FLUORIDE RELEASE FROM GLASS IONOMER CEMENTS CORRELATES WITH THE NECROTIC DEATH OF HUMAN DENTAL PULP STEM CELLS

Tatjana Kanjevac[1], Marija Milovanovic[2], Vladislav Volarevic[2], Nebojsa Arsenijevic[2],
[1]Department for Preventive and Pediatric Dentistry, Faculty of Medicine, University of Kragujevac,
[2] Centre for Molecular Medicine and Stem Cell Research, Faculty of Medicine University of Kragujevac

ABSTRACT

Glass ionomer cements (GICs) are commonly used as restorative materials. The effect of GICs on different cell types varies. Stem cells from Human Exfoliated Deciduous teeth, SHED are a source for dental tissue regeneration. The necrosis and inflammation that eventually follows necrosis can disturb this regenerative process.

We tested seven GICs including Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji triage and Vitrebond for their necrotic induction potential in human SHEDs. We also correlated these effects with eluate fluoride release. The toxicity of GICs was tested via a lactate dehydrogenase assay and flow cyto-metric analysis of propidium iodide and Annexin V stained cells. The concentration of fluoride was measured by HPLC. The Fuji I and Fuji II GICs had a significantly lower cytotoxic effect on SHEDs compared to other tested GICs, as evaluated by the LDH assay. The results obtained from the flow cytometric analyses were similar. The Fuji I and Fuji II eluates released the lowest concentrations of fluoride and induced the lowest percentages of SHED death. Fluoride release correlated with GIC cytotoxicity.

Keywords: glass ionomer cements, cytotoxicity, fluoride, Stem cells from Human Exfoliated Deciduous teeth

INTRODUCTION

Pulp has an important role in the formation of dentin. Dentin formation begins when dental pulp mesenchymal stem cells differentiate into odontoblasts and start the deposition of collagen matrix and subsequent mineralisation [1]. Dentin formation continues through life due to tooth aging, as well as in response to physical and/or chemical injuries [2]. The main goal of restorative dentistry is to restore teeth using adequate treatments that will protect pulp function. To avoid any additional damage to pulp tissue during operative procedures caused by the toxicity of restorative materials or the penetration of bacteria, several layers of a specific material between the restorative material and the dental tissue must be applied [3, 4]. Calcium hydroxide-based products, adhesive systems and glass ionomer cements (GICs) are typically used for this purpose. GICs were introduced by Wilson and Kent in 1971 as a mixture of a calcium or strontium alumino-fluoro-silicate glass powder (base) and a water-soluble polymer (acid) [5]. Several variations of glass-ionomer materials were subsequently developed. Later variants of GICs demonstrated enhanced flexural strength, diametral tensile strength, elastic modulus and wear resistance, but their main disadvantage is higher cytotoxicity in comparison with conventional GICs. The responses to GICs differ by cell type. Thus, it is important to evaluate the cytoxicity of GICs to SHEDs [6-9].

It has been suggested that the pattern of cell death pattern could be an important method of evaluating the irritation potential of dental materials. Apoptotic cells are removed by phagocytosis and with little inflammatory response, in contrast to the inflammation and injury to the surrounding tissues induced by the necrotic process [10]. As dental pulp stem cells are the main source for dental tissue regeneration, it is important to evaluate the potential of GICs to induce necrosis of SHEDs and subsequent inflammation in the surrounding tissue. We evaluated the potential of seven commonly used biomaterials, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji Plus, Fuji Triage and Vitrebond, to the induce necrosis of human SHEDs.

Correspondence to: Nebojša Arsenijević, MD, PhD, Centre for Molecular Medicine and Stem Cell Research,
Faculty of Medicine University of Kragujevac, 69 Svetozara Markovica Street, 34 000 Kragujevac, Serbia
phone: +381 34 306800; fax: +381 34 306800 ext 116; e-mail: arne@medf.kg.ac.rs

MATERIALS AND METHODS

Cell culture

Human SHEDs were purchesed from AllCells (Emeryville, California USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 IU/mL penicillin G and 100 µg/mL streptomycin (Sigma-Aldrich Chemical, Munich, Germany). SHEDs passaged 6 times were used throughout these experiments.

Tested materials

The cytotoxic effects of seven glass ionomer cements, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji Triage (GC America, Alsip, IL, USA) and Vitrebond (3M ESPE, London, UK), were tested in this study. Separate GIC samples were prepared according to the manufacturers' directions at room temperature and then placed into open plastic rings 5 mm in diameter by 2 mm deep. After consolidation, the samples were removed from rings and dry heat sterilised for 1 hour at 170 °C. The samples were then incubated in complete culture medium (150 µL per sample) for 72 hours at the 37°C and in a 5% CO_2 atmosphere. The sample dimensions and immersion conditions were chosen to approximate the GIC mass and the dentin-exposed surface area typically used in restorative dentistry patient procedures. The medium exposed to each GIC sample was used for testing in a cell-culture system.

Evaluation of toxicity using the LDH assay

The cytotoxicity of GICs was examined via a *Cytotoxicity Detection Kit* (LDH) (Roche Applied Science). SHEDs were diluted with DMEM medium to 1 x 10^5 cells/mL, and aliquots (1 x 10^4 cells/100 µL) were placed in individual wells in 96-well plates. The next day, the media were exchanged with media exposed to a GIC mixed with fresh medium in a 1:1 ratio to a final volume of 100 µL. Each eluate was tested in triplicate. Two groups of control wells were prepared: low control (medium was exchanged with fresh medium) and high control (medium was exchanged with medium containing 1% Triton X). The cells were incubated at 37°C in a 5% CO_2 incubator for 24 h. After treatment, the supernatant (100 µL) was transferred to a new plate and incubated with an equivalent volume of substrate solution. After incubating the plates for 30 minutes at RT, 50 µl/well of stop solution was added, and the plates were spectrophotometrically examined at 450 nm. The percentage of dead cells was calculated using the formula:

% of dead cells=
(exp. value-low control)/(high control-low control) x 100

Apoptosis assay

SHEDs exposed to GIC eluates were examined by flow cytometry using Annexin V FITC (BD Pharmingen, San Jose, CA, USA) Propidium Iodide (Sigma-Aldrich Chemical Company, Munich, Germany) staining. After the SHEDs reached subconfluency, the flask medium was replaced with mixture of medium exposed to GICs and fresh, complete DMEM (ratio 1:1) (volume, 4 mL). The SHEDs exposed to the GICs eluate were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The cultured cells were washed twice with cold phosphate-buffered saline (PBS, Sigma Aldrich) and resuspended in 1x binding buffer (10x binding buffer: 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1 x 10^6/mL. Annexin FITC (5 µL) and propidium iodide (PI) (5 µL, 50 µg/ml in PBS) were added to 100 µL of the cell suspension and incubated for 15 min at room temperature (25°C) in the dark. After incubation, 400 µL of 1 x binding buffer was added to each tube. The stained cells were analysed within 1 hour using FACS Calibur (BD, San Jose, USA) and CellQuest software. Because Annexin V FITC staining precedes the loss of membrane integrity that accompanies the later stage identified by PI, Annexin V FITC positive and PI negative staining indicates early apoptosis, whereas Annexin V FITC negative and PI negative staining indicates cell viability. Cells that are in late apoptosis or already dead are both Annexin V FITC and PI positive, and dead cells are PI positive only [11].

Quantification of fluoride in medium exposed to GIC

The fluoride (F) concentrations of each eluate were assayed by high performance liquid chromatography using a Chromeleon® Chromatography Workstation (Dionex, Wien, Austria) equipped with a GP50 gradient pump, conductivity detector, ASRS ultra 4 mm. An Ionpac AS15 column and an AG15 guard column were used. Potassium hydroxide was used as the eluent. The flow rate was 1.0 mL/min. All results were analysed on Chromeleon 6.7 Chromatography Management Software.

Statistical Analysis

The cytotoxicity was expressed as mean + standard deviation. One-way ANOVA tests and linear regression were used to analyse the data. A $p < 0.05$ was considered statistically significant.

RESULTS:

LDH assay

Stem cell death, as evaluated by an LDH test 24 hours after incubation in GIC eluates, indicated very similar cytotoxic effects for the Fuji VIII, Fuji IX, Fuji plus, Fuji triage and Vitrebond GICs. These eluates induced necrosis in approximately 50% of the SHEDs. The Fuji I (25.14% dead cells) and Fuji II (28.56% dead cells) GICs demonstrated significantly less cytotoxicity (Figure 1).

Fluoride release

We wanted to know if the leaching of ionic components into the biomaterial eluates could account for the cytotoxic effect of the GICs. For that purpose, one of the major ions present in all tested GICs, F⁻, was quantified in the eluates (Figure 2). There was a strong correlation between the cytotoxic effects of GICs and fluoride release. Pear-

son's correlation coefficient (r^2) value demonstrated a high correlation between F- release and cytotoxicity (r^2=0.848, p=0.003). The more cytotoxic GICs (Fuji Plus, Vitrebond, Fuji IX, Fuji triage and Fuji VIII) released more F- then the other tested GICs. The less cytotoxic materials (Fuji I and Fuji II) released less F-.
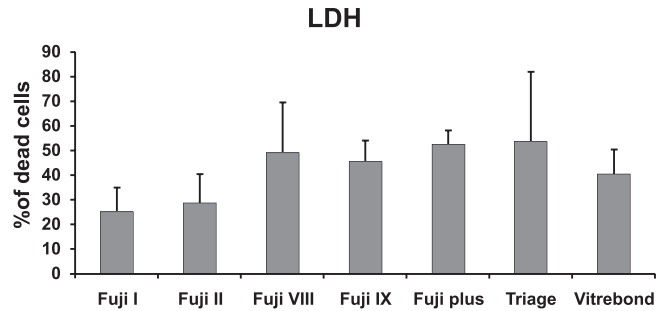
Apoptosis assay

To more closely investigate the effects of GICs on human SHEDs, we performed apoptosis assays. These assays confirmed the results obtained by the LDH test (Figure 3). All GICs induced permeabilisation of the SHED membranes (propidium iodide-positive cells). The highest percentages of dead cells were recorded after treatment with Fuji VIII, Fuji IX or Vitrebond (Figure 3). In addition, there was a good correlation between the percentage of dead cells as measured by the apoptosis assay and fluoride release (r^2 = 0.717, p=0.016).

Figure 1. Stem cell necrosis as evaluated by the LDH assay. Each column represents the percentage of dead cells (mean value and standard deviation of 3 experiments with 3 replicates).
The least cytotoxic GICs were Fuji I (25.14%) and Fuji II (28.56%), while Fuji Plus (52.48%), Vitrebond (54.18%), Fuji VIII (49.13%), Fuji IX (45.20%) and Fuji Triage (53.68%) demonstrated higher cytotoxic effects on SHEDs. There was a statistically significant difference between Fuji I and Vitrebond (p=0.04).

DISCUSSION

The biological compatibility of dental materials is essential for avoiding or limiting pulp tissue irritation or degeneration. GIC formulations contain organic monomers and different ions that may diffuse through the dentin tubules and reach the pulp tissue. These ions can affect the vitality of the odontoblast layer and interfere with pulp homeostasis and healing [12,13]. SHEDs are adult stem cells that are able to regenerate a dentin-pulp-like complex, composed of mineralised matrix with tubules lined with odontoblasts and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth [14]. Dentin formation continues through life in response to physical and/or chemical injuries and tooth aging. As SHEDs are involved in the damaged pulp repair processes, we selected them as an adequate cell culture system for testing the effects of GICs.
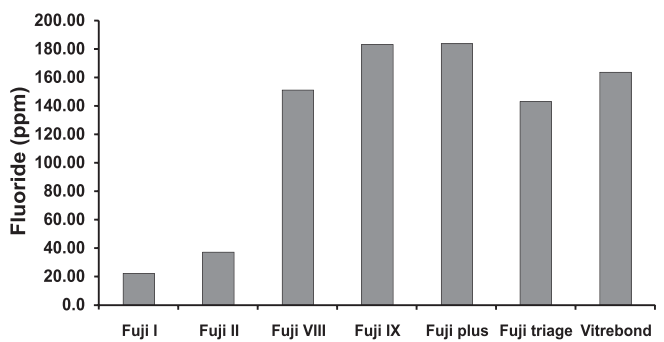
To explore the cell damage potential of GICs, we used an LDH assay. Lactate dehydrogenase is a cytoplasmatic enzyme released after cell membrane disruption. The LDH assay measures the activity of LDH in cell supernatants, thus, indirectly measuring cell death associated with a loss of membrane integrity [15]. The results of the LDH assay indicated that eluates from the Fuji VIII, Fuji IX, Fuji plus, Fuji Triage and Vitrebond samples were highly cytotoxic to human SHEDs (Figure 1), and there was no significant difference between them. The Fuji I and Fuji II eluates were slightly less cytotoxic, suggesting better biocompatibility. Our results are in agreement with previous reports showing that GICs are toxic to dental pulp [16] and pluripotent mesenchymal precursor cells [17].

GICs eluates induced similar toxicity as evaluated by flow cytometric analysis of PI-stained cells. These data indicated that the highest percentage of damaged cells was attained after treatment with Fuji plus, Fuji IX or Vitrebond GICs, a moderate percentage was attained after treatment with Fuji
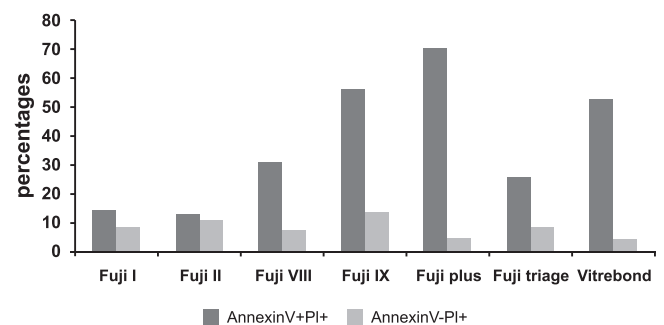

Figure 2. Quantification of Fluoride in the eluates of tested GICs.

Figure 3. The percentage of dead cells as evaluated by the apoptosis assay. The highest percentage of dead cells was attained after treatment with Fuji IX (69.6%), Vitrebond (57.20%) or Fuji Plus (74.9%). A moderate percentage of dead cells was attained after treatment with Fuji VIII (38.40%) or Fuji Triage (34.10%). The lowest percentage of necrosis was attained after treatment with Fuji I (22.8%) or Fuji II (24.3%). The results of a representative experiment are presented.

triage or Fuji VIII GICs and the lowest percentage was attained after treatment with Fuji I or Fuji II GICs (Figure 2).

These differences in cytotoxic effects between different GICs on human SHEDs appear to be related to the amount of fluoride released. The most toxic GICs, Fuji Plus, Vitrebond and Fuji VIII, released a higher amount of F anions than the other tested materials (Figure 3). In addition, low levels of released fluoride were noticed in the Fuji I and Fuji II eluates, which were the least cytotoxic GICs (Figure 2). Although it is known that the main advantage of using GICs as adhesive restorative materials is the long-term antibacterial effect due to fluoride release [3, 9, 18], we are the first group to demonstrate that the fluoride release of GICs has a direct correlation with cytotoxic effects on human SHEDs. GICs achieve maximum fluoride release 24 h after the initial setting [19], and that fluoride release has a significant potential for inducing pulpal toxicity [20]. The potential of fluoride anions to induce necrosis of Swiss-strain mouse hepatocytes [21] and primary culture of rat thymocytes [22] has been reported previously but in this study, we are first to show that fluoride release directly correlates with GIC cytotoxic effects in human SHEDs.

Acknowledgements

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