the vital dental tissues through the dentinal tubules of the prepared teeth [1-3]. Allergic and toxic effects mainly caused by polymerization deficiencies are associated with biocompatibility of these materials [3,4]. Recent studies have demonstrated the cytotoxic effects of unpolymerized free monomers such as 2-hydroxyethyl methacrylate (HEMA), bisphenol A-glycidylmethacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA) leached from inadequately polymerized resin cements [3,5-8].

Resin-based dual-polymerized conventional luting cements can be polymerized either in dual- or auto-polymerization mode as they contain a redox initiator system that triggers the polymerization in addition to photoinitiators [9]. The auto-polymerization mechanism is supposed to compensate for polymerization deficiencies in the areas that are not readily accessible to light or under restorations where the opacity and the thickness of the material attenuates the light transmission to the luting cement [10-12]. However, previous studies have reported remarkable differences in dual-polymerized luting cements in regards to degree of polymerization according to the polymerization protocol employed [2,3,5]. Insufficient degree of conversion may also compromise biological and mechanical properties of the luting cement [13].

Conventional resin luting cements require smear layer removal or modification of the smear layer prior to the application of resin-based material to dentin in order to obtain adequate bond strength between the prepared tooth surface and the restoration material. The use of acidic conditioners, primers, adhesive resins or their different combinations aim for preparing the dentin surface for the luting cement. However, such conditioners significantly increase the toxic potential of restorative procedure as they enhance dentin permeability and local humidity [3]. Due to the fact that their application is technique-sensitive and complicates clinical steps, cementation is usually time-consuming and susceptible to manipulation errors [11,13]. Within this context, self-adhesive resin cements simplify adhesive cementation in that they adhere to hard dental tissues without the need of complex conditioning steps [11,14]. Such cements include acidic and hydrophilic monomers in their composition that simultaneously demineralize and infiltrate enamel or dentin, resulting in strong bonding. Although it may not apply for all self-adhesive cements, a chemical reaction between the functionalized monomers of the cement and hydroxyapatite of dentin has been reported [15-18]. Moreover, chemical reactions between the acidic monomers and the basic inorganic fillers of the material have been stated to take place, leading to an additional acid-base setting reaction, apart from the free radical polymerization of the material [9,18,19].

Numerous previous studies have assessed the cytotoxicity of resin-based luting cements polymerized under various clinical scenarios [2-4,6,8]. However, little information is available regarding the time-dependent changes in cytotoxic effects of such materials, especially the newly introduced ones when they are used under restorative materials implying clinical conditions. Based on these considerations, this in vitro study aimed to evaluate the cytotoxic effects of different categories of resin-based luting cements on NIH/3T3 fibroblasts in their auto- and dual-polymerized modes when irradiated through ceramic or nano-ceramic restorative materials up to 7-day preincubation timepoints. The first research hypothesis was that there would be no difference between resin-based luting cements regarding their

cytotoxic effects as a function of timepoints. The second research hypothesis was that the interposition of ceramic or resin nano-ceramic restorative material would negatively affect the cell viability when exposed to luting cements.

Materials and methods

Specimen preparation

Two conventional dual-polymerized (RelyX ARC, 3M ESPE, St Paul, MN, USA, RA and Variolink N, Ivoclar Vivadent, Schaan, Liechtenstein, VL) and two self-adhesive resin cements (RelyX Unicem, 3M ESPE, RU and

Multilink Speed, Ivoclar Vivadent, MS) were tested in this study.

The specimens were polymerized using four different polymerization protocols simulating a variety of potential clinical situations:

Group D: Photo-polymerization directly from the top of the cement specimen.

Group E: Photo-polymerization through lithium disilicate ceramic (IPS Empress 2, Ivoclar Vivadent, Shade A2) disc (diameter: 10 mm; thickness: 2 mm).

Group L: Photo-polymerization through resin nano-ceramic disc (Lava Ultimate, 3M ESPE, Shade A2) disc (diameter: 10 mm; thickness: 2 mm).

Group C: Auto-polymerization without light activation.

The chemical composition and application protocol of resin-based luting cements used in this study are listed in Table 1.

The specimens prepared according to different polymerization protocols were then divided into four subgroups to be tested at 4 different preincubation times (0-, 1-, 2- and 7-days) (n=5). All materials were handled according to each manufacturer`s instructions under aseptic conditions in laminar air flow cabinet (Bilser LF 2000, EfLAB, Ankara, Turkey). Cement discs were shaped using heat-resistant

polytetrafluoroethylene moulds having cylindrical cavities (depth: 2 mm; diameter: 5 mm). High power mode (1200 mW/cm^2) of an LED (Bluephase 20i, Ivoclar Vivadent) photo-polymerization unit was used for the photo-polymerized groups. Prior to each polymerization step, the light intensity of the device was controlled through polyester film with an LED radiometer (LED Radiometer, SDI Limited Dental Products, Victoria, Australia). Immediately after preparation, the specimens were sterilized using ultraviolet light applied to both sides of cement discs for 45 minutes. The sterilized materials were immersed in culture medium and the extracts were collected to be tested.

Cell culture

Murine fibroblast cell line NIH/3T3 (ATCC CRL-1658, American Type Culture Collection, Manassas, USA) was used for the cell viability assay. The cells were maintained in Dulbecco's modified Eagle medium with stable glutamine (DMEM, Biochrom GmbH, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom GmbH) and penicillin (100 units/mL) and streptomycin (100 µg/mL) (Biochrom GmbH) at 37°C in a fully humidified atmospheric environment containing 5% CO_2. The Trypsin-EDTA solution [0.05 % trypsin (Biochrom GmbH) and 0.02% ethylene diamine tetraacetic acid (EDTA, Biochrom GmbH)] was used to passage cultures when grown to 80% confluence. Fibroblast cells from the fourth passage were thawed two weeks prior to each experiment and passaged twice before use. The cells in the logarithmic growth phase were used in this experiment.

Cell viability assay

Cell viability was tested using MTT (3-{4,5- dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide) assay. Exponentially growing cells were digested with the trypsin-EDTA solution and the supernatant fluid was decanted after centrifugation at 1200 rpm for 5 minutes. Culture medium was added to convert to a single cell suspension, and then cells were counted and adjusted to 3x10^4/mL. A total volume of 100 µL of cell suspension was added to 96-well plates (Cell star, Greiner Bio-one GmbH,

Frickenhausen, Germany). Afterwards, the culture plates were placed back into the incubator (Model MCO-18AIC, Sanyo Electric, Osaka, Japan) [20]. After 24 h, adherent cells were observed and the old culture medium was decanted, followed by adding culture medium with three replicas of each material extract (100 µL) obtained at 0-, 1-, 2- and 7 days.

MTT in vitro toxicology assay kit (Sigma-Aldrich, St. Louis, Missouri, USA) was used in the experiment according to the protocol of the manufacturer. Briefly, the solution of MTT was prepared in phosphate-buffered saline (5 mg/mL) and final concentration of 0.5 mg/mL was prepared in DMEM. Twenty-hours after the incubation of the cells, the medium was aspirated, and 100 ul of MTT solution (0.5 mg/mL) was added to each well of culture and the cells were incubated for 3 h. Then, the MTT solution was aspirated and replaced by 100 µL of isopropanol solution (%10 Triton X-100 and 0.1 N HCl) to dissolve the formazan crystals. After agitation for 10 minutes in an orbital shaker (Orbital Shaker-Incubator model ES-20, Biosan, Riga, Latvia), the optical density of formazan dye was read at 570 nm by ELISA reader (Synergy HT, Biotech Instrument, Winooski, Vermont, USA). Glass specimens were used as negative and Polyvinyl chloride strips (PVC, Smiths Industries Medical System, Portex Ltd. Hythe, Kent, UK) as positive controls. The viability of the control cells that were kept in culture medium only was defined as 100% and the relative cell viability (%) was calculated based on the absorbance.

The viability of cell growth was calculated according the following formula:

Cell viability % = 100 x (Optic density-OD- mean of test groups / OD mean of control groups)

Statistical analysis

Statistical analysis was performed using as statistical software (IBM SPSS Software V.19 for Windows, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to test normal distribution of the data. The effects of the material (2 levels: lithium disilicate versus resin nano-ceramic), polymerization

protocol (4 levels: D, E, L, C) and the timepoints (4 levels: 0-, 1-, 2- and 7 days) of the experiment on living cell ratios were statistically analyzed by using 3- way analysis of variance (ANOVA). As the binary and triple interactions were significant, comparisons based on material, polymerization protocol and timepoints were further analysed using 1-way ANOVA. Multiple comparisons were performed using Bonferroni post-hoc test ($p<0.05$).

Results

When the cytotoxic effects of tested luting cements were compared, variations in results were observed as a function of preincubation timepoints (Figs 1a-d) (Table 2). MS showed the highest cytotoxic effect among all tested materials regardless of the polymerization method at all the preincubation timepoints ($p<0.05$). At 0-day preincubation time, conventional resin cements recorded higher cell viability values than self-adhesive resin cements polymerized with different protocols ($p<0.05$). In addition, the cell viability values of conventional resin cements, RA and VL, photo-polymerized through ceramic restorations were not statistically different ($p>0.05$)

Considering 1-day preincubation timepoint, an alteration in the cytotoxic behaviour of the groups as to the previous preincubation timepoint was observed. Generally, RA and RU groups showed significantly higher cell viability than the other two cement groups tested. Directly photo-polymerized and auto-polymerized specimens of these cements showed no significant results ($p>0.05$). At this observation period, cell viability measurements recorded for the VL group photo-polymerized through ceramic restorative material was comparable with that of the corresponding RU group ($p>0.05$).

At 2-day preincubation timepoint, RU showed the highest cell viability for each polymerization protocol followed by in a rank order of RA, VL and MS, with significant differences ($p<0.05$).

After 7 days of preincubation, cell viability results of all the cement groups increased. At this observation period, results of auto-polymerized RA, VL and RU groups were not statistically different

from each other ($p>0.05$). Moreover, no significant difference was recorded between RA and VL groups directly photo-polymerized or polymerized through resin nano-ceramic material ($p>0.05$).

When cytotoxicity of each resin luting cement was evaluated regarding polymerization protocol (Figs. 2a-d), no statistically significant differences were observed between the groups of RA, VL and RU resin cements directly photo-polymerized or photo-polymerized through ceramic or resin nano-ceramic material at all the preincubation timepoints ($p>0.05$). However, only for RU cement, when directly photo-polymerized, showed higher cell viability than the groups photo-polymerized through ceramic material at 1- and 2-day preincubation timepoints ($p<0.05$). With this exception, the restorative material interposed between the light source and the cement or its composition did not affect the cell viability as opposed to their directly photo-polymerized counterparts. In contrast, when these resin cements were allowed to auto-polymerize, significant decreases in the cell viability was noted at all the preincubation timepoints ($p<0.05$).

MS, differing from the former resin cements, showed no significant differences in cytotoxicity between the polymerization protocols at 0- day preincubation time ($p>0.05$). However, auto-polymerized groups of this cement presented significantly less cell viability compared to the other polymerization protocols at 1- and 7- day preincubation timepoints ($p<0.05$). Except for directly photo-polymerized and photo-polymerized through ceramic material groups of MS ($p<0.05$), the cell viability values obtained were different from each other at 2nd day of preincubation regardless of polymerization protocol ($p<0.05$).

Considering preincubation times, conventional resin cements recorded significant decreases in cell viability from 0- to 2-day followed by significant increase after 7 days ($p<0.05$). However, the decreases between 0- and 1-day were not statistically different for RA regardless of the polymerization method ($p>0.05$). Likewise, auto-polymerized groups of VL showed no significant difference between 0 and 1 and 1 and 2-days. In contrast to conventional resin cements, self-adhesive cements demonstrated greater cytotoxicity initially, followed by gradual decrease up to 7-

days. Differences in cell viability values between 1- and 2-day for RU cement directly photo-polymerized, photo-polymerized through nano-ceramic material and auto-polymerized modes were not significantly different ($p>0.05$). Also, no significant difference was noted between 1- and 2-day preincubation timepoints for MS groups that were photo-polymerized through ceramic restorative material ($p>0.05$) (Figs. 3a-d).

Discussion

Biocompatibility is critical for restorative materials that are in direct contact with living tissues. Thus, luting cements are desired to be biocompatible and have low cytotoxicity, especially in cases where they are placed close to the pulp after tooth preparation due to less remaining dentin thickness. This study compared the cytotoxicity of commonly used resin luting cements using the MTT assay under different circumstances that simulate clinical conditions.

One disadvantage of the MTT assay is non-consistent estimation of number of viable cells in the resin content of tested materials [21] or the test itself [22] depending on the material tested, while several advantages of the test are obtaining rapid results, ease of application and visualization of cell density in small cell cultures [14,23,24]. In vitro studies that evaluated cytotoxicity of dental materials have commonly used fibroblast cells such as L929 and NIH/3T3 because of the reproducibility, availability and the resemblance of these cells to pulpal and gingival cells [1,6,24-27]. Especially, NIH/3T3 which is one of the most commonly recommended cells for MTT assay, is a continuous cell line meaning that they can be reproduced fast and easy [26]. Based on such advantages, in this study, the cytotoxicity of the tested resin cements were evaluated using NIH/3T3 (ATCC CRL-1658) fibroblast cell line.

Considering the previous studies, cytotoxic effects were evaluated at different timeperiods. While some studies have compared the cytotoxic changes of the dental cements immediately after

polymerization (0 or 1 day) and after 7 days [6,26,28], others have investigated the cytotoxicity after 24 h incubation time [3,7,14,20]. Schmid-Swap et al. have shown that resin cements present high cytotoxicity levels at the beginning of the polymerization process and reduction in cytotoxicity increases with time [8]. However, the cytotoxic behaviour through 1-week period of different resin cements tested in this study has never been investigated with the same methodology. In addition, since it is known that resin-based cements do show cytotoxic effects, clinicians should also be aware of the peak of the effect in terms of the response of pulp cells. For these reasons, this study evaluated the immediate cytotoxic effects of conventional and self-adhesive resin cements (0 day), early reaction to the cytotoxic substances (1-2 day) and the cytotoxicity levels at the end of 1-week period.

The results of the present study showed that all the tested luting cements reduced the viability of fibroblast cells at varying rates regarding time and polymerization conditions. Therefore, our first research hypothesis suggesting that all the tested luting cements would present similar cytotoxicity apart from polymerization protocol and preincubation timepoints was rejected.

According to ISO 10993-5:1999(E) [29], materials that promote reduction in cell viability by more than 30% are considered cytotoxic. In the present study, when the luting cements were directly photo-polymerized, most of the cement groups showed cell viability ranging between 74.4%-98.6% throughout the observation period and may be considered as non-toxic. However, RU at 0-day preincubation time, VL at 2-day preincubation time and MS at most of the preincubation timepoints presented cell reductions exceeding the safety limit of 30%. Pontes et al. reported slight cytotoxicity when extracts of RA specimens immersed in culture medium were applied to MDCP-23 and HDPCs for 1-day period [7]. Similar results were noted for VL exposed to L929-fibroblasts immediately after preparation and following 7-day preincubation time [8]. The results of the present study regarding conventional resin luting cements are similar to that of the aforementioned studies. This can be attributed to the similarity of the methodologies of those studies in which the test specimens were not

treated with bonding systems. However, other studies reported severe cytotoxic effects of RA on MDCP-23 and/or HDPCs after exposure of cultured cells to 24-h and/or 7-day extracts [3,28]. In these studies, simulating clinical conditions, a dentin barrier was used and dentin was pretreated with acid etching and bonding agent. HEMA, an acidic monomer included in the composition of bonding agent was related to the ascertained cytotoxicity [3,28,30]. Likewise, a moderate influence of bonding agents on the cytotoxicity was reported for the VL [6]. In contrast to these studies in which the clinical conditions were simulated by a combination of different approaches, this study compared the cytotoxic effects of resin cements alone. Experimental conditions including application protocol and test method might be responsible for the differences between our findings and those recorded in the above-mentioned studies reporting moderate to severe cytotoxic effects of the tested conventional resin-based cements.

In the present study, 1- and 2-day extracts of conventional resin luting cement groups showed significant reduction in cell viability compared to those of the control groups and other preincubation timepoints. However, most of the recordings were within the safety limit of 30%. The polymerization of dimethacrylates produces densely cross-linked network and, during polymerization period, part of the methacrylate groups involved in the formation of the cross-linked matrix remains unreacted, especially in the case of high-molecular-weight monomers [31]. The size and hydrophilicity of penetrating monomers are determining factors in their diffusion rate. Since bis-GMA, present in the composition of the conventional resin cements tested in the present study, is a monomer with low hydrophilicity and high molecular weight, one can suggest that its release to the medium may take longer leading to a significant reduction in cell viability on the first and/or second day [3,4]. Moreover, penetration of the solvent, in which the material is immersed might have intensified the diffusion of unreacted monomers and other leachable components by accelerating the degradation [32,33].

Regarding self-adhesive resin cements, both RU and MS showed low cell viability rates that indicate cytotoxicity at 0-day preincubation time. In accordance with our results, other studies reported significant reductions in cell viability for RU when extracts from freshly prepared specimens were cultured [5,6,8]. It has been suggested that self-adhesive resin luting cements may present lower rate of polymerization than the conventional resin cements, irrespective of the activation mode [9,34]. Although mechanism for both self-adhesive and conventional resin cements is reliant on free-radical polymerization, self-adhesive resin cements have monomers with acidic functionalities. Detrimental effect on the rate and extent of co-polymerization may occur due to acidic functional monomers with unmodified dimethacrylates, resulting incomplete polymerization of methacrylate acids [9,19]. Furthermore, high filler content of RU such as silane-treated silica may play an important role in the initial retention of free monomers [32]. Consequently, high amount of unpolymerized components and initially low pH of this material might have irritated the fibroblasts resulting in higher cell death at 0-day preincubation period. RU has no HEMA in its composition as declared by its manufacturer and contains sodium persulfate as a chemical initiator and possess a high filler content which decreases the mobility and so the reactivity of polymer radicals. These facts associated with the self-neutralizing mechanism of self-adhesive cements might have prevented any further hydrolysis of components, impairing the later release of unreacted monomers [32]. Thus, a gradual increase in the rate of viable cells was recorded for self-adhesive cement groups from 0- to 7-preincubation. These results are in line with those of other studies that reported high survival rates for RU in cell cultures preincubated for 24 h or longer periods [3,7,32]. Nonetheless, the findings of this study should be considered with caution since binding mechanism of RU to dentin depends partly on the interactions between Ca^{+2} ions and acidic monomers [15-17]. Absence of a hard tissue barrier between cement extracts and cultured cells might have interfered with the polymerization process and influenced the results of the present study.

Although similar cytotoxic patterns were observed, there were significant differences in terms of cytotoxicity between the two self-adhesive resin cements teste where MS showed significantly lower cell viability rates than that of the RU at all the preincubation timepoints. Information available for this luting cement is limited but it can be assumed that the different cytotoxicity values may result from dissimilar chemical content and monomer ratios of these cements.

In the present study, the tested resin luting cements RA, VL and RU photo-polymerized through ceramic and resin nano-ceramic restorative materials presented slight toxicity at the 0-, 1- and 2-day preincubation times compared to the controls. However, cytotoxic values of these groups were mostly within the safety limit of 30% and were not significantly different from those of directly irradiated groups. Thus, the second research hypothesis suggesting interposition of ceramic or resin nano-ceramic restorative material would negatively affect the cell viability is also rejected. Our results corroborate with those of Nocca et al. [35], who investigated degree of conversion, monomer release and cytotoxicity of two resin-based luting cements irradiated through ceramic and nano-ceramic restorative materials of 1.5 mm thickness. The authors demonstrated that despite reductions of degree of conversion values and increase of toxic substances into the culture medium, both barriers provoked similar alterations in the tested cements regardless of their chemical nature without any significant change in cytotoxicity. One other study that investigated the impact of light exposure time on the cytotoxicity of resin luting cements including RA, and RU photo-polymerized through 2 mm thick IPS Empress 2 disc with a high-power LED polymerization unit [36] showed slight reductions compared to the controls in terms of cell viability confirming the results of the present study.

Efficacy of the auto-polymerization mode of dual-polymerized resin cements is still controversial and varies from one material to another [37]. Although the number of viable cells increased at the end of 7 days of preincubation, significantly higher cytotoxicity levels were observed for the auto-polymerized resin luting cement groups than the photo-polymerized ones in the present study. The extent to which

the mode of polymerization affects cytotoxicity is related to the initiator system each material contains, which could favour auto- or photo-polymerization. Dual-polymerized resins have a limit to the number of auto-polymerization initiators that can be added to the material in order not to impair their working time [37]. Therefore, depending on the formulation, the deficiency of auto-polymerization component can result in higher concentration of unreacted double bonds and higher solubility of the cement, which results with an increase in cytotoxicity [11]. The low cell survival rates of auto-polymerized resin cements presented in our study can be explained by this phenomenon. These results agree with the findings of previous studies reporting high photo-activation dependence for resin cements [6,13,14,31,37]. In clinical situations where light attenuation is expected and dual polymerization of resin cement would not be possible due to opacity of the restoration, clinicians should use the cement that shows the least cytotoxic effect in auto-polymerization mode. Although tested auto-polymerized resin cements showed different cytotoxic effects regarding varying polymerization times, Rely X ARC may be considered to be the least cytotoxic cement tested.

Another finding that draws attention of the present study is the difference in cytotoxic patterns of the resin cements tested. Previous studies have emphasized that cytotoxicity decreases over time [4,8]. In this study, the time-dependent changes in cytotoxic pattern within a particular period were not tested. For self-adhesive resin cements (RU and MS), a gradual increase in the rate of viable cells was recorded from 0- to 7-preincubation days. On the other hand, conventional resin cements tested (RA and VL) showed the highest cytotoxicity at the 2nd day of preincubation, an effect that decreased after 7 days. The clinical significance of this situation might be a delayed sensitivity that occur several days after cementation when conventional resin cements are used. Conversely, in the use of self-adhesive resin cements, clinicians may expect an immediate sensitivity that diminishes over time.

Despite the fact that our results point out slight to significant cytotoxicity of tested resin luting cements depending on polymerization protocol and cement type, the limitations of in vitro studies should be

taken into consideration when reflecting the results to clinical implications. First, specimens were sterilized with UV application which would interfere with the polymerization process of resin materials [38]. Second, the direct application of material extracts on cultured cells represents an extreme challenge since no defensive mechanisms are available such as cytoplasmic elongations of odontoblasts and collagen in dentin tubules, as well as transdentinal movement of dentin fluid which would moderate the damage to the pulp cells [1,2]. Furthermore, contrary to the clinical situations, incubation of conventional resin cements with cultured cells without the use of an adhesive system is another limitation to be considered. Therefore, future studies should assess the cytotoxicity of resin luting cements to confirm the relevance of the present results on clinical applications.

Conclusions

From this study, the follow could be concluded:

1) The cytotoxicity levels of resin-based luting cements tested were material-dependent and decreased after 7 days preincubation.

2) Self-adhesive resin cements showed the most cytotoxic effect at 2nd day, while conventional resin cements presented immediate cytotoxicity.

3) Auto-polymerized resin specimens and especially Multilink Speed, demonstrated the most cytotoxic effect regardless of the preincubation time implying the impostance of light application in reducing the cytotoxic effect.

4) Interpositioning of 2 mm thick ceramic or resin nano-ceramic restorative material did not significantly affect the cytotoxicity of the resin-based cements.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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Ethical approval: This article does not contain any studies with living human participants or animals. Institutional approval was given.

Informed consent: Not required.

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Captions for tables and figures:

Tables:

Table 1. Brands, chemical compositions, shades, types, application protocols and group abbreviations of luting cements.

Table 2. Cell survival rates of the tested resin luting cements polymerized with different protocols with regard to the preincubation timepoints. See Table 1 for group abbreviations.

Figures:

Figures 1a-d. Comparison of cell survival rates regarding different resin-based cements polymerized with the same polymerization protocol at **a)** 0-, **b)** 1-, **c)** 2- and **d)** 7-day preincubation timepoints. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.

Figures 2a-d. Comparison of cell survival rates regarding different resin-based cements polymerized with different polymerization protocol at **a)** 0-, **b)** 1-, **c)** 2- and **d)** 7-day preincubation timepoints. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.

Figures 3a-d. Comparison of cell survival rates regarding different resin-based cements polymerized within the same preincubation timepoint. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.

Tables:

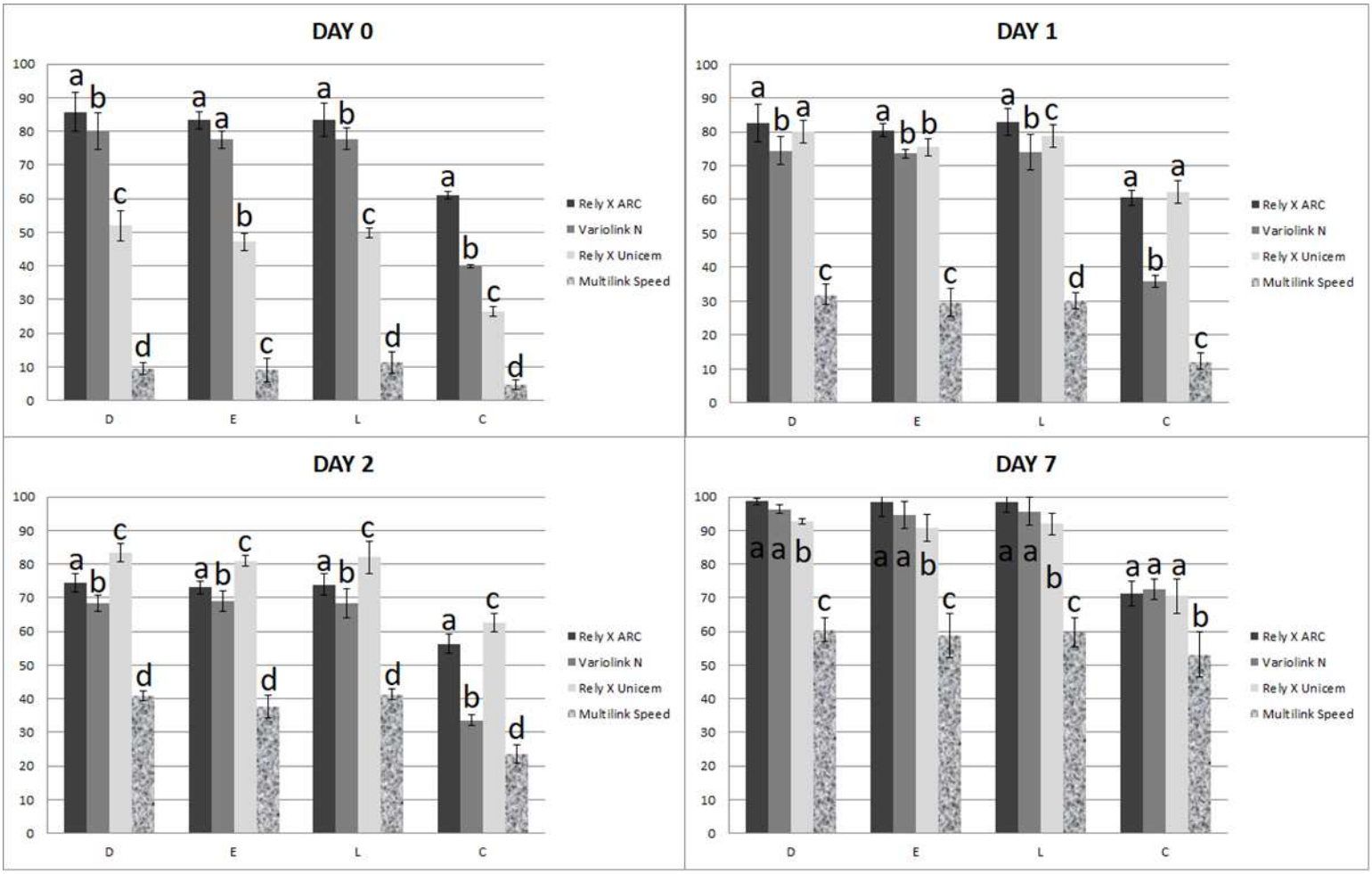
Brands	Chemical Composition	Type	Application Protocols (According to manufacturer`s instructions)	Group Abbrevia-tion
RelyX ARC (RA)	TEGDMA/bis-GMA silanated filler, functionalized dimethacrylatepolymer (Shade: Transparent)	Conventional resin composite cement	Directly photo-polymerized for 40 s Photo-polymerized through ceramic disc for 40 s Photo-polymerized through resin nano-ceramic disc for 40 s Auto-polymerized for 10 min	RAD RAE RAL RAC
Variolink N (VL)	bis-GMA, TEGDMA, UDMA, inorganic fillers, ytterbiumtrifluoride, initiators, stabilizers and pigments, Benzoylperoxide (Shade A1)	Conventional resin composite cement	Directly photo-polymerized for 10 s Photo-polymerized through glass ceramic disc for 10 s Photo-polymerized through resin nano-ceramic disc for 10 s Auto-polymerized for 10 min	VLD VLE VLL VLC
Rely X Unicem (RU)	Powder: glass powder, silica, calcium hydroxide, pigment, substituted pyrimidine, peroxycompound and initiator Liquid: methacrylatedphosphoric ester, dimethacrylate, acetate, stabilizer and initiator (Shade: Translucent)	Self-adhesive resin cement	Directly photo-polymerized for 20 s Photo-polymerized through glass ceramic disc for 20 s Photo-polymerized through resin nano-ceramic disc for 20 s Auto-polymerized for 10 min	RUD RUE RUL RUC
Multilink Speed (MS)	UDMA, TEGDMA, polyethylene glycol dimethacrylate, ytterbium trifluoride copolymer, disperse silica, glass filler, adhesive monomer, stabilizer (Shade: Transparent)	Self-adhesive resin cement	Directly photo-polymerized for 20 s Photo-polymerized through glass ceramic disc for 20 s Photo-polymerized through resin nano-ceramic disc for 20 s Auto-polymerized for 8 min	MSD MSE MSL MSC

Table 1. Brands, chemical compositions, shades, types, application protocols and group abbreviations of luting cements.

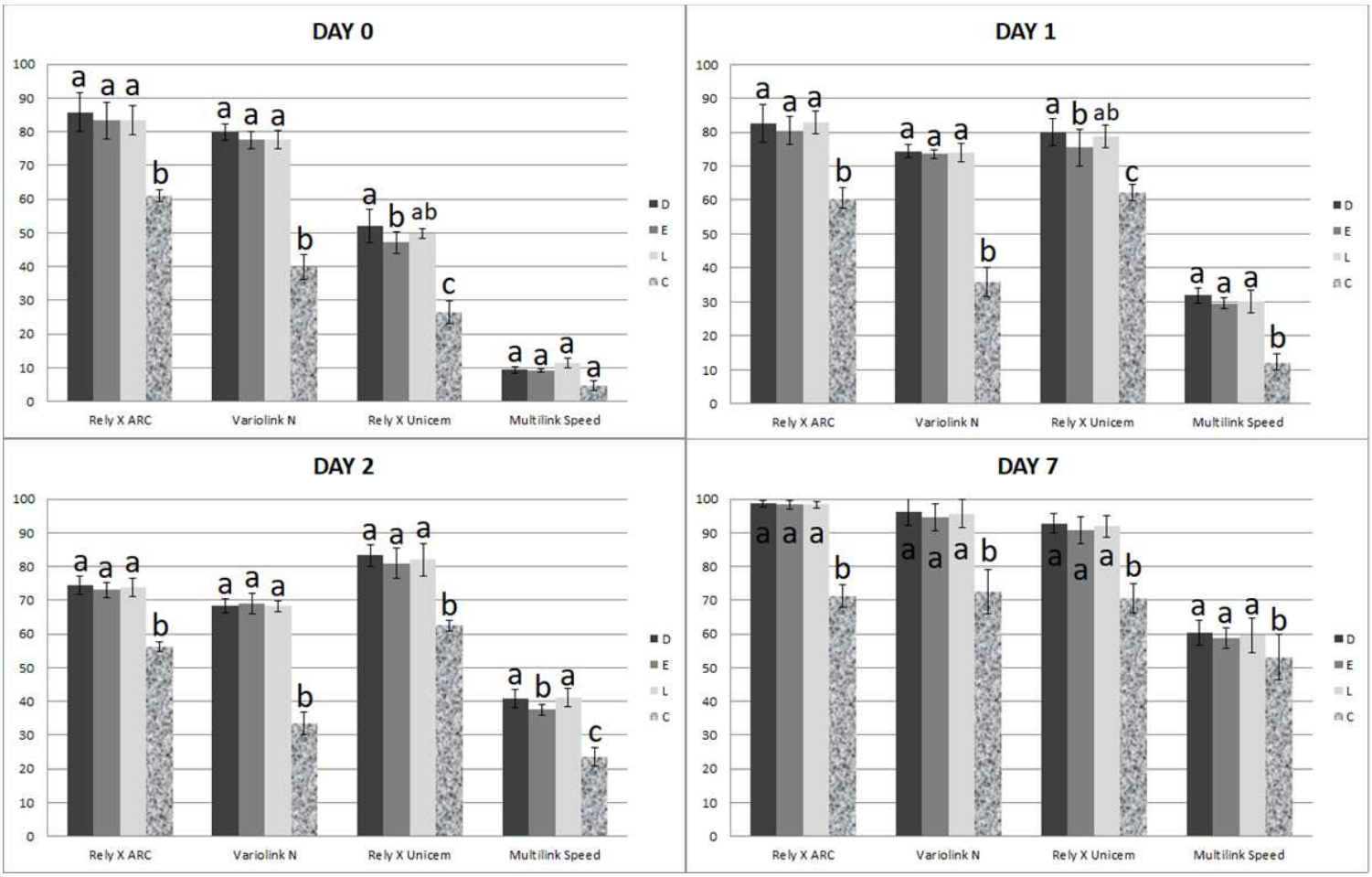
Test Groups	Polymerization Method	Preincubation Timepoints (Days)			
		0	1	2	7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RA	D	85.8 ± 5.8	82.5 ± 5.6	74.4 ± 2.8	98.6 ± 1
	E	83.3 ± 5.4	80.4 ± 4.2	73 ±2.4	98.3 ± 1.2
	L	83.3 ± 4.4	82.9 ± 3.3	73.9 ±2.8	98.3 ± 0.9
	C	61 ± 1.7	60.5 ± 3.1	56.3 ± 1.4	71.2 ± 3.4
VL	D	80 ± 2.4	74.4 ±1.9	68.3 ± 2	96.3 ±4.2
	E	77.5 ± 2.6	73.5 ± 1.2	69 ± 3	94.7 ± 4.1
	L	77.7 ± 2.7	74 ± 2.6	68.3 ±1.5	95.7 ± 4.1
	C	39.9 ± 3.5	35.8 ± 4.2	33.6 ±3.3	72.4 ± 6.6
RU	D	51.9 ± 4.9	79.9 ± 3.9	83.4 ± 3.2	92.8 ± 2.9
	E	47.1 ± 3.1	75.5 ± 5.3	81 ± 4.3	90.8 ± 4.1
	L	49.9 ± 1.5	78.8 ± 3.4	82 ± 4.7	92 ± 3.2
	C	26.4 ± 3.2	62.3 ± 2.3	62.5 ± 1.6	70.5 ± 4.3
MS	D	9.4 ± 1	31.9 ± 2.2	40.9 ± 2.8	60.5 ± 3.7
	E	9 ± 0.5	29.6 ± 1.7	37.5 ± 1.7	58.7 ± 3
	L	11.4 ± 1.5	30.2 ± 3.3	41.2 ± 2.8	59.6 ± 5.1
	C	4.7 ± 1.4	12.1 ± 2.4	23.5 ± 2.6	53.1 ± 6.7

Table 2. Cell survival rates of the tested resin luting cements polymerized with different protocols with regard to the preincubation timepoints. See Table 1 for group abbreviations.

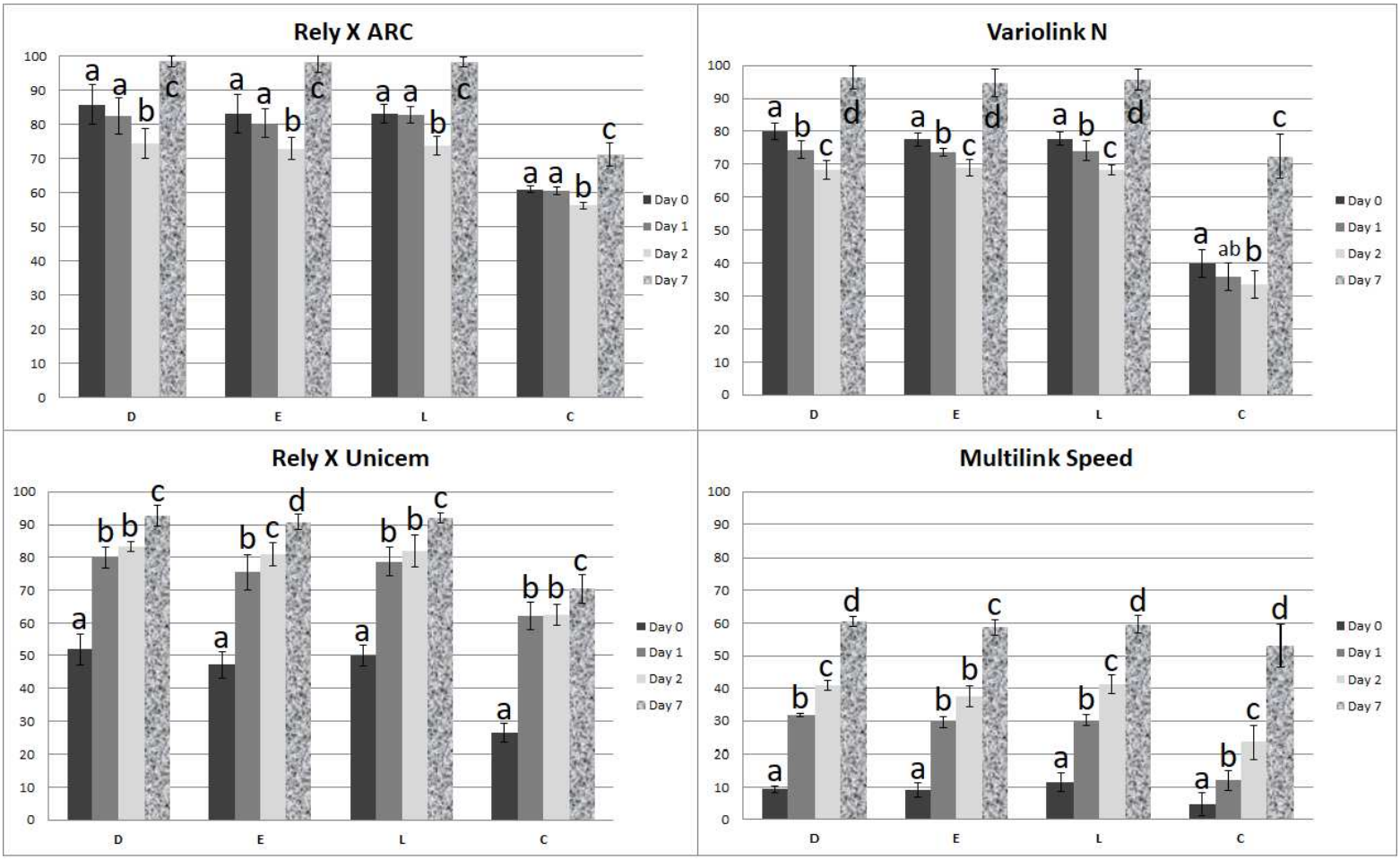
Figures:



Figures 1a-d. Comparison of cell survival rates regarding different resin-based cements polymerized with the same polymerization protocol at **a)** 0-, **b)** 1-, **c)** 2- and **d)** 7-day preincubation timepoints. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.



Figures 2a-d. Comparison of cell survival rates regarding different resin-based cements polymerized with diffrent polymerization protocol at **a)** 0-, **b)** 1-, **c)** 2- and **d)** 7-day preincubation timepoints. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.



Figures 3a-d. Comparison of cell survival rates regarding different resin-based cements polymerized within the same preincubation timepoint. *Different superscript letters represent statistically significant difference ($p<0.05$); D: Photo-polymerization directly from the top of the cement specimen; E: Photo-polymerization through lithium disilicate ceramic; L: Photo-polymerization through resin nano-ceramic disc; C: Auto-polymerization without light activation.