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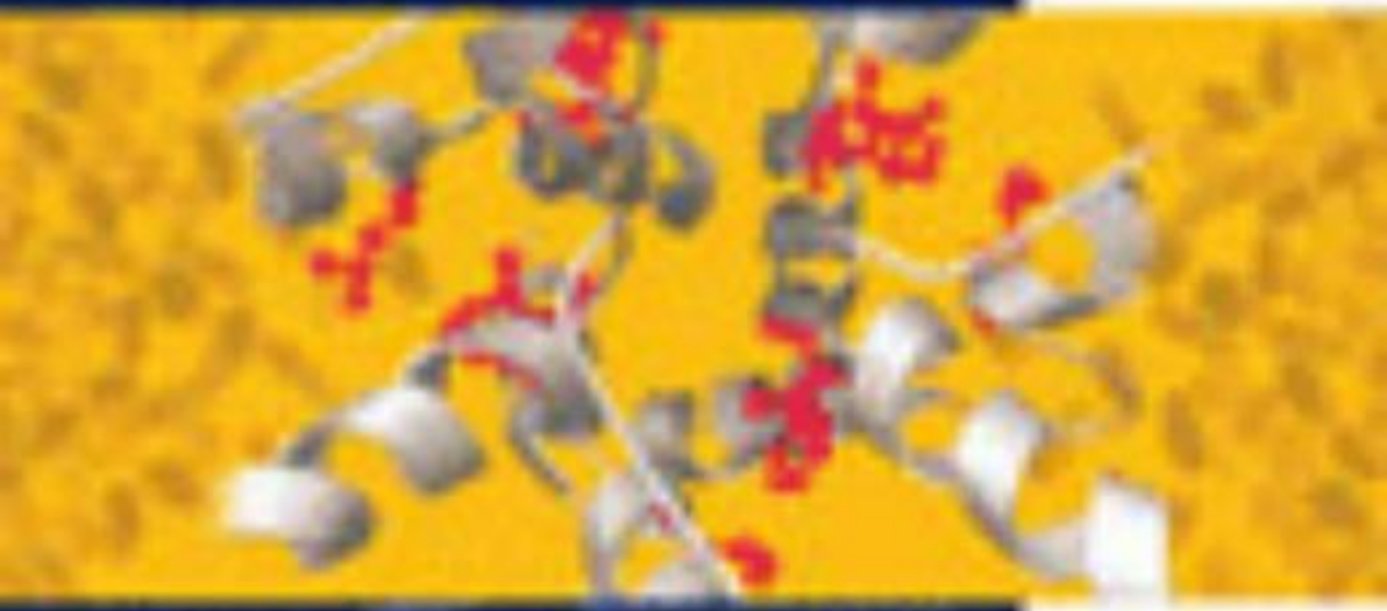

Cytotoxic Effects of Glass Ionomer Cements on Human Dental Pulp Stem Cells Correlate with Fluoride Release

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Abstract:

Objectives: Glass ionomer cements (GICs) are commonly used as restorative materials. Responses to GICs differ among cell types and it is therefore of importance to thoroughly investigate the influence of these restorative materials on pulp stem cells that are potential source for dental tissue regeneration.

Eight biomaterials were tested: Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji Plus, Fuji Triage, Vitrebond and Composit. We compared their cytotoxic activity on human dental pulp stem cells (DPSC) and correlated this activity with the content of Fluoride, Aluminium and Strontium ions in their eluates.

Methods: Elution samples of biomaterials were prepared in sterile tissue culture medium and the medium was tested for toxicity by an assay of cell survival/proliferation (MTT test) and apoptosis (Annexin V FITC Detection Kit). Concentrations of Fluoride, Aluminium and Strontium ions were tested by appropriate methods in the same eluates.

Results: Cell survival ranged between 79.62% (Fuji Triage) to 1.5% (Fuji Plus) and most dead DPSCs were in the stage of late apoptosis. Fluoride release correlated with cytotoxicity of GICs, while Aluminium and Strontium ions, present in significant amount in eluates of tested GICs did not.

Significance: Fuji Plus, Vitrebond and Fuji VIII, which released fluoride in higher quantities than other GICs, were highly toxic to human DPSCs. Opposite, low levels of released fluoride correlated to low cytotoxic effect of Composit, Fuji I and Fuji Triage.

Keywords: Glass ionomer cements, cytotoxicity, fluoride, human dental pulp stem cells.

INTRODUCTION

In restorative dentistry, the protection of the dentin-pulp complex consists of the application of one or more layers of specific materials (varnishes, calcium hydroxide-based products, glass ionomer cements (GICs) and adhesive systems) between the restorative material and dental tissue to avoid additional damage of pulp tissue caused by operative procedures, toxicity of restorative materials and bacteria penetration due to microleakage [1-2]. GICs, invented and originally described by Wilson and Kent [3], are consisted of basic glass powder (calcium or strontium aluminofluorosilicate) and a water-soluble acidic polymer, such as polyacrylic acid [4]. GICs are classified into three categories: conventional, metal-reinforced and resin modified GICs [5-6]. Metal-reinforced GICs are strengthened by the inclusion of finely divided metal powers, typically the silver-tin alloy of dental amalgams [7].

Although conventional GICs, because of their biocompatibility, elasticity similar to dentin and ability to release fluoride, have advantages in comparison with other materials used in restorative dentistry, they have several limitations such as susceptibility to dehydration and poor physical properties (high solubility and slow setting rate) [1, 8-9].

The incorporation of polymerizable water-compatible monomers such as 2-hydoxyethyl methacrylate (HEMA) led to the introduction of hybrid versions of conventional GICs, named resin-modified GICs (RMGICs) [1, 7]. In comparison with conventional GICs, RMGICs show enchanced flexular strength, diametral tensile strength, elastic modulus and wear

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resistance and are widely used in restorative dentistry [10]. The main disadvantage of RMGICs is their higher cytotoxicity in comparison with conventional GICs [11-12].

Responses to GICs differ among cell types [13-17], and thus, it is of importance to thoroughly investigate the influence of these restorative materials on pulp stem cells that are potential source for dental tissue regeneration. Dental pulp stem cells (DPSCs), self-renewable multipotent stromal cells of the pulp, due to their high differentiation potential and immunomodulatory characteristics [18] have the best therapeutic potential in the stimulation and modulation of local response in microenvironment of damaged pulp [19].

Based on these findings, we decided to evaluate the potential cytotoxic effects of eight commonly used biomaterials: Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji Plus, Fuji Triage, Vitrebond and Composit on human DPSCs.

MATERIALS AND METHODS

Culture of Human DPSCs

Human DPSCs derived as previously described by Gandia *et al.* [20] were fully characterized and tested for their ability to differentiate into ectodermal, endodermal and mesodermal lineages. 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). DPSCs in passage 6 were used throughout these experiments.

Tested Materials

Eight biomaterials: Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji Triage (GC America, Alsip, IL, USA), Vitrebond (3M ESPE, London, UK) and Composit were used in this study.

Elution samples of GICs were prepared in sterile tissue culture medium and the medium was tested for toxicity in a cell culture system. Separate samples of the seven GICs and Composit were mixed according to the manufacturers' directions at room temperature, and then placed into open plastic rings 5 mm in diameter by 2 mm deep during the interval 30 to 60 sec after initiation of mix. Sixteen samples were prepared for each material. The samples so formed were removed from rings and dry heat sterilized for 1 hour at 170 ^{o}C. Then the samples were immersed in separate tubes in which 2.4 mL of tissue culture medium was previously added. The sample dimensions and conditions of immersion were chosen in order to approximate the mass of GIC and the surface area exposed to dentin usually used in restorative dentistry in patients. The samples were held in medium for 72 h and then removed and discarded. The GICs eluates were retained for toxicity testing.

Evaluation of Cytotoxicity Using MTT Test and Phase Contrast Microscopy (PCM)

The effects of GICs on DPSC viability were determined using MTT colorimetric technique [21]. DPSCs were diluted with DMEM medium to 1 x 10^5 cells/mL and aliquots (1 x 10^4 cells/100μL) were placed in individual wells in 96-multiplates. The next day medium was exchanged with 100μL of each GICs eluate, which had been serially diluted 1:1 in DMEM medium. Each eluate was tested in triplicate. Cells were incubated at 37°C in a 5% CO_2 incubator for 24h. After incubation GICs eluate was removed and MTT solution (5 mg/mL in PBS, 10 μL) was added to each well. After additional 4 h of incubation 37°C in a 5% CO_2 incubator, medium with MTT was removed and DMSO (150 μL) with glycine buffer (20 μL) was added to dissolve the crystals. The plates were shaken for 10 min. The optical density of each well was determined at 595 nm. The percentage of cytotoxicity was calculated using the formula:

% cytotoxicity = 100-((TS−BG0)−E/(TS−BG0) × 100) where BG0 is for background of medium alone, TS is for total viability/spontaneous death of untreated target cells, and E is for experimental well.

DPSCs cultured in GIC's eluates for 24 hours were observed using PCM. DPSCs cultured in DMEM served as controls.

Apoptosis Assays

For detection of apoptosis the Annexin V binding capacity of treated cells was examined by flow cytometry using Annexin V FITC Detection Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's protocol. Since externalization of phosphatidylserine occurs in the early stages of apoptosis, Annexin V FITC staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

After DPSC reached the subconfluency, medium was replaced with GICs eluate diluted 1:1 in complete DMEM (volume, 4 mL). DPSC exposed to GICs eluate were placed at 37°C in a 5% CO_2 incubator for 24h. Cultured cells were washed twice with cold phosphate-buffered saline (PBS, Sigma Aldrich) and resuspended in 1 x binding buffer (10x binding buffer: 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at concentration 1 x 10^6 / mL. Annexin FITC and propidium iodide (PI) were added to the 100 μL of 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 and stained cells were analyzed within 1 hour using FACS Calibur (BD, San Jose, USA) and WinMDI software. Since Annexin V FITC staining precedes the loss of membrane integrity that accompanies the later stage identified by PI, Annexin V FITC positive, PI negative indicates early apoptosis, while viable cells are Annexin V FITC negative, PI negative. Cells that are in late apoptosis or already dead cells are both Annexin V FITC and PI positive [22].

Quantification of Fluoride, Aluminium and Strontium Concentration in the Eluates

The Fluoride (F) concentrations of each eluate were assayed by Chromeleon® Chromatography Workstation (Dionex, Wien, Austria) with Chromeleon 6.7 Chromatography Management Software while Aluminium (Al) and Strontium (Sr) concentration were assayed by Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, UK) according to manufacturer's instruction.

Fig. (1). The stem cell viability evaluated by MTT assay. Each column represents percentage of dead cells (mean value and standard deviation of 3 experiments with 3 replicates).
The least cytotoxic GICs were Fuji Triage (20.38%), Composit (20.76%) and Fuji I (25.88%) while Fuji Plus (98.5%), Vitrebond (97.92%), Fuji VIII (92.06%), Fuji IX (82.71%) and Fuji II (71.07%) showed high cytotoxic effect on MSCs.
Cytotoxic effects of Fuji I was significantly lower than Fuji Plus, Vitrebond and Fuji VIII ($p<0.05$) while Fuji Triage was significantly less cytotoxic then Vitrebond, Fuji VIII and Fuji II ($p<0.01$). There was significant difference in cytotoxic effects between Composit and Fuji Plus, Vitrebond, Fuji VIII and Fuji II ($p<0.01$).

Statistical Analysis

The cytotoxicity was expressed as mean + standard deviation. Statistical analysis was performed by using ANOVA test, Linear regression and $p < 0.05$ was considered as statistically significant.

RESULTS

MTT Test

Stem cell survival, as evaluated by MTT test, after 72 hour incubation in GIC eluates indicated wide range of GIC cytotoxic activity. The most cytotoxic GICs were Fuji Plus (98.5% dead cells) and Vitrebond (97.92% dead cells) while Fuji Triage (20.38% dead cells), Composit (20.76% dead cells) and Fuji I (25.88% dead cells) were the least cytotoxic materials (Fig. **1**).

Phase Contrast Microscopy

During 24 hours, DPSCs cultured in the eluates of Composit, Fuji I and Fuji Triage exhibited spindle to polygonal shapes similar to the untreated cells cultured in DMEM medium. (Fig. **2**) On contrary, DPSCs cultured in the eluates of Fuji Plus, Vitrebond, Fuji II, Fuji VIII and Fuji IX were mostly detached, grew poorly, lost normal spindle shape and acquired round and/or collapsed appearance (Fig. **2**).

Fluoride, Aluminium and Strontium Release

We wanted to know if ionic components leached into biomaterial eluates could account for the cytotoxic effect of GICs. For that purpose, three major ions present in all tested GICs (F^-, Al^{3+}, Sr^{2+}) were quantified in the eluates (Fig. **3**).

There was a strong correlation between cytotoxic effects of GICs and Fluoride release. Pearson's correlation coefficient (r) value demonstrated a high correlation between F^- release and cytotoxicity ($r=0.777$, $p<0.05$). The most cytotoxic GICs (Fuji Plus, Vitrebond and Fuji VIII) released more F^- then other tested GICs. The least cytotoxic materials (Composit, Fuji I and Fuji Triage) demonstrated low amounts of F^- release.

There was no correlation between Al^{3+} release and cytotoxicity ($r=0.139$) and between Sr^{2+} release and cytotoxicity ($r=0.197$)

Apoptotic Assays

Apoptosis assays showed that all tested GICs induced apoptosis of DPSCs (Fig. **4**).

There was a high correlation between percentage of dead cell measured by MTT test and apoptotic assay ($r = 0.808$, $p<0.05$). The most of dead DPSCs were in the stage of late apoptosis (Annexin V+PI+ cells) 24h after exposure to tested materials (Fig. **4**).

The highest percentage of late apoptotic (Annexin V+PI+) cells was noticed after treatment with Fuji IX, Vitrebond and Fuji Plus (Fig. **4**).

DISCUSSION

The growing use of GICs for crown cementation, intracoronal restoration, and cavity lining makes studies of their pulp effects of great interest. Here we demonstrated that GICs have cytotoxic effects on DPSCs, stem cells that are involved in reparation processes of damaged pulp [19].

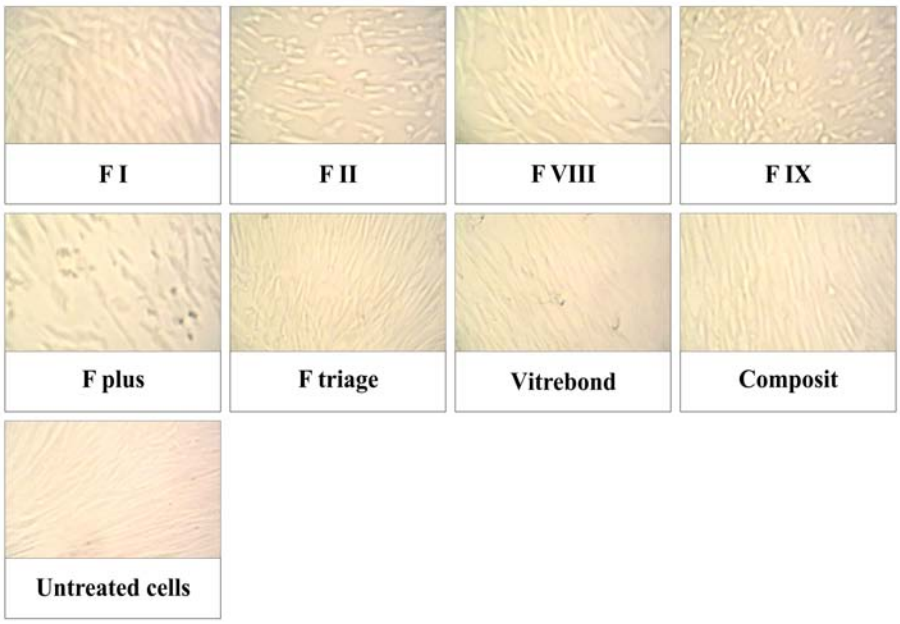
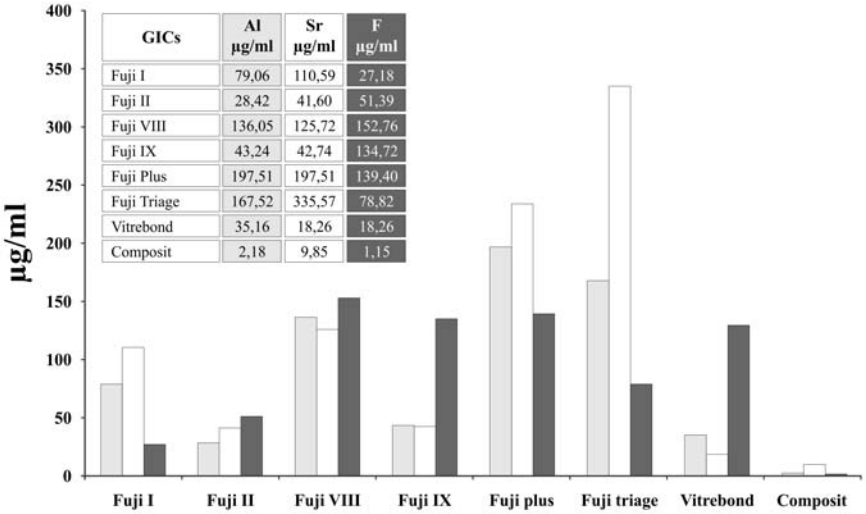


Fig. (2). Phase contrast microscopy of DPSCs cultured in the eluates of tested GICs or DMEM medium for 24 hours. DPSCs cultured in eluates of Fuji I, Fuji Triage and Composit maintained spindle to polygonal shapes like untreated cells cultured in DMEM medium. DPSCs cultured in the eluates of Fuji Plus, Vitrebond, Fuji II, Fuji VIII and Fuji IX acquired round and/or collapsed appearance.



GICs	Al µg/ml	Sr µg/ml	F µg/ml
Fuji I	79,06	110,59	27,18
Fuji II	28,42	41,60	51,39
Fuji VIII	136,05	125,72	152,76
Fuji IX	43,24	42,74	134,72
Fuji Plus	197,51	197,51	139,40
Fuji Triage	167,52	335,57	78,82
Vitrebond	35,16	18,26	18,26
Composit	2,18	9,85	1,15

Fig. (3). Quantification of Fluoride, Aluminium and Strontium concentration in the eluates of tested GICs. The Fluoride release ranged from 1,15 µg/ml (Composit) to 152.76 µg/ml (Fuji VIII). The Aluminium release ranged from 2.18 µg/ml (Composit) to 197.51 µg/ml (Fuji Plus). The Strontium release ranged from 9.85 µg/ml (Composit) to 335.57 µg/ml (Fuji Triage).

Phase contrast microscopy revealed that Composit, Fuji I and Fuji Triage supported the attachment and proliferation of DPSCs, while significantly fewer cells with impaired morphologies attached to flask surfaces during culture in the eluates of Fuji Plus, Vitrebond, Fuji II, Fuji VIII and Fuji IX (Fig. **2**).

The results of the MTT assays, which measured mitochondrial dehydrogenase activity and reflect cell viability [21], corresponded with those of the PCM evaluations, showing a significant inhibition of DPSCs growth after treatment with Fuji Plus, Vitrebond, Fuji II, Fuji VIII and Fuji IX. Eluates from these GICs were highly cytotoxic to human DPSCs (Fig. **1**). Fuji Plus and Vitrebond were the most toxic restorative material among tested GICs, while Fuji VIII, Fuji IX and Fuji II showed similar and moderately high cytotoxic effects. Imazato *et al.* previously found that resin based GICs, particularly Fuji II LC, suppressed proliferation capacity, ALP activity and attenuated differentiation potential of pluripotent mesenchymal precursor cells [23]. Here we demonstrated that GICs, particularly resin-modified GICs, were higly toxic for DPSCs, stem cells responsible for restoration of damaged pulp.

However, we also showed that among GICs, there are materials (Fuji I, Fuji Triage and Composit) with low cyto-

Fig. (4). A. The percentage of dead cells using apoptotic assay. The high percentage of late apoptotic cells (Annexin V+PI+) was noticed after treatment with Fuji IX (68.18%), Vitrebond (66.93%), and Fuji Plus (53.57%). The moderate percentage of apoptotic cells was seen after treatment with Fuji II (43.51%) and Fuji VIII (41.90%). The low percentage of apoptotic cells was noticed after treatment with Fuji I (21.96%), Fuji Triage (26.24%) and Composit (22.40%). The values are shown as mean + standard deviation. B. The representative dot plots (a. untreated cells, b. Fuji IX treated cells, Fuji Triage treated cells). The most of untreated and Fuji Triage-treated cells were viable while the most of Fuji IX-treated cells were in the stage of late apoptosis.

toxic effect on DPSCs suggesting their better biocompatibility.

Eluates of all tested GICs induce apoptotic cell death of DPSCs and most of GIC- treated DPSCs were in the stage of late apoptosis (Fig. **4**). The data from apoptotic assay showed that the highest percentage of viable cells was noticed after treatment of Composit, Fuji I and Fuji Triage that was in a strong correlation with results of MTT test and PCM observation.

These differences in cytotoxic effects of different GICs on human DPSCs appear to be related to the amount of fluoride released. Although it is known that main advantage of using GICs as adhesive restorative materials is long-term fluoride release because of their antibacterial effects [1, 8-9], we are first to demonstrate that fluoride release of GICs were in direct correlation with their cytotoxic effects on human DPSCs.

The most toxic Fuji Plus, Vitrebond and Fuji VIII released F^- ions in higher amounts then other tested materials (Fig. **3**). In addition, low levels of released fluoride were noticed in the eluates of Composit, Fuji I and Fuji Triage which were the least cytotoxic GICs (Fig. **3**). It is known that GICs achieved a maximum fluoride release 24 h after initial setting [24] and that fluoride release has significant potential for pulpal toxicity [25]. Previous studies showed that fluoride has cytotoxic effects to cultured human pulp cells by inhibiting cell growth, proliferation, mitochondrial activity, and protein synthesis [25] and is able to induce programmed cell death through mitochondrial/caspase-9/caspase-3-dependent pathway [26].

Along with F^-, Al^{3+} and Sr^{2+} are the main ions present in the eluates of tested GICs. Although Al^{3+} and Sr^{2+} release varied among tested materials (Fig. **3**), there was no correlation between quantities of Al^{3+} and Sr^{2+} ions in GICs eluates and their cytotoxic effects on human DPSCs. Al^{3+} and Sr^{2+} ions were present in significant amounts in the eluates of tested GICs but were not responsible for their cytotoxic effects in agreement with previously reports by Elshahawy *et al.* [27] and Isaac *et al.* [28].

CONCLUSIONS

Fluoride release was in direct correlation with cytotoxic activity of GICs on human DPSCs. Fuji Plus, Vitrebond and Fuji VIII, which released fluoride in higher quantities than other GICs, were highly toxic to human DPSCs. Opposite, low levels of released fluoride correlated to low cytotoxic effect of Composit, Fuji I and Fuji Triage on human DPSCs.

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