Cytotoxicity of Fast-set Conventional and Resin-modified Glass Ionomer Cement Polymerized at Different Times on SHED

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Objectives: *To compare the cytotoxicity of conventional GIC and Resin Modified GIC (RMGIC) polymerized at 2 different times on stem cells from human exfoliated deciduous teeth (SHED).* ***Study design:*** *The conventional GIC (Fuji IX GP Extra) and RMGIC (Fuji II LC) were mixed and incubated in a prepared Dublecco's Modified Eagle Medium (DMEM) for seven days. After seeding the characterized SHED for 24 hrs, six replicates of seven serially diluted extracts of each group were added and incubated for 72 hrs. MTT test was used for cytotoxicity evaluation and the data were analysed using Kruskal-Wallis followed by Mann-Whitney test, with the statistical significance set at P<0.05.* ***Results:*** *The half maximal inhibitory concentration (IC_{50}) was found at 45.0 mg/ml, 45.0 mg/ml and 31.25 mg/ml for Fuji IX, Fuji II LC (40s) and Fuji II LC (20s), respectively. Significantly different cytotoxic effects were found between Fuji II LC polymerized at 20 secs and 40 secs, and between Fuji IX and Fuji II LC (20s) (P<0.05), and these were observed in all concentrations except for 50 mg/ml.* ***Conclusions:****RMGIC polymerized at 20 secs exhibited the least favorable cell viability among all groups. Nevertheless, the cell viability was comparable to conventional GICs when the manufacturer's recommended time was doubled (40 secs).*

Key words: *Glass ionomer cement, resin modified glass ionomer cement, polymerization time, cytotoxicity, SHED.*

INTRODUCTION

Dental restorative materials with different formulations are continuously being developed and introduced to the market for a variety of dental applications. Conventional glass-ionomer cement (GIC) is the basic product of an acid–base reaction between acid-decomposable fluoraluminosilicate glass powders and an aqueous solution of polyacrylic acid.[1] Over the past decades, GIC has gained clinical popularity for several of its important properties, such as fluoride release,[2] coefficient of thermal expansion and modulus of elasticity similar to dentin,[3] bonding to both enamel and dentin,[4] and good biocompatibility.[5–7] Despite these favorable properties, conventional GIC exhibits some limitations primarily because of its susceptibility to dehydration, high solubility, and slow setting rate. These limitations compromise the physical and mechanical properties of GIC and restrict its adoption for wider clinical applications.[1,3]

Further improvement in GIC has facilitated the development of resin-modified glass-ionomer cement (RMGIC). RMGICs were introduced to the market to enhance the physical and mechanical properties of GIC by adding hydrophilic monomers (HEMA) and polymerization initiators into the components of conventional GICs,[8] as confirmed in previous studies[3,9,10]. Based on these advantages, RMGIC is indicated for use as restoration material in the cervical and sub-gingival areas and is recommended for use as a substitute for amalgam restorations in pediatric dentistry.[11]

Despite these improvements, many commercial RMGICs showed more intense cytotoxic effects and have been regarded as more cytotoxic than conventional GICs.[5,11–13] These findings are mainly attributed to the release of leachable resin components, such as HEMA, during initial setting and with the degradation of the material over time.[14–17] Studies have shown that polymerization times and methods influence the cytotoxicity of resin composite.[18,19] Nevertheless, little data are available on similar investigations conducted for glass ionomer-based materials with emphasis on restorative-grade RMGIC, particularly on human cell cultures of odontogenic origin. Therefore, this study aimed to evaluate, in an in vitro model, the cytotoxic effects of conventional and RMGIC restorative-based materials polymerized at two different times on stem cells from human exfoliated deciduous teeth (SHED).

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MATERIALS AND METHOD

This study was approved by the Human Ethical Committee of Universiti Sains Malaysia (vide reference USM 236.4. (2.12) USMKK/ PPP/JEPeM dated 23rd April 2011). The stem cells used in this study were cryopreserved SHED obtained from the Cranio-facial Science Laboratory, School of Dental Sciences, Universiti Sains Malaysia, which were established from the dental pulp of human extracted deciduous teeth of 9 to 11 years old children, based on the detail isolation protocols and culture procedures mentioned by Lutfi *et al.*[20]. The cryopreserved SHED were then thawed and cultured in Dulbecco's Modified Eagle's Medium (DMEM)(Gibco, USA) containing L-Glutamine and supplemented with Fetal Bovine Serum (FBS) (Gibco, Invitrogen, USA) and 1 % Penicillin, 1 % Streptomycin (Gibco, Invitrogen, USA) at 37^0C and 5% CO_2. Cell cultures of the fifth passage were used in this study (Figure 1a).

SHED characterization

Once confluent, the cells were detached using 0.25% Trypsin (Invitrogen, Denmark) and centrifuged. After cell counting, the SHED were then placed into the bottom of the round flow cytometry tube ((BD FACS Canto II, Canada) and 20 µl of a stem cell surface marker, CD90 (PE stain, BioLegend, San Diego, USA) was used for cell characterization by adding it on the cell pellet. The tube was vortexed for 10 seconds and then incubated in the dark at room temperature for 30 minutes. After incubation, the cells were washed with 2 ml of Phosphate Buffer Saline (PBS) by vortexing at 1500 rpm for 5 minutes. The supernatant was discarded carefully leaving the cell pellet at the bottom of the tube. A total of 0.5 ml of PBS was added to resuspend the cells and gently re-vortexed. Subsequently, the PBS was removed and cells fluorescence were evaluated using flow cytometry (BD FACS Canto II, Canada).

Materials and extracts preparation

Fast set conventional GIC (Fuji IX GP Extra, GC, Japan) and RMGIC (Fuji II LC, GC, Japan) were used in this study (Table 1). The materials were divided into three experimental groups. The fast set conventional (Fuji IX GP Extra, GC, Japan) served as group 1 and RMGIC (Fuji II LC, GC, Japan) polymerized at 20 secs and 40 secs served as group 2 and 3, respectively.

Prior to material mixing, paraffin wax moulds with a dimension of 8 mm diameter and 2 mm depth, supported by sterile stainless steel ring were prepared as described by Ahmed *et al.*[6]. Then, both materials were mixed in a capsule mixer according to the manufacturer's instructions (10 secs), and subsequently syringed into the moulds (Figure 1b).

The materials were divided into three groups, according to the types of material used and polymerization times. Fuji IX was allowed to set chemically while the polymerization of RMGIC was achieved at 20 secs (manufacturer's recommended time, MRT) and 40 secs (double MRT), respectively, using a visible light curing unit (Bluedent LED Smart, BG Light LTD, Malaysia). The light intensity was calibrated using light intensity meter, situated on the charging station of the Bluedent LED Smart (BG Light LTD, Malaysia, 800 W/cm^2). During polymerization, the tip of the light curing unit was held approximately 1 mm away from the top surface of the moulds containing RMGIC. Subsequently, each material was retrieved from the mould, sterilized using alcohol[21], weighed and introduced into a sterile glass bottle. A prepared Dulbecco's Modified Eagle's Medium

Figure 1: a) Confluent SHED (Passage 5)
b) Paraffin wax supported by stainless steel mould.

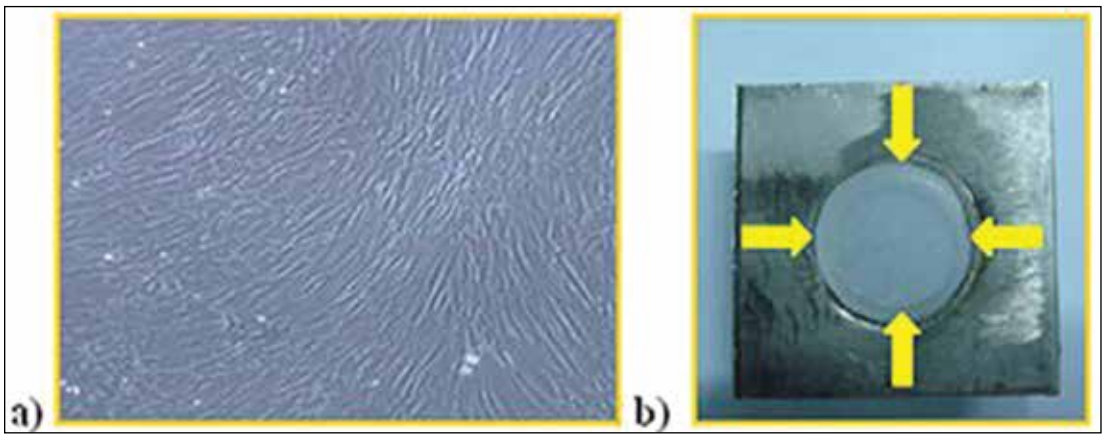


Table 1: List of restorative materials used in the study.

Material	Manufacturer	Description	Polymerization	Main components
Fuji IX	GC Corporation, Tokyo, Japan	Conventional Glass Ionomer Cement	Chemical cured	100% aluminium silicate, 65-70% polyacrylic acid,
Fuji II LC	GC Corporation, Tokyo, Japan	Resin Modified Glass Ionomer Cement	Light cured	100% aluminium silicate, 65-70% polyacrylic acid, 8-10% 2- hydroxyethyl-methacrylate (HEMA)

(DMEM) (Gibco, USA) was then added into each glass bottle at a concentration of 200 mg/ml. The immersed samples were incubated for seven days at 37^{0}C. After incubation, the material extracts were obtained after passing through a 0.45 µm filter (Pall, USA) into another sterile glass bottle. Each material extract was then prepared at concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.625 mg/ml, which was achieved by means of diluting the each material extracts serially into seven concentrations with a prepared DMEM (Gibco, USA) using sterile pipette tips (Eppendorf, Germany).

SHED inoculation and extracts application

SHED were seeded in 96-well tissue culture plates at 5 x 10^3 cells per well and incubated in complete DMEM for 24 hours at 37^{0}C and 5% CO_2. Six replicates of each material extract (seven concentrations each), were added into the cell culture plates with the last group served as the negative control group (100 % culture media with no material extract). The plates were then incubated for 72 hours at 37^{0}C and 5% CO_2.

Cytotoxicity testing using MTT assay

After removal of the culture media from each well, 3-(4,5-dimethyl-thizoyl)-2,5-diphenyl-tetrazolium bromide (MTT) succinate was prepared at 5 mg/ml and 30 µl of MTT solution was then added into each well and incubated for 3-4 hours at 37^{0}C and 5% CO_2. Subsequently, the wells were evacuated and 200µl of dimethyl sulphoxide (DMSO) was then introduced. The optical density (OP) of the solution was measured spectrophotometrically using an ELISA reader (Sunrise, Tecan) at a test and reference wavelength of 570 nm and 600 nm, respectively. Control cells without material extracts served as cell viability of 100%, and the cytotoxicity of the extracts was then calculated using the following formula:

Cell viability (%) = (OP of Test Group – OP of DMSO / OP of Control Group – OP of DMSO) X 100.

Following that, the level of cytotoxicity of the materials were classified into severe, moderate, slight and non-cytotoxic based on classification described by Bryan et al.[22]. The half maximal inhibitory concentration (IC_{50}) values, which represents the concentration of the material extracts/media that reduce the cell viability value by 50%, were then determined for each group. This is established by presenting all cell viability values in a graph where the x-axis stands for concentration and y-axis stands for cell viability values.

Statistical analysis

The data were entered using Predictive Analytics Software (PASW) Statistics version 18.0 (SPSS Inc., Chicago IL) and the analyses were performed using Kruskal-Wallis, followed by multiple Mann-Whitney tests with Bonferroni correction. The level of significance was set at $P<0.05$.

RESULTS

Flow cytometry analysis demonstrated that the cryopreserved stem cells from human exfoliated deciduous teeth was positively expressed for CD90 cell surface marker (98.5%) (Figure 2).

For cytotoxicity evaluation, the results showed that the cell viability values were proportional to the materials' extracts concentrations whereby all the tested groups exhibited severe cytotoxic activity at their maximum concentration (100 mg/ml). The cytotoxic activity is improved from moderate to slight when the extract concentrations are reduced. Fuji IX and Fuji II LC 40 secs were slightly cytotoxic at concentrations ≤25 mg/ml, while Fuji II LC 20 secs demonstrated its slight cytotoxicity only at ≤6.25 mg/ml (Figure 3).

The half maximal inhibitory concentration IC_{50} for each material was observed at 45.0 mg/ml, 31.25 mg/ml and 45.0 mg/ml for Fuji IX, Fuji II LC 20 secs and Fuji II LC 40 secs respectively (Figure 4).

Data analysis using Kruskal-Wallis test shows significant differences in cell viability among the three groups at all concentrations ($P<0.05$), except for 50 mg/ml (Figure 3). Accordingly, further pairwise comparison using multiple Mann-Whitney test with Bonferonni correction was performed. The results were significantly different between Fuji IX vs. Fuji II LC 20 secs and Fuji II LC 20 secs vs Fuji II LC 40 secs ($P<0.05$). In the meantime, a significant difference in cell viability was found between Fuji IX and Fuji II LC 40 secs, at their maximum concentration (100 mg/ml) ($P<0.05$), which were not observed at any other concentrations (Table 2).

Figure 2: a) Flow cytometry of the control group
b) Flow cytometry showing the positive expression of CD90 (98.5%).

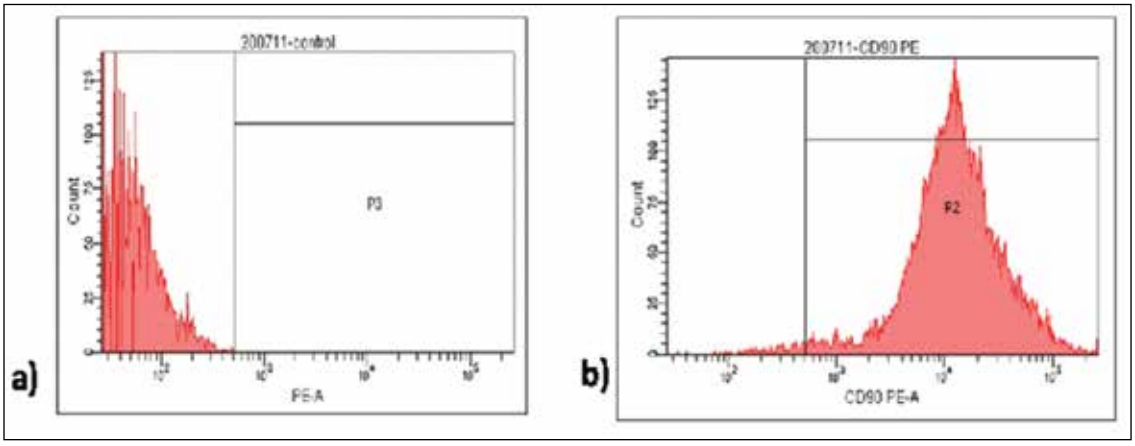


Figure 3: a) Analysis of cell viability values among Fuji IX, Fuji II LC (20secs, 40secs) on SHED using Kruskall-Wallis test.*Statistically significant *P*-value ≤ 0.05.

b) Classification of cell viability: severe, moderate, slight and non-cytotoxic (Bryan *et al* [22]).

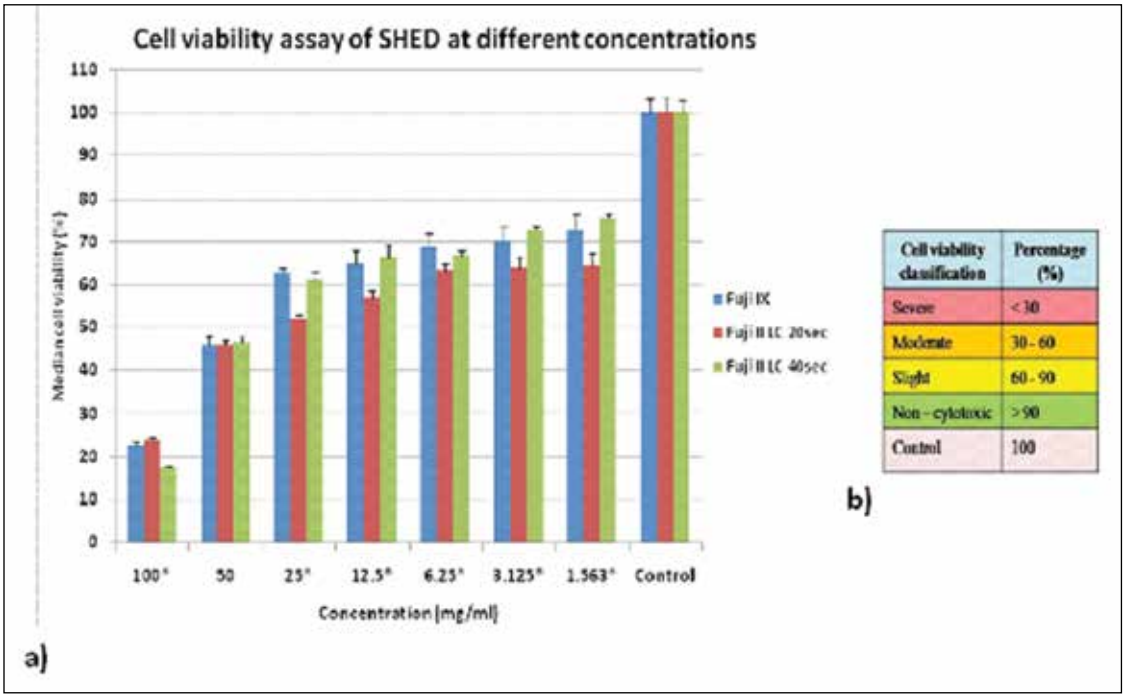


Table 2: Comparative cell viability analysis among Fuji IX, Fuji II LC (20secs, 40secs) on SHED using Mann Whitney test. *Statistically significant *P*-value ≤ 0.017.

Conc.	n	Comparison	z-stat	*P*-value
100	6	Fuji IX vs Fuji II LC 20sec	2.576	0.010*
	6	Fuji IX vs Fuji II LC 40sec	2.898	0.004*
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.903	0.004*
25	6	Fuji IX vs Fuji II LC 20sec	2.887	0.004*
	6	Fuji IX vs Fuji II LC 40sec	1.796	0.072
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.934	0.003*
12.5	6	Fuji IX vs Fuji II LC 20sec	2.882	0.004*
	6	Fuji IX vs Fuji II LC 40sec	0.480	0.631
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.882	0.004*
6.25	6	Fuji IX vs Fuji II LC 20sec	2.887	0.004*
	6	Fuji IX vs Fuji II LC 40sec	1.446	0.148
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.727	0.006*
3.125	6	Fuji IX vs Fuji II LC 20sec	2.567	0.010*
	6	Fuji IX vs Fuji II LC 40sec	1.286	0.199
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.887	0.004*
1.563	6	Fuji IX vs Fuji II LC 20sec	2.887	0.004*
	6	Fuji IX vs Fuji II LC 40sec	0.966	0.334
	6	Fuji II LC 20sec vs Fuji II LC 40sec	2.892	0.004*

DISCUSSION

The current literature provides scientific evidence that modified formulations of GIC can achieve reasonable long-term success in pediatric dentistry.[23,24] This clinical advantage justifies the use of SHED in this experimental investigation, particularly because these cells are capable of proliferation, can easily be expanded *in vitro*, and readily accessible from young patients.[25] In addition, *in vitro* cytotoxicity tests should be performed with the most appropriate cells, that is, cells homologous to human tissues of ultimate concern.[13] Indeed, the use of primary oral cells from human sources may correlate better with the *in vivo* situation with respect to the cytocompatibility of dental materials than using permanent cell lines derived from animals[26]. Thus, a primary culture of stem cells from human exfoliated deciduous teeth have been selected in this study, particularly when with the increase in the popularity of GIC-based restorative materials used in pediatric dentistry.

This study demonstrated that none of the study groups was deemed non-cytotoxic, irrespective of the material type and polymerization time. However, higher cell viability values were achieved when the polymerization time of RMGIC was doubled. Considering that conventional GIC also exerted some degree of cytotoxicity on SHED and does not contain HEMA, we assume that the presence of some other components in this material might produce a cytotoxic effect to the cultured cells. Ions, such as fluoride (F^-), aluminum (Al^{3+}), and strontium (SR^{2+}), were speculated to cause cytotoxic effects. However, Stanislawski *et al* [10] found that the concentrations of these ions are extremely low and that zinc (Zn^{2+}) was the only component found to be at a sufficiently high concentration to induce cytotoxicity.

Numerous *in vitro* studies assessed the cytotoxicity of conventional GIC and RMGIC. Most of these studies supported the concept

that the leachable components of these materials are responsible for a wide range of cytotoxic effects.[9,15,27] The findings and theory on the release of HEMA have also been corroborated by other authors using different cell types.[10,14,28,29] Nevertheless, most of these studies were notably conducted on RMGIC materials used as a base/liner rather than as restorative materials. Palmer *et al.*[29] observed that a larger amount of HEMA was released when the liner/base materials were tested (Vitrebond, Fuji Lining LC) than when restorative materials were used (Fuji II LC, Vitremer). This finding can be attributed to the fact that the liner/base-grade materials generally contain a significantly higher composition of HEMA than restorative materials, thus resulting in a higher percentage of HEMA release[29]. In addition, Geurtsen et al.[15] and Leyhausen *et al* [28] suggested that the severe cytotoxicity of RMGIC lining materials (Vitrebond) may be produced by chlorine benzene, iodine benzene, and bromide benzene, which are the decomposition products of their photo-initiator, diphenyl iodonium chloride.

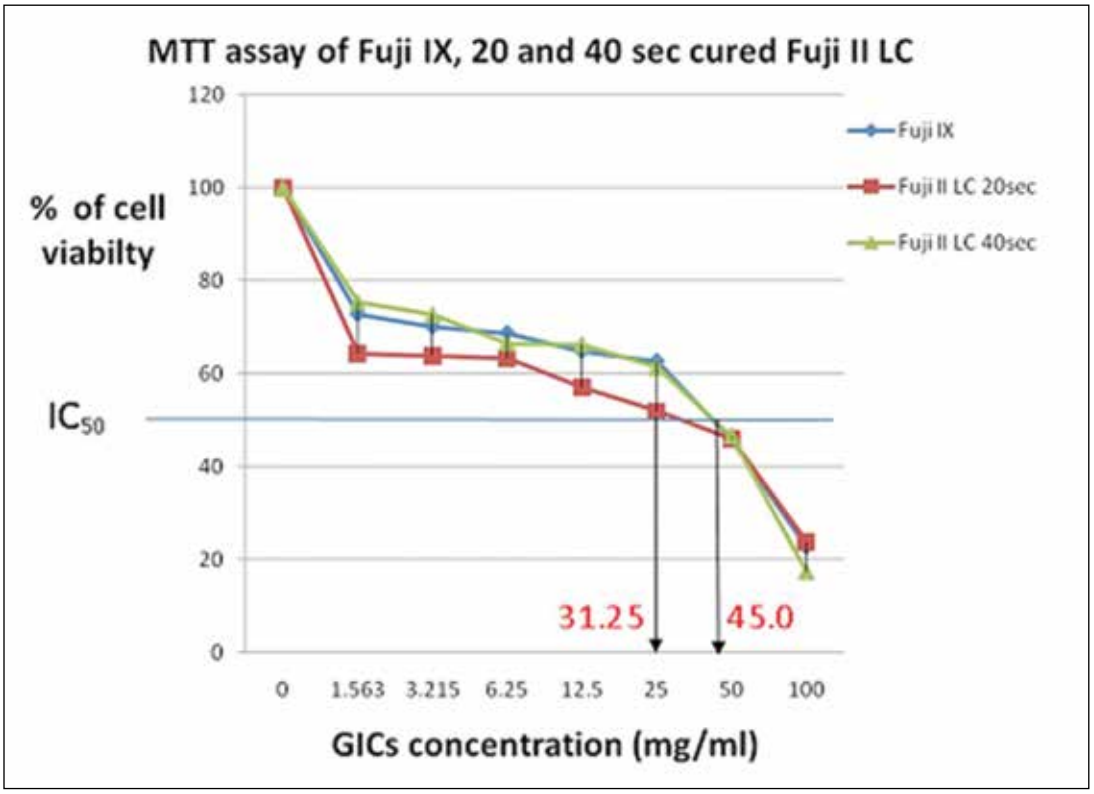
This study did not assess the leachable components in the culture medium, but we speculate that unpolymerized HEMA is responsible for the toxicity of RMGIC. Several studies have demonstrated that measurable quantities of HEMA are released into the storage solutions used,[10,14,15,29] which may induce a series of intracellular mechanisms when added to a cell culture, thus resulting in cell death via apoptosis[30]. This finding has recently been proven by Bakapoulou *et al* [31], who observed a significant cytotoxic effect of HEMA on SHED that can severely disturb the odontogenic differentiation potential of HEMA, thus compromising pulp tissue homeostasis and repair.

At a 25 mg/mL concentration and below, a significant difference in cell viability values was demonstrated between Fuji IX versus Fuji II LC (20 s) and between Fuji II LC (20 s) versus Fuji II LC (40 s). This finding indicates that extending the polymerization time of Fuji II LC causes less cell cytotoxicity than 20 s of the manufacturer's recommended time (MRT). This finding might correspond to a study by Palmer *et al.*[29], who found that over-cured specimens treated at 1.5 times longer than the recommended minimum time (RMT) (30 s) released significantly less HEMA than those cured for either RMT (20 s) or 0.5 RMT (10 s). This observation could be explained by the fact that a greater degree of monomer conversion might have occurred upon over-curing, thus reducing the concentration of HEMA release. However, the study by Palmer *et al.*[29] was notably unassociated with cytotoxicity evaluation.

By contrast, Aranha *et al* [32] found that the variation of polymerization time did not influence the cytotoxicity of RMGIC, even when half the MRT was used. In addition to the difference in materials and cell line used, the contradiction between our results and those by Aranha *et al* [32] might be attributed to the difference in methodological procedures. In the study by Aranha *et al.*[32] the samples were rinsed with phosphate-buffered saline and culture medium for 5 min, which may have removed a considerable amount of HEMA, thus masking the actual cytotoxic activity of the test materials. In our study, the samples were only swabbed with alcohol for sterilization before being added to the culture medium.

Notably, while all materials were deemed severely and moderately cytotoxic at 100 and 50 mg/mL concentrations, respectively, Fuji II LC 20 s exhibited slight cytotoxicity effects at a significantly lower concentration than Fuji IX and Fuji II LC 40 s. A low conversion rate of monomer into the polymerized end product attributed to insufficient or sub-optimal polymerization time could possibly

Figure 4: Cell viability and IC_{50} of Fuji IX, Fuji II LC (20secs, 40secs) on SHED after 72 h.



explain this observation, as such condition may cause a greater amount of toxic monomer to leach out of the materials and thus be released in the culture medium.

Based on these results, we find that the polymerization time of RMGIC should be optimized to reduce the cytotoxic effect, which could possibly aid in reducing the release of residual or free HEMA. Therefore, within the limitations of this study, doubling the polymerization time of RMGIC (Fuji II LC) beyond the MRT is advised to reduce the material cytotoxic effect. However, more in vitro and in vivo studies should be conducted to validate these findings further.

CONCLUSIONS

Different types of GICs may cause specific cytotoxic effects on SHEDs. RMGICs polymerized at 20 s exhibited the least favorable cell viability among all groups. Nevertheless, the cell viability of these RMGICs was comparable with that of conventional GICs when the manufacturer's recommended polymerization time was doubled (40 s).

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