Archives of Oral Biology 86 (2018) 108–115

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

# Live cell imaging reveals different modes of cytotoxic action of extracts derived from commonly used luting cements

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ARTICLE INFO

*Keywords:*
Luting cements
Gingival fibroblasts
Cytotoxicity
Live-cell imaging

ABSTRACT

*Objective:* To compare cytotoxicity of extracts derived from commonly used luting cements: Hoffmann's Zinc Phosphate (ZPC), GC Fuji Plus Resin Modified Glass Ionomer (RMGIC) and 3 M ESPE RelyX Unicem Resin Cement (RC) on primary human gingival fibroblasts (HGFs).

*Design:* HGFs were exposed to different concentrations of the ZPC, RMGIC and RC extracts. The cytotoxicity was assessed with the PrestoBlue Cell Viability Reagent and viable cells were counted by a haemocytometer using the trypan blue exclusion test. In order to determine the primary mechanism of the cell death induced by extracts from different luting cements, the real-time monitoring of caspase-3/-7 activity and membrane integrity of cells was employed.

*Results:* The extracts from the RMGIC and ZPC decreased the metabolic activity and numbers of viable cells. Unexpectedly, the extracts from the RC evoked only small effects on the metabolic activity of HGFs with a decreasing number of viable cells in a dose-and time-dependent manner. The live cell imaging revealed that the apoptosis was the primary mechanism of a cell death induced by the extracts derived from the RMGIC, whereas the extracts from the RC and ZPC induced a cell death through a necrotic and caspase-independent pathway.

*Conclusions:* The apoptosis was the primary mechanism of the cell death induced by the extracts derived from the RMGIC, whereas the extracts from the RC and ZPC induced a cell death via a necrotic pathway. We suggest that metabolic assays commonly used to assess the cytotoxicity of luting cements should be validated by alternative methods.

## 1. Introduction

Glass ionomer cements (GIC) and resin-modified GICs (RMGIC) are widely used in the modern clinical dentistry as luting agents. Despite the recent improvements of resin-based restorative materials, the biocompatibility still represents an important problem (Lad, Kamath, Tarale, & Kusugal, 2014). Unreacted components of resin-based materials are released into an oral cavity due to the incomplete polymerization and therefore, they are cytotoxic and genotoxic (Schweikl, Spagnuolo, & Schmalz, 2006). These elutable substances represent residual monomers such as 2-hydroxyethylmethacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), bisphenol A-glycidyl methacrylate (Bis-GMA), as well as other components, such as initiating substances (Schweikl et al., 2006). The cytotoxic properties of different resin monomers depend on the chemical structure and concentration (Issa, Watts, Brunton, Waters, & Duxbury, 2004). Thus, more lipophilic monomers are usually more cytotoxic. Lower concentrations of monomers suppress the metabolic activity of cells by blocking the activity of mitochondrial dehydrogenases, while at higher concentrations these monomers interact with phospholipid bilayers by altering an integrity and increasing the permeability of cellular membranes (Schuster, Caughman, Rueggeberg, Lefebvre, & Cibirka, 1999). Several studies demonstrated that resin monomers induced an oxidative stress primarily via a depletion of intracellular levels of glutathione (Engelmann et al., 2004) (Volk, Engelmann, Leyhausen, & Geurtsen, 2006). Furthermore, the cytotoxic effects were inhibited in a presence of reactive oxygen species (ROS) scavengers like N-acetylcysteine, ascorbate, or vitamin E (Spagnuolo et al., 2006). However, the exact mechanism by which resin monomers induce the generation of ROS in cells is not yet clear. Another study demonstrated, that HEMA/Bis-GMA induced DNA double strand breaks through, at least in part, oxidative mechanisms (Blasiak et al., 2012). Furthermore, methacrylic acid, a product of

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https://doi.org/10.1016/j.archoralbio.2017.11.011
Received 1 January 2017; Received in revised form 19 November 2017; Accepted 26 November 2017

HEMA degradation also displayed genotoxic effects (Szczepanska et al., 2012). It should be noted, however, that dental resins release a complex mixture of different methacrylates, therefore it is difficult to precisely evaluate their cytotoxic mechanisms.

Apoptosis and necrosis represent two major mechanisms of cell death and both are involved in the cytotoxic action of resin monomers. Although initially considered as mutually exclusive states, the apoptosis and necrosis are frequently regulated by similar signalling pathways, thus they may act in a complementary fashion (Nikoletopoulou, Markaki, Palikaras, & Tavernarakis, 2013). It is also important to discriminate between the caspase-independent primary necrosis and the post-apoptotic secondary necrosis. The latter occurs when the late apoptotic cells lose the membrane integrity and release the cytoplasmic contents into the culture medium. Importantly, the appearance of some apoptotic markers such as active executioner caspases 3/7 may be transient and detectable only within a limited window of time. Therefore, methods allowing constant and long-term monitoring of cellular responses may increase our understanding about the dynamics and primary mechanisms of cell death induced by resin monomers. Live-cell imaging enables a real-time examination of dynamic processes in living cells under normal and experimental conditions. This method allows a detection of early changes in morphology, migration and heterogeneity of cells in response to the cytotoxic substances. Novel live cell-permeable fluorogenic substrates enable a real-time monitoring of caspase-3/-7 activity in cell cultures helping to determine if and when apoptosis occurs (Shi et al., 2012). At the same time the membrane integrity of cells can be monitored by using cell-impermeable fluorescent dyes such as propidium iodide (PI), or 7-aminoactinomycin D (7-AAD) allowing the distinction between necrosis, apoptosis and secondary necrosis. Thus, the combination of live-cell imaging together with other commonly used cytotoxicity assays such as tetrazolium, or resazurin reduction tests, may provide additional important information about cytotoxic properties of different dental resin monomers.

In the present study we compared the cytotoxic effects of extracts derived from three commonly used luting cements: Hoffmann's Zinc Phosphate (Hoffmann's **ZPC**), GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus **RMGIC**) and 3 M ESPE RelyX Unicem Resin Cement (RelyX Unicem **RC**). For this purpose, we continuously analysed and compared changes in the metabolic activity and cell viability. The live cell imaging was employed for the detection of dynamic changes of cellular morphology. In addition, a simultaneous real-time monitoring of caspase-3/-7 activity and membrane integrity of cells was performed in order to determine the primary mechanism of cell death induced by the extracts derived from different luting cements.

## 2. Material and methods

### 2.1. Preparation of extracts from luting cements

The following luting cements were tested: Hoffmann's Zinc Phosphate (Hoffmann's **ZPC**) (Hoffmann Dental Manufaktur), GC Fuji Plus Resin Modified Glass Ionomer (Fuji Plus **RMGIC**) (GC Corporation, Tokyo 174-8585, Japan) and 3 M ESPE RelyX Unicem Resin Cement (RelyX Unicem **RC**). All three materials were handled and proportioned according to the manufacturer's instructions. Hoffmann's ZP, Fuji Plus RMGI were mixed manually and RelyX Unicem RC in a high-frequency mixing unit. Luting materials were placed at a bottom of a 12-well cell culture plate (Orange Scientific, Braine-l'Alleud, Belgium). Then, RelyX Unicem RC specimens were light cured from one side with a curing unit (3 M ESPE Elipar FreeLight). The amount of luting cement used to cover the bottom of each well and to get 3 ml of extract was comparable to the amount needed to cement three single crowns in a routine clinical practice. The chemical composition of luting cement materials is presented in Table 1.

The prepared luting cements were sterilized with an ultraviolet (UV) light for 1 h. Then, each well with a test specimen was covered with a 3 ml cell culture medium (low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine all from Biochrom, Berlin, Germany) and incubated for 1 h in a humidified atmosphere of 95% air/5% $CO_2$ at 37 °C. After the incubation, the extracts were collected and filtered through 0.2 μm filter (Sarstedt, Nümbrecht, Germany). For the cytotoxicity studies we used 100% and half-diluted extracts (dilution with a cell culture medium, a final concentration of FBS-10%).

### 2.2. The establishment and culturing of the human gingival fibroblast cell line

Primary human gingival fibroblast (HGF) cells were derived from a healthy gingival tissue obtained from a 21 years-old Caucasian female with a written informed consent undergoing a canine tooth exposure surgery because of orthodontic treatment. Material was collected under the approval of Lithuanian Bioethics Committee. Primary HGFs were isolated using an explant outgrowth method. Using surgical blades the gingival tissues were dissected from a gingival epithelium and cut into three pieces (3–4 $mm^3$ in size), then placed into 35 mm diameter culture dishes (Orange Scientific) and cultured in a cell culture medium (see above). Explant cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$ with changing the medium routinely twice a week. For the passaging, the cells were washed 3 times with PBS, harvested with a 0.25% trypsin/1 mM EDTA solution (Gibco, Life technologies), suspended into the culture medium and plated onto the cell culture flasks for expansion. The HGFs from the 4th, 5th and 6th passages were used for the experiments.

### 2.3. Assessment of metabolic activity and counting of viable cells

The HGF cells were seeded at a density of $2 \times 10^3$ cells into each well of a 96-well plate (Orange Scientific) and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. Then, the culture medium was removed and the cells were exposed to 200 μl extracts of different cements (ZPC, RMGIC, RC) at a concentration of 100% and 50%, or to a fresh culture medium (control group).

The cytotoxicity was then assessed with PrestoBlue Cell Viability Reagent (Life technologies) according to the manufacturer's protocol. Fluorescence was detected with the Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at the excitation of 544 nm and emission of 620 nm. The fluorescence intensity was examined after 1, 6, 12 and 24 h after the incubation with extracts from different luting cements.

In parallel, under the identical conditions, viable cells were counted by the haemocytometer (Fast-Read 102, Biosigma) using the trypan blue (Sigma, St Louis, MO) exclusion test.

The measurements were carried out in triplicate for the two independent experiments. The data were normally distributed. All of the samples passed the Anderson-Darling normality test ($p > 0.05$). Results were statistically analysed by the two-way ANOVA test using the MaxStat Pro Statistics Software (Version 3.6).

### 2.4. Live cell imaging

For the confocal microscopy imaging, the HGF cells were seeded on the twenty-four well plates (Greiner Bio-One, Frickenhausen, Germany) at a density of $10^4$ cells per well and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. Then, the culture medium was removed and 600 μl of extracts (100%, or half-diluted) derived from different luting cements (ZPC, RMGIC, RC) were added into the wells. The same amount of a fresh culture medium was used for the control group.

The real-time monitoring of apoptosis and the assessment of the membrane integrity of cells was performed using the CellEvent™ Caspase-3/7 green detection reagent for apoptosis (Life technologies)