

Biocompatibility of glass ionomer cements with and without chlorhexidine

Sultan Gulce Iz¹, Fahinur Ertugrul², Ece Eden², S. Ismet Deliloglu Gurhan¹

Correspondence: Dr. Sultan Gulce Iz
Email: sultangulce@gmail.com

¹Department of Bioengineering, Faculty of Engineering, Ege University, Izmir, Turkiye,

²Department of Pedodontics, Faculty of Dentistry, Ege University, Izmir, Turkiye

ABSTRACT

Objective: The aim of the present study is to evaluate the biocompatibility of glass ionomer cements (GICs) with and without chlorhexidine (CHX) as well as coated with varnish or not using *in vitro* cytotoxicity test. **Materials and Methods:** Biocompatibility of Fuji IX, Fuji IX with varnish, Fuji IX with 1% CHX diacetate and Fuji IX with 1% CHX diacetate with varnish was determined with *in vitro* cytotoxicity assay by using L929 mouse connective tissue fibroblasts. After 72 h, cell viabilities were evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay to determine the effects of the cements on the mitochondrial function and microscopic images were taken by scanning electron microscopy. **Results:** Statistical analysis was performed by one-way analysis of variance followed by the Bonferroni *post-hoc* test at a significance level of $P < 0.05$. 72 h after treatment, there were statistically significant differences between Fuji IX and Fuji IX-CHX ($P < 0.001$). In addition, the reduction of the cytotoxicity by coating the GICs with varnish was indicative and increased the cell viability ratio ($P < 0.001$). **Conclusions:** Fuji IX coated with varnish was found to be the most biocompatible one among others. Thus adding CHX significantly reduced the cell viability, it is assumed that, due to the leakage of CHX and the other components of the GICs to the cell culture medium, the cell viabilities were decreased, so it is highly recommended to use varnish not only to reduce the water loss from the GICs, but also to reduce the cytotoxicity of the GICs.

Key words: Biocompatibility, chlorhexidine, glass ionomer cements

INTRODUCTION

Glass ionomer cements (GICs) are widely used dental materials first introduced to dentistry in 1972 by Wilson and Kent.^[1] GICs are especially used as restorative materials in children dentistry, as well as liners and bases.^[2] To improve the physical efficacy of the GICs a variety of filling materials were used such as bioactive glass and hydroxyapatite.^[3] In addition to improve the anti-bacterial properties of GICs, anti-bacterial materials such as cetylpyridinium chloride, cetrimide and benzalkonium chlorhexidine (CHX) were used.^[4-6] Nowadays, novel anti-bacterial agents like titanium dioxide nanoparticles are also used as anti-bacterial filling materials.^[3]

Amongst the anti-bacterial applications CHX is accepted to be the gold standard in dentistry.^[6] As well as fluoride is a possible agent for preventing dental caries.^[7] Fluoride containing GICs has gained great importance because of the sustained levels of fluoride release over-time.^[7] For this reason, most GICs include fluoride however fluoride is not enough to combat the bacterial growth.^[8] For this reason, to increase the anti-bacterial properties of GICs, the controlled release of CHX from GICs that include fluoride is a potent solution.^[6]

The low molecular weight of CHX results in slow release, which is also desired to control the bacterial growth for a longer duration compared to fluoride.^[8,9] In an experimental CHX release study, 10% of the

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total CHX diacetate loaded in the GICs (11.28%) were released after 240 days.^[5] Fluoride can also be encapsulated into chitosan microparticles to achieve controlled and long term release.^[10] Furthermore adding 1% CHX diacetate into Fuji IX GICs reduced the bacterial growth.^[11]

Thus, to increase the anti-bacterial properties of GICs, formulations that include both fluoride and CHX are promising. However, it is also important to determine the biocompatibility properties of new formulations. Adding CHX to GICs may alter the *in vitro* biocompatibility properties. CHX has cytotoxic effects on a variety of eukaryotic cells, which can alter membrane permeability, protein synthesis, lysosomal enzyme release, mitochondrial function disturbance, intracellular Ca increase and oxidative stress.^[12-14]

In this study, biocompatibilities of GICs that include fluoride either with CHX or without CHX were evaluated. Various cells are used for *in vitro* test systems to evaluate the biocompatibility of GICs such as osteoblasts, fibroblasts, bone marrow and osteoclasts.^[15] In this study, L929 mouse connective tissue fibroblasts were used for *in vitro* cytotoxicity studies due to its fibroblastic nature to be a model of gingival fibroblasts.^[16]

MATERIALS AND METHODS

Test materials, chemicals and reagents

L929 mouse connective tissue fibroblasts were obtained from HUKUK (Foot and Mouth Disease Institute, Animal Cell Culture Collection, Ankara, Turkey). Cell culture medium Roswell Park Memorial Institute (RPMI 1640), L-glutamine, gentamicine, fetal bovine serum (FBS) were obtained from Biochrom (Germany). Trypsin and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromid) were from Sigma (Germany) and Trypan blue and dimethyl sulphoxide (DMSO) (dimethylsulfoxide) were from Applichem (Germany). The test materials were Fuji IX and Fuji IX-CHX (GC Corporation, Japan).

Cylindrical specimens (5 mm in diameter × 5 mm thick) of each GIC material were prepared by using teflon molds. All cement pastes were dispensed and evenly mixed according to the manufacturers' instructions. All specimens were shaped as described by Lin *et al.*, 2008. Briefly, GICs were filled into the cylindrical teflon molds (which were autoclaved for sterilization before), sandwiched between 2 glass plates and separated by cellophane strips. Excess

cement was removed and the specimens were allowed to completely set according to the time recommended by the manufacturer.^[17] There were four experimental groups; Fuji IX, Fuji IX with varnish, Fuji IX-CHX and Fuji IX-CHX with varnish containing three samples were tested for three replicates.

In vitro cytotoxicity test

L929 mouse connective tissue fibroblasts were routinely cultivated in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/mL) at 37°C and 5% CO₂. 50000 cells were seeded onto 12 mm diameter round shaped coverslips (Menzel-Glaser, Germany), which are placed in 24 well plates and incubated for 24 h at 37°C. After overnight attachment of the cells, 0.4 µm, 0.5 cm² polycarbonate tissue culture inserts (Nunc, Germany) were placed on the coverslips and the GICs specimens were placed on the tissue culture inserts. After 72 h, the inserts and cements were discarded from the culture plates and cell viabilities were determined by using MTT assay to evaluate the effects of the cements on the mitochondrial function [Figure 1]. In addition, cells remained attached on the coverslips were scanned by electron microscopy [Figure 2].

MTT is a colorimetric assay, which measures the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product. After discarding the exposure medium, 0.5 mg/mL of MTT were added to each well and incubated at 37°C, and 5% CO₂ for 4 h. After that, 200 µl DMSO was added to each well to dissolve the formazan salts. The absorbance was immediately determined at 570 nm using an ultraviolet-visible single beam spectrophotometer (Jenway, 6400, UK).

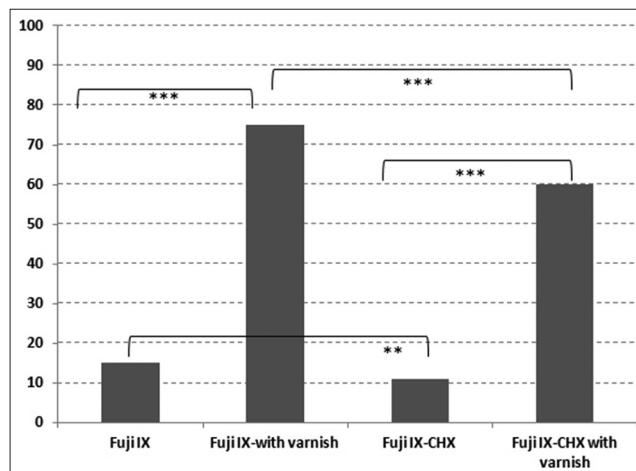


Figure 1: Cell viability rates after treatment with glass ionomer cements samples

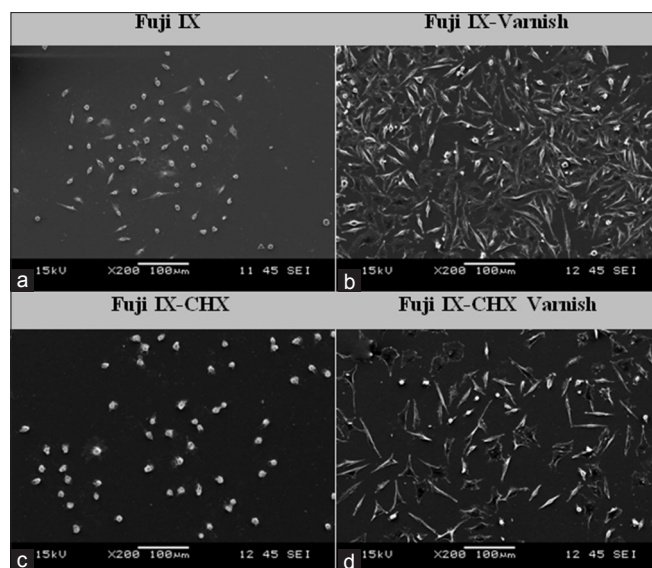


Figure 2: Scanning electron microscopy images taken after 72 h treatment period. (a) Fuji IX. (b) Fuji IX-varnish. (c) Fuji IX- chlorhexidine (CHX)-varnish. (d) Fuji IX-CHX-varnish ($\times 200$ magnification)

Scanning electron microscopy analysis

In order to examine the morphology, especially the shape and attachment properties of the L929 cells on the coverslips, SEM were used. Briefly, cells on the coverslips were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 30 min, post-fixed in 1% osmium tetroxide in distilled water for 30 min, dehydrated through a graded ethanol series, desiccated for 5 min in hexamethyldizilasane, followed by air drying for 30 min and incubation in a desiccator with phosphorus pentaoxide overnight. Finally, samples were coated with a thin layer of gold by ion sputtering. Samples were then examined using a JEOL JSM-6060 SEM (Tokyo, Japan).

Statistical analysis

All assays were repeated at least three times to ensure reproducibility and three replicates of each group were performed in each test. The significance of difference between GIC types with and without CHX as well as varnish coated or not was analyzed by one-way analysis of variance followed by Benferroni's *post-hoc* comparison by Graphpad Prism version 5.0 for Windows (Graphpad Software, USA). A $P < 0.05$ was considered to be statistically significant.

RESULTS

GICs are widely used in dentistry so their biocompatibility is a very important issue. In this study, the biocompatibilities of Fuji IX with and without CHX as well as with and without varnish

coating were examined by L929 cells. The cell viability ratios were determined by MTT assay while the morphologies of the cells were scanned by SEM.

Cell viability rates after incubated with different GIC samples were determined by MTT assay. The absorbances of the control cells not incubated with any GIC were considered to be one hundred. For each GIC sample, the absorbances were converted into percentages relative to control cells. There were statistically significant differences among GICs with and without CHX after 72 h ($P < 0.001$) [Figure 1]. The highest cell viability rate was achieved by Fuji IX with varnish (75%), the lowest cell viability rate was achieved by Fuji IX-CHX without varnish (11%). Cell viabilities of Fuji IX compared with Fuji IX with varnish and Fuji IX-CHX compared to Fuji IX-CHX with varnish were significantly lower ($P < 0.001$) [Figure 1].

Fuji IX without varnish demonstrated 15% cell viability while Fuji IX with varnish demonstrated 75% cell viability with a significant difference ($P < 0.001$). Fuji IX-CHX without varnish demonstrated 11% cell viability while Fuji IX-CHX with varnish demonstrated 60% cell viability ($P < 0.001$). In addition, cell viabilities of the GICs that are containing CHX were significantly lower compared with GICs without CHX. The cell viability ratio difference of Fuji IX without varnish compared with Fuji IX-CHX without varnish was significant ($P < 0.05$). Similarly, there was a statistically significant difference among Fuji IX with varnish compared with Fuji IX-CHX with varnish ($P < 0.001$).

Cell morphologies were examined by SEM, 72 h after treatment; $\times 200$ magnifications of the images are shown on Figure 2. Fuji IX and Fuji IX-CHX with varnish the morphologies of the cells were elongated flattened healthy fibroblastic nature and the confluency ratio of the cells on the coverslip was reflecting almost the same ratios obtained by MTT assay. However, Fuji IX and Fuji IX-CHX without varnish, the cells were detached from the surface with a round unhealthy nature, which were also correlating with the cell viability rates obtained by MTT assay.

The cytotoxicities of GICs *in vitro* were mostly attributed to the leachable materials from them. On Figure 3 ($\times 750$ magnification), the leachable components from Fuji IX (A) and Fuji IX-CHX (C) without varnish were shown like small dots. However, using varnish prevents the leakage of the GIC components to the cell culture medium, there were not any debris as seen on the Fuji IX (B) and Fuji IX-CHX (D).

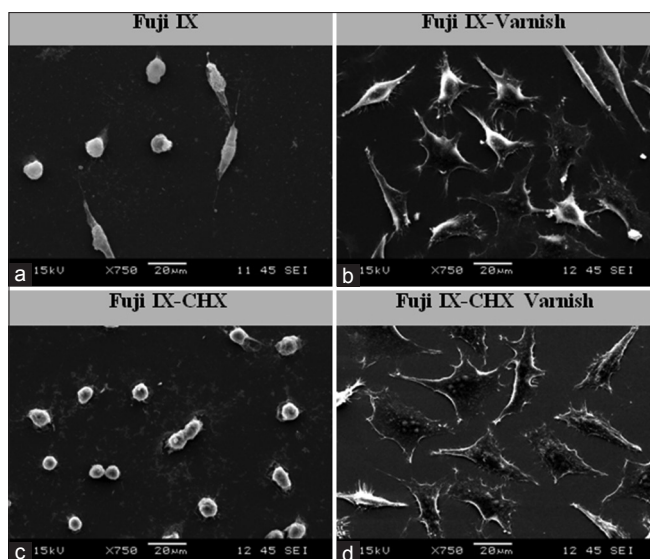


Figure 3: Scanning electron microscopy images taken after 72 h treatment period to show glass ionomer cements competent release. (a) Fuji IX. (b) Fuji IX-Varnish. (c) Fuji IX-chlorhexidine. (CHX)-varnish. (d) Fuji IX-CHX-varnish ($\times 750$ magnification)

DISCUSSION

To increase the anti-bacterial properties of GICs different formulations are under development. Amongst them formulations that include both fluoride and CHX are promising. However, it is also important to determine the biocompatibility properties of new formulations. Adding CHX to GICs may alter the *in vitro* biocompatibility properties.

In this study, the biocompatibilities of Fuji IX with and without CHX as well as with and without varnish coating were examined by L929 cells. The highest cell viability ratio was obtained by Fuji IX with varnish followed by Fuji IX-CHX with varnish ($P < 0.001$). The lowest cell viability ratio was obtained by Fuji IX-CHX without varnish followed by Fuji IX without varnish ($P < 0.05$). Thus adding CHX significantly reduces the cell viability ($P < 0.05$). However using varnish significantly increases the cell viability ($P < 0.001$).

Especially, varnishes were applied to the GICs when they were used as restorative materials in clinical use to prevent water loss from the immature GICs.^[18] In addition, this study shows coating the GICs with varnish reduce the cytotoxicities of GICs by preventing the component leakage from them.

In this study, adding CHX to GICs reduced the cell viability. Apart from anti-bacterial studies, various studies were done to determine *in vitro* and *in vivo*

cytotoxicities of CHX. It was shown that CHX has serious toxic effects on several cells such as gingival fibroblasts, endothelial cells, alveolar osteoblast cells and odontoblast-like cells *in vitro*^[14,19,20] and on rat blood and kidney cells *in vivo*.^[21]

In this study, Fuji IX coated with varnish is found to be the most biocompatible one among others. It is assumed, due to the leakage of CHX and the other components of the GICs the cell viabilities are decreased.

Several modifications are carried out to enhance the mechanical and physical properties of GICs as well as anti-bacterial properties. To increase the anti-bacterial properties of GICs, formulations that include CHX are promising. However, it is also important to determine the biocompatibility properties of new formulations. Adding CHX to GICs may alter the *in vitro* biocompatibility properties of GICs.

Various *in vitro* tests are used to evaluate the biocompatibility of GICs but Hatton *et al.*, 2006 stated that GICs are classified as bioactive rather than bioinert. Thus, the results of the *in vitro* tests are not reflecting the real *in vivo* situation.^[15]

CONCLUSION

In dentistry, *in vitro* cell culture studies are important tools to evaluate the cytotoxicities of restorative dental materials before *in vivo* applications. However, *in vitro* test systems have several limitations in testing surface bioactive materials like GICs,^[15] thus the correlation of *in vitro* laboratory tests with *in vivo* clinical trials are inevitable. To achieve this correlation, clinical trials, which last at least 10-20 years must be done to see the real efficacy of the dental materials on the target tissue and patient because of the limitations of the *in vitro* test systems.^[21-23]

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