

## Cytotoxicity of commonly used luting cements —An *in vitro* study

Rita TRUMPAITE-VANAGIENE<sup>1</sup>, Virginija BUKELSKIENE<sup>2</sup>, Jolanta ALEKSEJUNIENE<sup>3</sup>, Alina PURIENE<sup>1</sup>,  
Daiva BALTRIUKIENE<sup>2</sup> and Vygandas RUTKUNAS<sup>1</sup>

<sup>1</sup> Institute of Odontology, Faculty of Medicine, Vilnius University, Zalgirio str. 115, 08217 Vilnius, Lithuania

<sup>2</sup> Department of Biological models, Institute of Biochemistry, Vilnius University, Mokslininku str. 12, 08662 Vilnius, Lithuania

<sup>3</sup> Department of Oral Health Sciences, Faculty of Dentistry, University of British Columbia, 2199 Westbrook Mall, Vancouver, BC V6T 1Z3, Canada  
Corresponding author, Rita TRUMPAITĖ-VANAGIENĖ; E-mail: rita.vanagiene@odontalis.lt

The study aimed to 1) evaluate the cytotoxicity of luting cements: Hoffmann's Zinc Phosphate (Hoffmann's ZP), GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus RMGI) and 3M ESPE RelyX Unicem Resin Cement (RelyX Unicem RC) and 2) test if pre-washing reduces the cements' cytotoxicity. *In vitro* human gingival fibroblast (HGF) culture model was chosen. The cytotoxicity was evaluated by MTT test, the cell viability —by staining the cells with AO/EB dye mixture. The means±SD of Cell Survival Ratio (CSR%) were compared among different cement types under two testing conditions, with or without cement pre-washing. The CSR% were compared by ANOVA and linear multiple regression (LMR). Hoffmann's ZPC was less cytotoxic, while Fuji Plus RMGI and RelyX Unicem RC were more cytotoxic (ANOVA,  $p < 0.001$ ). The type of cement and cement pre-washing jointly explained 90% of cell survival (LMR,  $p < 0.001$ , adjusted squared  $R = 0.889$ ). The commonly used luting cements such as Hoffmann's ZP, Fuji Plus RMGI and RelyX Unicem RC may have a cytotoxic potential.

**Keywords:** Dental luting cements, Cytotoxicity, Gingival fibroblasts, Cell culture, *In vitro* testing

### INTRODUCTION

The clinical success of fixed prosthodontics is influenced by many factors, including the choice of an appropriate luting material. A wide variety of luting cements is currently available from conventional water-based to contemporary adhesive resin cements. However, there is no single luting material capable of meeting all the desired requirements<sup>1</sup>. Zinc phosphate cement, one of the oldest materials still widely used, is traditionally applied as a luting agent or a temporary restorative<sup>1</sup>. Resin-modified glass ionomer cements are the latest generation of glass ionomers containing various resins that improve their mechanical properties. However, these modifications may affect their biocompatibility<sup>2</sup>. Self-adhesive resin cements based on filled polymers designed to adhere to the tooth structure without a separate adhesive or etchant have gained popularity due to their simplicity of application, improved mechanical properties and adhesion to various substrates<sup>3</sup>. A primary important consideration is the biocompatibility of dental cements, which, when in contact with oral soft tissues including the periodontium, may evoke adverse clinical reactions of a toxic and/or allergic nature<sup>4,5</sup>. Cytotoxicity may be evoked due to chemical irritation from the materials as well as to pH changes occurring in the vicinity of the materials during their setting<sup>6</sup>. Dental materials vary in their release of substances before and/or after their setting<sup>7</sup> or in their surface characteristics<sup>8</sup>. Consequently, one should expect varying biological responses with different cement types. As acidity of dental materials may contribute to cytotoxicity<sup>6</sup>, it is important to determine which luting cements produce the most acidic environments.

Given there is numerous evidence regarding the overall cytotoxicity of dental materials, it is also important to find out if simple procedures such as thorough pre-washing of cements can reduce cytotoxicity.

The present study aimed (1) to evaluate the cytotoxicity of three commonly used luting cements, namely Hoffmann's Zinc Phosphate (Hoffmann's ZP), GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus RMGI) and 3M ESPE RelyX Unicem Resin Cement (RelyX Unicem RC) as well as (2) to test if pre-washing of cements reduces their cytotoxicity.

The following null hypotheses were formulated:

- Hypothesis 1: Cell survival is comparable with and without exposure to luting cements.
- Hypothesis 2: There is no difference in cytotoxicity between different types of luting cements.
- Hypothesis 3: Luting cements do not create acidic environments on their surfaces.
- Hypothesis 4: Cell survival after exposure to luting cements is similar at primary and later stages.
- Hypothesis 5: Cell survival is similar whether cells are exposed to unwashed or pre-washed cements.

### MATERIALS AND METHODS

The present *in vitro* study was approved by the Ethics Committee of Vilnius University, Lithuania (No. 158200-11-116-28).

The *in vitro* cell culture model provides a controllable and reproducible method to study the potential toxicity of dental materials<sup>9-13</sup>. Many *in vitro* studies have

utilized fibroblast cell lines because these cells represent a common cell type in pulp and gingival tissues and because fibroblasts have reproducible growth rates<sup>9-13</sup>.

#### Preparation of specimens

Each specimen included a cement, medium and fibroblast cells except for the control specimens where cements were not added.

The following types of luting cements were tested: Hoffmann's Zinc Phosphate (Hoffmann's ZP) (Hoffmann Dental Manufaktur, Berlin, Germany), GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus RMGI) (GC Corporation, Tokyo, Japan) and 3M ESPE RelyX Unicem Resin Cement (RelyX Unicem RC) (3M ESPE). The chemical composition of these materials is presented in Table 1.

All three materials were handled and proportioned according to the manufacturers' instructions; Hoffmann's ZP, Fuji Plus RMGI were mixed manually and RelyX Unicem RC was mixed in a high-frequency mixing unit. Luting materials were placed at the bottom of 96- and a 24-well cell culture plate (Orange Scientific, Belgium) insert wells. The RelyX Unicem RC specimens were light cured from one side with a curing unit (3M ESPE Elipar FreeLight). A 96-well cell culture plate was used for cell viability tests. A 24-well cell culture plate was used for pH measurements and scanning electron microscopy (SEM).

Three groups of 24 specimens were prepared separately for each testing condition and for each of the follow-ups (1, 6 and 12 h). Each group of specimens included the following set: six specimens for Hoffmann's ZP, six specimens for Fuji Plus RMGI, six specimens for RelyX Unicem RC and six negative controls. This way, 72 specimens were obtained to test the cytotoxicity of luting cements without pre-washing and 72 specimens were obtained to test the cytotoxicity of pre-washed luting cements.

To test whether cements exhibit varying acidity on their surfaces at different time periods, the pH measurements were taken at 1, 3, 6, 12, 24 and 48 h.

#### Establishment of primary cell line (Cell culture)

Human gingival fibroblasts (HGFs) were obtained from a gingival biopsy of a healthy female patient by the explants method<sup>14</sup>. Immediately after biopsy, a sample of tissue 2–4 mm<sup>3</sup> in size was placed in Dulbecco's modified Eagle's medium (Invitrogen/Life Technologies, USA) enriched with 250 U/mL Penicillin (Invitrogen/Life Technologies, USA), 0.25 mg/mL Streptomycin (Invitrogen/Life Technologies, USA), and 200 U/mL Nystatin (Sigma-Aldrich Inc., USA) for transportation. Then, the subepithelial tissue specimen was minced under sterile conditions, the suspension diluted in Iscove's modified Dulbecco's media (IDMEM; Sigma-Aldrich Inc., USA) supplemented with 10% of foetal calf serum (FCS; Invitrogen/Life Technologies, USA) and antibiotics, and cultured at 37°C in 5% CO<sub>2</sub> and 95% relative humidity atmosphere. HGFs which grew out of the explants were sub-cultured and maintained. The cells from the passages between six and ten were used in the experiment.

#### Testing conditions

Two testing conditions were prepared by exposing fibroblasts to either pre-washed (condition 1) or to unwashed luting cements (condition 2). For the pre-washing, the copious rinsing with the saline of the cement surface prior to exposing them to fibroblasts cell suspension was employed.

#### Outcome measurements

To thoroughly assess the potential of cement's cytotoxicity, cross validation employing both quantitative and qualitative methods was used. The SEM and microscopic cell images were used to acquire qualitative

Table 1 Chemical formulations of test luting cements

Cement	Manufacturer	Chemical composition
Hoffmann's Zinc Phosphate*	Hoffmann Dental Manufaktur, Berlin, Germany	Powder: zinc oxide, magnesium oxide; Liquid: o-phosphoric acid.
GC Fuji PLUS**	GC CORPORATION, Tokyo, Japan	Conditioner: citric acid, distilled water, ferric chloride, food additive Blue no.1.; Powder: fluoro alumino-silicate glass (amorphous); Liquid: 2-hydroxyethylmethacrylate, distilled water, polyacrylic acid, urethanedimethacrylate.
RelyX Unicem***	3M ESPE, St. Paul, MN, U.S.A.	Powder: alkaline (basic) fillers, silanated fillers, initiator components, pigments; Liquid: methacrylate monomers containing phosphoric acid groups, methacrylate monomers, initiator components, stabilizers.

\*Product information;

\*\*GC Materials Safety Data List: <http://www.gcamerica.com/downloads/sds/index.php> accessed April 3, 2015.

\*\*\*Technical Product Profile RelyX Unicem: *RelyX Unicem\_TPP\_EBU\_final.pdf*

outcomes. The acidity of the specimens' vicinity and cell survival were used as quantitative outcomes.

In the SEM images, the vital fibroblasts were seen as outstretched cells indicating cell survival, while the absence of outstretched cells or rounded cells implied cells death. Another visual assessment was microscopic images of stained cells distinguishing between the vital (green) and dead cells (orange)<sup>15</sup>.

Two quantitative outcomes were used. The acidity in the vicinity of the luting cements was measured as an indicator of a potentially cytotoxic environment. The cell survival expressed as optical density (OD) indicated the proportion of vital cells.

#### Scanning electron microscopy (SEM)

The HGFs cell suspension ( $6-7 \times 10^4$  cells/mL) was placed into each well on the cement surface. HGFs maintained in polystyrene plate wells without luting cements served as control specimens. After the 6 h exposure, the specimens were prepared for the SEM examination (Hitachi, TM-1000). The cell growth medium was removed and the wells rapidly filled with the glutaraldehyde (2.5% in water; Sigma-Aldrich Inc., USA) fixative. The samples were incubated for 60 min at room temperature and rinsed three times for 15 min with distilled water. Subsequently, the samples were fixed in  $\text{OsO}_4$  (Sigma-Aldrich Inc., USA) for 60 min at room temperature, rinsed with distilled water three times for 15 min, dehydrated through an ethanol series beginning with 25% and changing to solutions of 50% and 75% over 10 min and then 96% for 2 h. Finally, the samples were dried in a critical point dryer (K850, Quorum Technologies) and coated with 20 nm of gold using a sputter coater (Q150R, Quorum Technologies).

#### Cell viability test

Half of the test specimens were washed with  $20 \times 200$   $\mu\text{L}$  saline solutions (PBS). A total of 100  $\mu\text{L}$  of the HGF cell suspension was placed onto cement specimens in the 96-well cell culture plate. After 1, 6 and 12 h, the cell monolayer was dispersed by trypsin-EDTA (Life Technologies Corp., USA) mixture (1:9), the suspension was centrifuged and the pellet suspended in 25  $\mu\text{L}$  of growth medium. Then, 2  $\mu\text{L}$  of acridine orange (AO) and ethidium bromide (EB) (Life Technologies Corp., USA) dye mix (100  $\mu\text{g}/\text{mL}$  of each was prepared in PBS and mixed in equal proportions) was added to 10  $\mu\text{L}$  of test cell suspension. AO is cell-permeable and it interacts with DNA and RNA. When bound to DNA, viable cell nuclei look like well-formed round fluorescent green structures. EB intercalates DNA, providing a fluorescent red-orange stain. EB does not stain healthy cells, thus it is used to identify cells that have permeable membranes. This EB/AO combined stain enables to distinguish between the vital (green color) and dead, *i.e.* apoptotic and necrotic cells (orange color)<sup>15</sup>. The images were viewed using an Eclipse TS100 (Nikon) inverted microscope. The pictures were taken with a digital camera.

#### pH measurements

Half of the test specimens were washed 20 times with 1,000  $\mu\text{L}$  of PBS. Then, 500  $\mu\text{L}$  of cell growth medium (IDMEM) was poured over every cement specimen as well as into control wells. The pH measurements were taken at 1, 3, 6, 12, 24 and 48 h using pH-meter Knick, type 766 Calimatic.

#### Assessment of cytotoxicity (cell survival)

Half of the test specimens were pre-washed with  $20 \times 200$   $\mu\text{L}$  saline solution (PBS). All specimens were sterilized with ultraviolet (UV) light for 15 min. The HGF cell suspension ( $6-7 \times 10^4$  cells/mL) was prepared and 100  $\mu\text{L}$  of it was placed onto cement specimens. Then, the cells were incubated for 1, 6 and 12 h. The HGF, maintained in the control polystyrene plate wells without luting cements, served as controls.

The toxic effect of luting cements on the HGF cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich Inc., USA) in a direct-contact format according to the ISO-10993-5:1999 specifications<sup>16</sup>. After exposure to the materials for 1, 6 and 12 h, the number of vital HGF cells was detected using the MTT test. The MTT test is based on the ability of a vital cell's mitochondrial succinate dehydrogenase to reduce the yellow MTT dye into insoluble blue formazan crystals. The amount of formazan is directly proportional to the number of vital cells. After adding the solvent, the optical density (OD) of the resulting solution was measured with a microtiter plate reader (TECAN Infinite 200 PRO) at 570 nm. The OD value indicates the proportion of vital cells. Based on the optical density comparing test ( $\text{OD}_{\text{test}}$ ) and control specimens ( $\text{OD}_{\text{control}}$ ), the cell survival ratio % (CSR%) was calculated. The CSR% is a percent ratio, where cell survival in wells with cement and fibroblasts is expressed as the percent of cell survival in a control well containing only fibroblasts<sup>17</sup>. A higher CSR% value indicates a higher cell survival, concomitantly less cytotoxicity.

#### Statistical analysis

Univariate analyses were employed to calculate the means and dispersion of CSR%. As CSR% did not present skewed distributions, only parametric tests were employed in both bivariate and multivariate analyses. The bivariate analyses (one way ANOVA with Post Hoc Bonferroni adjustment) were used to compare CSR% among the three different luting cements and under two different testing conditions. The linear multiple regression was used to test the combined effect of cement type and washing. The threshold for significance for all tests was set at  $p < 0.05$ .

## RESULTS

#### Qualitative findings

##### 1. SEM assessments of cell survival

Figure 1 illustrates an example of SEM images taken after 6 h of human gingival fibroblast (HGF) exposure to luting cements in the cell growth medium. One can

see that outstretched cells were found only on the ZPC surface (Fig. 1A) while the SEM image of a control shows many fibroblasts (Fig. 1B).

2. Cell viability test

Cell survival was evaluated qualitatively under two testing conditions (pre-washed vs unwashed cements). The overall finding was that pre-washing of the cements

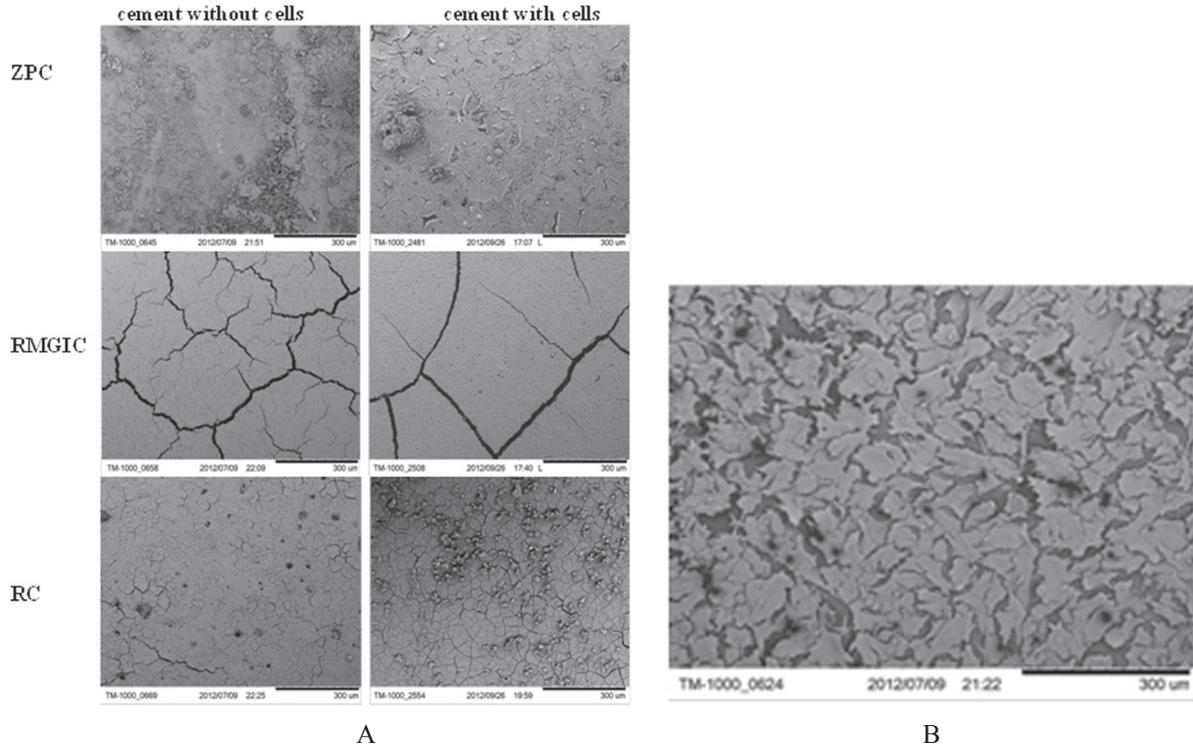


Fig. 1 SEM view (6th h follow-up). A: ZPC (Hoffmann’s Zinc Phosphate Cement), RMGIC (Fuji Plus Resin Modified Glass Ionimer), RC (RelyX Unicem RC) specimens without and with HGFs (human gingival fibroblasts) B: Control (HGFs without cement)

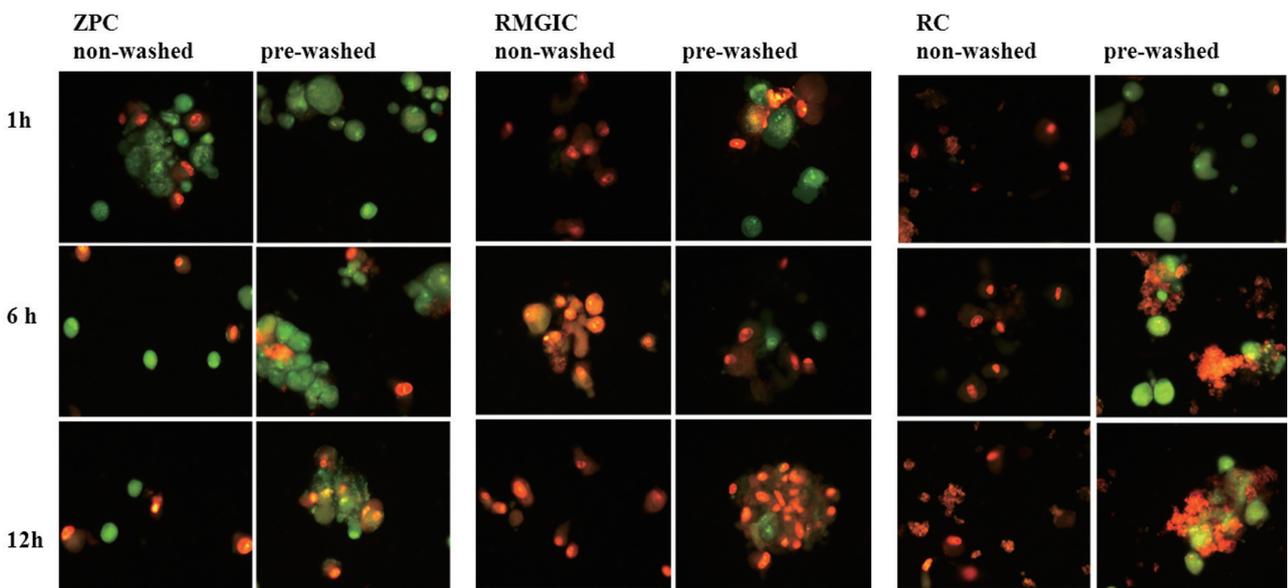


Fig. 2 HGFs maintained 1, 6 and 12 h on the ZPC, RMGIC and RC non-washed and pre-washed surfaces, collected by using trypsin /EDTA solution, then suspended in growth medium and stained with AO/EB stain solution.

had some protective effect, particularly at the early stages of observation. As it can be observed in Fig. 2, more vital cells (green) are seen in the images of pre-washed samples than in unwashed samples. However, after 12 h incubation, dead (red) cells were dominant in almost all samples.

*Quantitative findings*

Significance of pH

The acidity on cement surfaces at different follow-ups is presented in Fig. 3. As one can see, all the cements tested created acidic environments on their surfaces. The pH

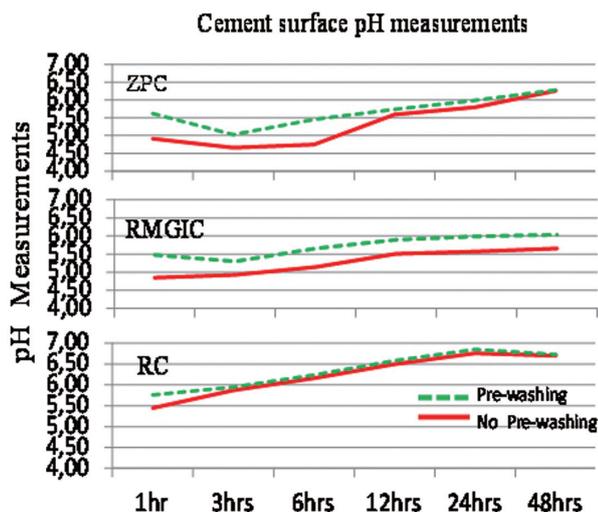


Fig. 3 Cement surface pH measurements. ZPC: Zinc phosphate, RMGIC: Resin modified glass ionomer cement, RC: Resin cement.

values at the vicinity of the cements were below neutral (pH=7.0) throughout the whole 24-h observation. As an overall pattern, the pH values on the cement surfaces were lower in earlier observations (e.g. 1 h and 3 h) than in later follow-ups. The pH started to return towards neutral at approximately the 3 h observation.

*Quantitative analyses of cell survival*

Bivariate analyses

The percentage cell survival ratio (CSR%) was used to quantitatively assess cell survival on luting cements. Table 2 presents the results of multiple comparisons demonstrating the cytotoxicity as a function of three factors: type of cement (1), time after exposure to cements (2) and pre-washing of cements (3). Pre-washing as compared to no pre-washing contributed to higher numbers of surviving cells. Statistically significant differences were observed among different types of luting cements in the unwashed cement group only at a 1 h follow-up. In the pre-washed cement group there were consistent statistically significant differences among the different types of luting cements at different follow-up times (1, 6 and 12 h).

Three trends can be identified. Firstly, all three luting cements, Zinc Phosphate Cement (ZPC), Resin Modified Glass Ionomer Cement (RMGIC) and Resin Cement (RC) presented substantial decreases of cell survival. For example, a 1-h observation showed that without pre-washing, only a relatively small percentage of fibroblasts survived in the cement cultures; 15.4%, 1.6% and 1.2% for the ZPC, RMGIC and RC test specimens, correspondingly. The second trend was that cytotoxicity differed depending on the type of cement studied, where a lower cytotoxicity was observed for the ZPC and a higher cytotoxicity was observed for RMGIC

Table 2 The cytotoxicity of luting cements with or without pre-washing

Cement type & Condition	Follow-up			Significance
	1 h	6 h	12 h	
	mean±SD#	mean±SD#	mean±SD#	
<b>No pre-washing</b>				
ZPC*	15.4±9.5	11.2±12.6	9.8±6.9	p=0.047
RMGIC*	1.6±1.7	1.3±1.4	3.6±4.0	p=0.278
RC*	1.2±1.4	5.9±6.8	10.1±6.0	p=0.035
Significance	p<0.001	p=0.153	p=0.129	—
<b>With pre-washing</b>				
ZPC*	48.7±8.1	23.4±11.9	20.0±5.4	p<0.001
RMGIC*	21.5±4.4	3.4±2.3	8.3±6.4	p<0.001
RC*	6.4±3.3	14.2±6.2	8.7±10.2	p=0.109
Significance	p<0.001	p=0.002	p=0.028	—

and RC. Thirdly, pre-washing of the luting cements had a positive effect on cell survival *i.e.* the overall cell survival after exposure to the pre-washed luting cements was higher compared to the cell survival in the non-washed cement groups. For example, there was a statistically significant difference in cell survival when cells were exposed to a pre-washed ZPC, where around 40% of cells survived in the vicinity of a pre-washed ZPC as compared to only 15% of surviving cells where no pre-washing of ZPC was employed.

Multivariate analyses

To study the joint effect of aforementioned factors, multivariate statistics was employed (Table 3). Linear multiple regression analysis estimated the magnitude of cytotoxicity taking into consideration two factors: the type of cement and the effect of pre-washing. In order to enable the simultaneous inclusion of information about cements into linear multiple regression analysis, three dummy variables were created, one for each luting cement. To ensure that the inclusion of three dummy variables did not violate the important assumption of independence among predictors, collinearity testing was employed (Table 2). The collinearity testing showed that the assumption of independence was fulfilled as tolerance of individual predictors was relatively high and variance inflation values low.

The linear multiple regression (LMR) model was highly statistically significant ( $p < 0.001$ ) and three out of four individual predictors were statistically significant ( $p < 0.05$ ). Jointly, the type of cement and washing procedure explained 90% (LMR, adjusted R Square=0.898) of the variation in cell survival (CSR%). The ranking of tested cements according to their decreasing cytotoxicity was as follows: RC, followed by RMGIC and ZPC. For the cell survival, pre-washing had the strongest independent positive effect (LMR,  $\beta = 0.599$ ,  $p < 0.001$ ). The type of cement was also important: ZPC showed higher levels of cell survival as compared to the two other cements ( $\beta = 0.543$ ;  $p < 0.001$ ), which indicated that ZPC cement was

substantially less toxic than either RMGIC or RC luting cement. Concomitantly, the RC as compared to the two other cements and when controlled for pre-washing still had an independent statistically significant negative effect on cell viability ( $\beta = -0.169$ ,  $p < 0.001$ ).

Figure 4 visualizes the main findings of the present experiment. Each of the cements are represented with two box-plots, one to demonstrate the cytotoxicity of a non-washed cement and one to illustrate how cytotoxicity is reduced when a cement is pre-washed prior to its exposure to fibroblasts.

There was a clear trend that pre-washing improved cell survival. The highest variation and the most considerable improvement of pre-washing were observed for the ZPC.

DISCUSSION

To thoroughly assess the potential cytotoxicity of

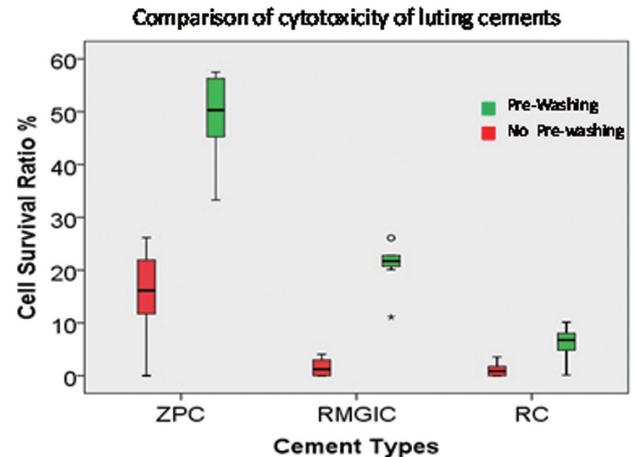


Fig. 4 Comparison of cytotoxicity of luting cements. ZPC: Zinc phosphate, RMGIC: Resin modified glass ionomer cement, RC: Resin cement.

Table 3 The cytotoxicity as a function of luting cement type and exposure to pre-washing

Predictors	Outcome: % Cell Survival Ratio					Collinearity Statistics	
	Unstandardized Coefficients	Standardized Coefficients	<i>t</i>	Sig.	Tolerance	VIF#	
	B	Beta					
ZPC vs. RMGIC & RC*	23.657	0.543	9.291	0.000	0.667	1.500	
RMGIC vs. ZPC & RC*	1.813	0.042	0.712	0.481	0.667	1.500	
RC vs. RMGIC & ZPC*	-7.384	-0.169	-2.900	0.006	0.667	1.500	
PRE-WASHING	19.476	0.599	7.949	0.000	0.400	2.500	

Model Summary: Method: enter, Adjusted R Square=0.898;  $p < 0.001$

VIF# Variance Inflation Factor

ZPC\* Hoffmann’s Zinc Phosphate Cement, RMGIC\* Fuji Plus RMGI, RC\*RelyX Unicem RC.

commonly used luting cements, the cross validation employing both quantitative and qualitative assessments was chosen in our *in vitro* study. Three commercial brands representing three types of luting cements were selected and the following cements were tested: 1) Hoffmann's Zinc Phosphate (Hoffmann's ZP), 2) GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus RMGI) and 3) 3M ESPE RelyX Unicem Resin Cement (RelyX Unicem RC). The cytotoxicity of these luting cements was evaluated as a function of three factors: cement type, pre-washing and time. Our findings support the importance of all these factors. Based on our findings, all null hypotheses had to be rejected. Therefore, we suggest alternative hypotheses:

Hypothesis 1: Hoffmann's Zinc Phosphate, GC Fuji Plus Resin Modified Glass Ionomer and 3M ESPE RelyX Unicem Resin Cement have cytotoxic potential.

Hypothesis 2: The severity of cytotoxicity of luting cements is type-dependent.

Hypothesis 3: All luting cements tested create acidic environments potentially contributing to cytotoxicity.

Hypothesis 4: Cytotoxicity of luting cements is time dependent and is more expressed at primary stages.

Hypothesis 5: Pre-washing of cements reduces cytotoxicity.

The findings will be further discussed in more detail.

The present experiment has demonstrated substantial cytotoxicity for all luting cements tested, namely Hoffmann's ZP, Fuji Plus RMGI and RelyX Unicem RC. This inference is based on the observation that survival of human gingival fibroblasts in all cement-containing specimens was substantially lower than in control specimens without a presence of luting cements. This finding is in accordance with other *in vitro* experiments reporting that a number of dental materials exhibit cytotoxic effects and that cytotoxicity varies among the brands of dental materials<sup>4,5,9-12,18-21</sup>.

The reasons for cytotoxicity might be several. Cytotoxicity of phosphate cements has been attributed to the release of zinc ions and other chemical substances<sup>22</sup>. Similarly, it has been suggested that GICs, which released fluoride in high quantities, were highly toxic to human dental pulp stem cells<sup>23</sup>. Contrarily, Stanislawski *et al.* reported that the concentration of  $F^-$ ,  $Sr^{2+}$  and  $Al^{3+}$  in the elutes of GICs was too low to be cytotoxic and suggested that the principal compounds responsible for cytotoxicity of RMGICs are unpolymerized resin monomers<sup>2</sup>. Furthermore, Goldberg reported that the mechanisms of cytotoxicity relate firstly to the short-term release of free monomers occurring during the monomer-polymer conversion and, secondly, to long-term release of leachable substances generated by erosion and degradation over time<sup>24</sup>. Early cytotoxic effects of luting cements could also be attributed to the acidity of the materials<sup>25,26</sup>. This initial acidity, involving a prolonged period of acidic pH, acid diffusions from luting cements

through dentine coupled with cytotoxicity of chemicals, may lead to damaging effects particularly when tooth preparation and cementation procedures are less than ideal<sup>6,25,26</sup>.

It has been demonstrated that even low concentrations of 2-hydroxyethyl methacrylate (HEMA) can significantly alter the morphology of human gingival fibroblasts<sup>27</sup> and primary pulp cells<sup>28</sup>. Comparable observations were reported by Becher and co-authors: extracts of some compomers as well as monomers TEGMA, HEMA and GDMA have the capacity to induce cell death in macrophages *in vitro*<sup>29</sup>.

It is important to consider that insufficient curing or long setting time of materials may increase cytotoxicity. For example, dual-cured specimens of resin-based cements showed significantly lower toxicity than chemically cured specimens<sup>4</sup>. Thus, the importance of a setting mode as well as its timing should not be underestimated for the cytocompatibility of resin-based materials<sup>5,30</sup>.

The present experiment also found that cytotoxicity of luting cements was highest at the first hours after setting. This trend toward a decreasing cytotoxicity over time has been also observed in previous investigations<sup>9,11,12</sup> and were attributed to the reduced leakage as the most of the leachable components are released within the first hours<sup>7</sup>. Our experiment has also demonstrated that pre-washing of cements helps cells to survive in the vicinity of luting cements. We can speculate that this positive effect may be explained by the fact that substantial amounts of leaking substances were washed away.

The promising trend is that new materials are under development and several new formulations claim to reduce unpolymerized residual mass<sup>31-34</sup>. Although our findings clearly point to substantial cytotoxicity of luting cements, it is important to acknowledge the limited nature of our *in vitro* experiment. The biological biocompatibility of these commonly used luting cements will be further explored in an experiment where the genotoxicity of the same luting cements will be tested *in vitro*.

Given the inherent limitation of the *in vitro* experiment, the clinical relevance of the present findings can only be speculated. In contrast to the present experiment, oral environment contains a number of protective mechanisms that could counterbalance the cytotoxic effects experienced due to presence of luting cements particularly during their setting stage. Concomitantly, dental clinicians should be aware of the potential cytotoxicity, consequently handle luting cements with caution and follow the handling instructions with an utmost care. Further clinical follow-up studies are needed to answer the question if the cytotoxicity observed in the present *in vitro* experiment also has clinical implications.

## CONCLUSION

The present *in vitro* testing shows that three of the

most common types of luting cements exhibit potential cytotoxicity. The pH values at the vicinity of the cements were below neutral (pH=7.0) throughout the whole 24-h observation. Among the luting cements studied, Hoffmann's ZP cement indicated less cytotoxicity than Fuji Plus RMGI or and RelyX Unicem RC. Pre-washing of luting cements reduced the cytotoxicity albeit not to the levels of the controls where there was no exposure to luting cements.

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