Cytotoxic Effect of a Novel Glass Ionomer Nano Zirconia-Silica-Hydroxyapatite Hybrid Material on Human Gingival Fibroblasts

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Abstract

In dentistry, there is an increased use of particles of nano scale to enhance certain traits of dental material, especially conventional glass ionomer cement (cGIC). A novel GIC-nanozirconia-silica-hydroxyapatite (GIC nanoZrO_2-SiO_2-HA) was developed and morphologically studied with the help of transmission electron microscopy (TEM), which showed significant improvement in physico-mechanical, chemical and aesthetics properties. This study was done to assess the cytotoxicity of GIC nanoZrO_2-SiO_2-HA on human gingival fibroblasts (HGFs). The HGFs were subcultured within 96 well culture plates. The material extracts of cGIC and GIC nanoZrO_2-SiO_2-HA were prepared by placing measured quantity of set samples in complete growth medium. The extracts were then added at varying concentrations into the plates and incubated for 24 h and 72 h. The viability of cell culture was then determined using MTT assay. The results show that both GIC nanoZrO_2-SiO_2-HA and cGIC showed cell viability more than 50% at all concentrations of the material extracts except 200 mg/ml. Therefore, both GIC nanoZrO_2-SiO_2-HA demonstrated cytotoxicity only at 200 mg/ml concentration. However, a statistically significant difference in cell viability was seen at both 100 and 200 mg/ml concentrations for both the incubation periods.

Keywords: Glass ionomer cement, hydroxyapatite, zirconia, cytotoxicity.

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Introduction

Nowadays there is an urgent demand for the use of tooth-coloured dental restoratives such as conventional glass ionomer cement (cGIC) and composite resins. However, off late the usage of cGIC has been hampered and seen a decline owing to its below par strength [1-3]. Recently, the addition of alumina, silica, hydroxyapatite, zirconia and glass fibres into cGIC were attempted in order to improve the clinical usage of cGIC [4-10].

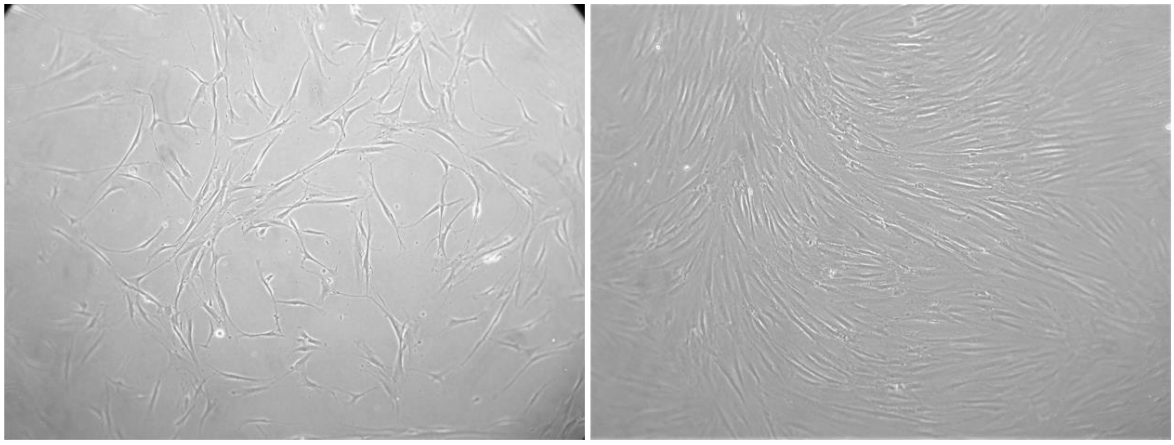
Recently at the School of Dental Sciences, Universiti Sains Malaysia, a novel nano zirconia–silica–hydroxyapatite (nanoZrO_2-SiO_2-HA) composite was synthesized by one-pot synthesis and incorporated into cGIC. The effect induced by the phenomenon of adding nanoZrO_2-SiO_2-HA to the cGIC on its physico-mechanical properties was analysed. The addition of nanoZrO_2-SiO_2-HA produced surprising improvements in the mechano-chemical properties of cGIC. It also possessed a mean roughness same as that of cGIC [11, 12]. In an earlier study from our research group, the authors found that the GIC nanoZrO_2-SiO_2-HA exhibited improved microhardness as compared to the cGIC [13]. However, concerns regarding the biocompatibility of the nanoZrO_2-SiO_2-HA and specifically the cytotoxic potential when added to cGIC needed to be addressed. Therefore, this study was done to assess the cytotoxicity of the GIC nanoZrO_2-SiO_2-HA on the HGFs employing a colorimetric assay (MTT) for the safety of its future usage.

Materials and Methods

The nanoZrO_2-SiO_2-HA powder was synthesized via one pot sol-gel technique based on previous studies [11, 13]. The synthesized nanoZrO_2- SiO_2- HA powder was morphologically characterized using transmission electron microscopy (TEM) and then the nanoZrO_2- SiO_2- HA powder was incorporated into cGIC (Fuji IX, GC Corp.; Japan), following which cytotoxicity was evaluated using MTT assay. Commercially available HGF(CRL-2014™), minimal essential medium alpha (MEM-α), foetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS) and trypsin-EDTA (0.25%) were obtained from ATCC®, USA. For MTT assay, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] powder and dimethyl sulfoxide (DMSO) were obtained from Invitrogen™, (USA).

Cell culture

The frozen HGFs were thawed, centrifuged and cultured in a T-25 flask (Nunc™ EasYFlask™, ThermoFisher, USA) containing 5 ml of complete growth medium (CGM) (MEM-α + supplements) at 37˚C with 5% CO_2. The growth of the HGF culture Cell growth was periodically observed under an inverted phase contrast microscope (Zeiss, Germany) after seeding (Figure 1a). Once the HGFs reached confluence (Figure 1b), they were passaged and subcultured in a T-75 flask. The cultures derived from the 5th and 6th passages were selected to carry out this experiment.



(a)Photomicrograph of HGF a few hours after seeding in a T-25 culture flask

(a)Photomicrograph of HGF at confluence

Figure1a & b: Inverted phase microscope images of HGFs at 400x magnification

Material extract preparation

Samples measuring 10 mm × 2 mm of cGIC and GIC nanoZrO_2-SiO_2-HA were prepared by mixing the powder and liquid (P/L=1:1) and loaded in a PTFE mould. After 15 min, samples were removed from the mould and UV sterilized. They were then immersed in 5 ml of CGM (200 mg/ml concentration) and incubated for 72 h at 37 ˚C with 5% CO_2 [14]. Following which the extract from both the groups was filtered and serially diluted. The serially diluted extracts were then pipetted into 96 well culture plates (200µl/well) containing HGF cultures ($5x10^3$ cells/well).

Cytotoxicity evaluation using MTT assay

The MTT assay was conducted as per the protocol laid out in a previous study [14]. The culture medium devoid of HGF or extract was designated to be negative control. A pair each of 96 well culture plate were prepared for the cGIC and the hybrid material extracts and one plate each of the pair were incubated for 24 h and 72 h respectively at 37º C with 5% CO_2 [14, 15]. Following the incubation period, the effect of the material extract on the mitochondrial dehydrogenase enzyme was measured by MTT test. All data were statistically analysed using IBM SPSS version 23 (IBM Corp., USA). The Mann-Whitney test was used to determine overall statistical significance between cGIC and GIC nanoZrO_2-SiO_2-HA at each concentration and for each incubation period with the significance level set at $p \leq 0.05$.

Results

TEM evaluation of nanopowder

Comparative morphologies of nano ZrO_2, nano SiO_2 and nano HA prepared by the modified sol gel technique as observed under TEM are shown in Figure 2(a & b). The TEM micrograph confirms the presence of asymmetrically elongated rod shaped HA particles represented by yellow arrows (Figure 2 b), similar to past studies [5, 9]. The smaller spherical/oval structures interspersed between and along the HA are the SiO_2 and ZrO_2 represented by white arrows (Figure 2 b) [8, 16-18]. Measurements on the micrographs confirmed that the spherical and oval particles were in the nano scale range. This show that the

synthesis of the nanopowder was successful in producing homogenous and non-agglomerated powder[11].

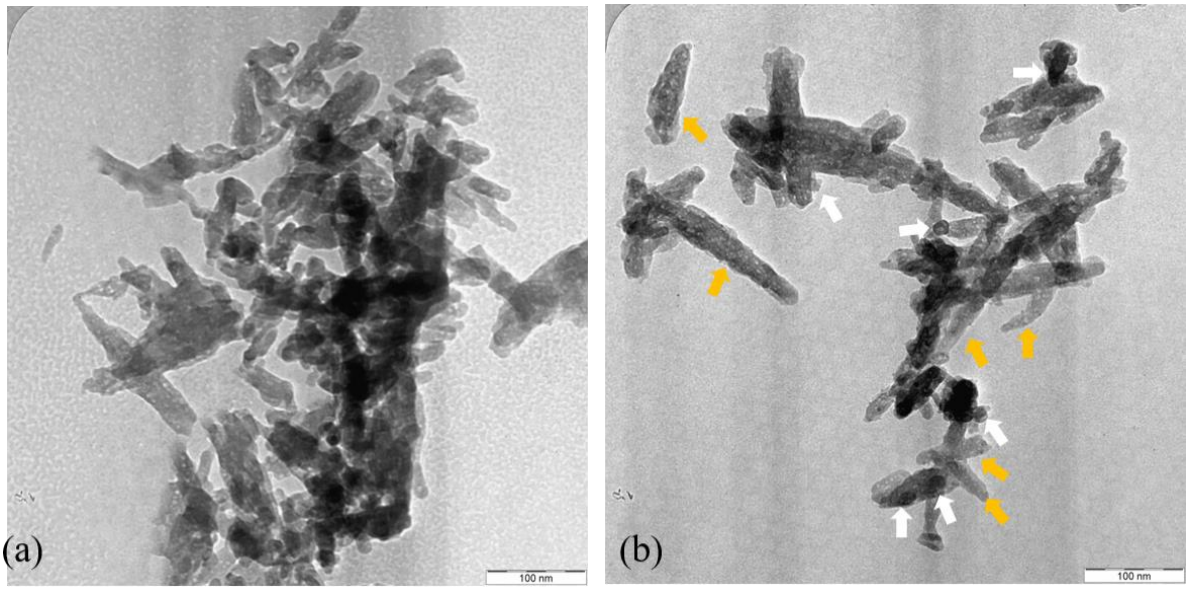


Figure 2 a & b: Transmission electron microscope images (100nm scale) of nanoZrO_2-SiO_2-HA at 100000x

Cytotoxicity evaluation of GIC nanoZrO_2-SiO_2-HA

The data analysis to measure the cell viability (%) of the two groups after 24 h and 72 h incubation period using Mann Whitney test is summarized in **Error! Not a valid bookmark self-reference.** I and Table II. The MTT assay is usually done after 24 h of incubation in order for the cells seeded in the 96 well plates to attach themselves to the culture well and attain confluence. Based on previously published studies evaluating the cytotoxicity of GICs using MTT assay, we chose to perform the MTT assay at 24 h and 72 h after incubation [15, 19-24]. The cell viability (%) after 24 h incubation period was found to be highest with cGIC material extract at higher concentrations of 25, 50, 100 and 200 mg/ml (Figure 3). Both cGIC and GIC nanoZrO_2-SiO_2-HA showed more than 50% cell viability at all concentrations except 200 mg/ml. However, there was statistically significant difference between cGIC and GIC nanoZrO_2-SiO_2-HA at 100 and 200 mg/ml ($p \leq 0.05$) for the 24 h incubation period as shown in Table I. There was decrease in cell viability with increase in concentrations of the material extract.

The cell viability (%) after 72 h incubation period was found to be highest with GIC nanoZrO_2-SiO_2-HA material extract at the higher concentrations of 50, 100 and 200 mg/ml (Figure 4). Both GIC nanoZrO_2-SiO_2-HA and cGIC showed more than 50% cell viability at all concentrations except 200 mg/ml. However, there was statistically significant difference between GIC nanoZrO_2-SiO_2-HA and cGIC at only 100 and 200 mg/ml for the 72 h incubation period ($p \leq 0.05$) as shown in Table 2. There was decrease in cell viability with increasing concentrations of the material extract.

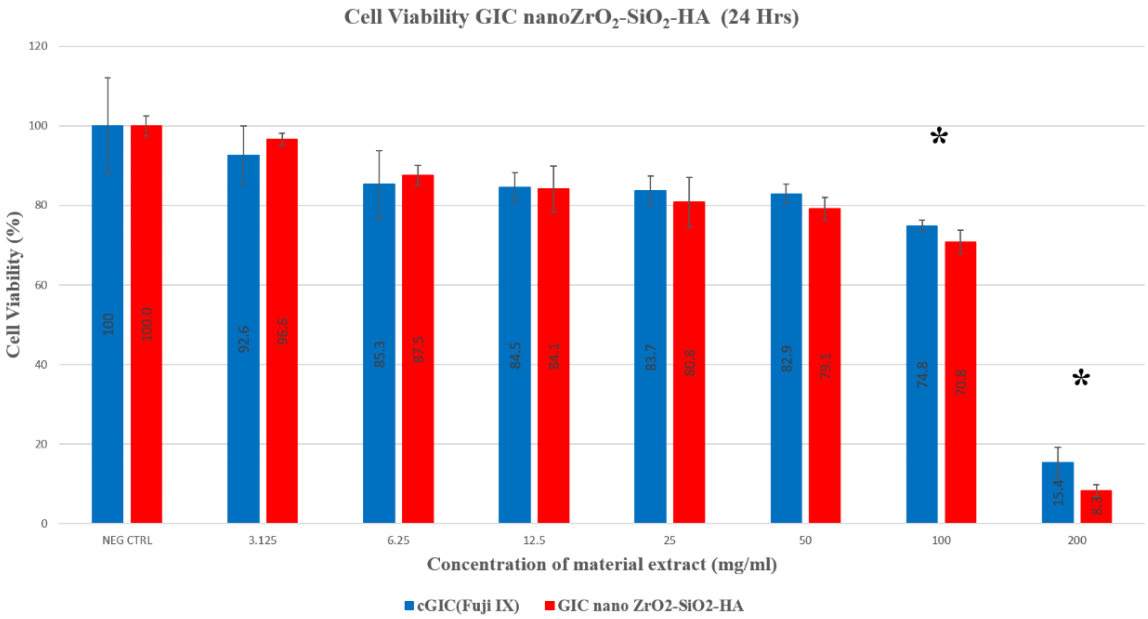


Figure 3: Cell viability percentage of HGFs after 24 h incubation with GIC nanoZrO$_2$-SiO$_2$-HA and cGIC

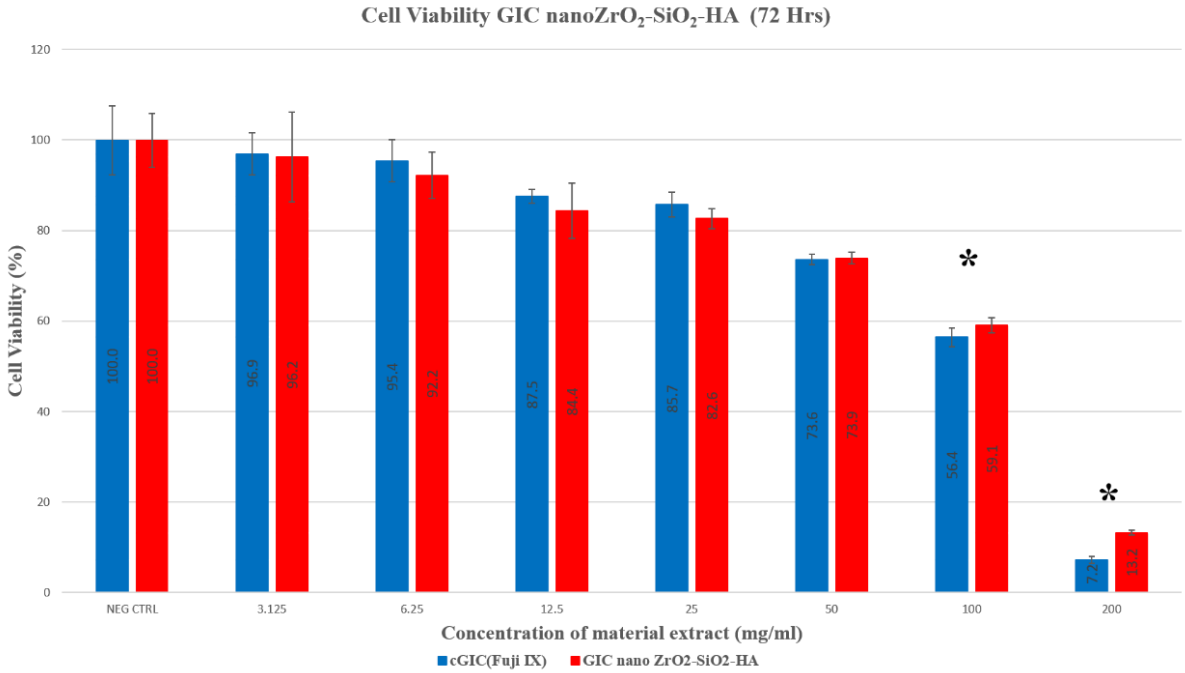


Figure 4: Cell viability percentage of HGFs after 72 h incubation with GIC nanoZrO$_2$-SiO$_2$-HA and cGIC

Table I: Cell viability test results for 24 h incubation period

Concentration mg/ml	Material (n=15)	Mean (SD)% 24 h	Median (IQR)% 24 h	*P* value
200	cGIC	15.45 ± 7.32	14.63	0.046*
	GIC nanoZrO_2-SiO_2-HA	8.33 ± 1.44	7.50	
100	cGIC	74.80 ± 8.45	75.61	0.043*
	GIC nanoZrO_2-SiO_2-HA	70.83 ± 2.89	72.50	
50	cGIC	82.93 ± 3.73	82.93	0.121
	GIC nanoZrO_2-SiO_2-HA	79.17 ± 2.89	77.50	
25	cGIC	83.74 ± 3.73	82.93	0.275
	GIC nanoZrO_2-SiO_2-HA	80.83 ± 6.29	80.00	
12.5	cGIC	84.55 ± 2.44	85.37	0.825
	GIC nanoZrO_2-SiO_2-HA	84.17 ± 5.77	87.50	
6.25	cGIC	85.37 ± 1.41	80.49	0.507
	GIC nanoZrO_2-SiO_2-HA	87.50 ± 2.50	87.50	
3.125	cGIC	92.68 ± 3.73	92.68	0.507
	GIC nanoZrO_2-SiO_2-HA	96.67 ± 1.44	97.50	
Negative Control	cGIC	100.81 ± 12.03	95.12	0.513
	GIC nanoZrO_2-SiO_2-HA	100.00 ± 2.50	100.00	

* The mean difference is significant at $p \leq 0.05$

Table II: Cell viability test results for 72 h incubation period

Concentration mg/ml	Material (n=15)	Mean (SD)% 72 h	Median (IQR)% 72 h	*P* value
200	cGIC	7.29 ± 0.78	7.04	0.050 *
	GIC nanoZrO_2-SiO_2-HA	12.72 ± 0.59	12.84	
100	cGIC	56.44 ± 2.07	57.04	0.050 *
	GIC nanoZrO_2-SiO_2-HA	59.69 ± 1.66	59.14	
50	cGIC	73.61 ± 1.12	73.42	0.827
	GIC nanoZrO_2-SiO_2-HA	73.57 ± 1.36	73.15	
25	cGIC	85.71 ± 2.78	85.66	0.275
	GIC nanoZrO_2-SiO_2-HA	83.82 ± 2.22	82.60	
12.5	cGIC	87.57 ± 1.53	87.14	0.827
	GIC nanoZrO_2-SiO_2-HA	84.47 ± 6.10	87.94	
6.25	cGIC	88.80 ± 4.67	87.14	0.275
	GIC nanoZrO_2-SiO_2-HA	92.26 ± 5.05	89.88	
3.125	cGIC	96.95 ± 4.64	99.63	0.825
	GIC nanoZrO_2-SiO_2-HA	96.29 ± 9.93	92.61	
Negative Control	cGIC	99.92 ± 7.65	103.70	0.827
	GIC nanoZrO_2-SiO_2-HA	99.92 ± 5.97	97.28	

* The mean difference is significant at the $p \leq 0.05$

Discussion

The cGIC and GIC 5% nanoZrO_2-SiO_2-HA exhibited cell viability greater than 50% except for the highest extract concentration (200mg/ml) at both 24 h and 72 h incubation. Similar findings have been reported previously by other researchers [14, 23, 25, 26]. The cell viability (%) after 24 h incubation period at 200 mg/ml and 100 mg/ml concentration of the extract was found to be significantly lower for GIC 5% nanoZrO_2-SiO_2-HA as compared to cGIC ($p< 0.050$). Similar finding has been reported by Noorani, *et al.* [15] in their study on a HA-Si-GIC material. They speculated that the incorporation of nano-HA-Si into GIC may have resulted in the formation and release of by products or components that may be toxic to the cells at highest concentration (200 mg/ml).

Previous studies on GIC cytotoxicity have demonstrated a correlation between the increased initial cytotoxicity of GIC and its initial F^- ion burst [27, 28]. Since in a recently published work [12], we have already established that F^- ion release of GIC nanoZrO_2-SiO_2-HA group was significantly higher than the cGIC especially during the first 24 h to 48 h ($p<0.05$). Therefore, it is reasonable to accept that the reason for significantly lower cell viability (%) after 24 h incubation period for GIC nanoZrO_2-SiO_2-HA at 200 mg/ml and 100 mg/ml concentration as compared to cGIC is indeed to a certain extent due to the higher F^- ion elusion. This initial F^- elusion could have suddenly changed the pH of the culture medium, resulting in more cell deaths [29]. In line with this reasoning, cell viability (%) after 72 h incubation period at 200 mg/ml and 100 mg/ml concentration of the extract was found to be significantly higher for GIC nanoZrO_2-SiO_2-HA as compared to cGIC ($p< 0.050$), as the initial massive F^- ion elusion tapers out after the first 24 h. In contrast, another study by Noorani, *et al.* [15] found that, the cell viability (%) at 200 and 100 mg/ml concentration of the extract was lower for the HA-Si-GIC material when compared to cGIC even after 72 h.

A few recent studies that assessed the cytotoxicity of nanoHA or HA- SiO_2 alone or in combination of cGIC reported a moderate to low cytotoxic response only at the highest concentration of the material extract. At all other lower concentrations, there was no statistical difference between the cytotoxicity of the experimental GIC and the cGIC [15, 21, 30]. Similarly, in this study, GIC nanoZrO_2-SiO_2-HA also demonstrated increased cytotoxicity compared to cGIC at 200 mg/ml at 24 h incubation. However, at 72 h incubation, results of MTT assay showed that GIC nanoZrO_2-SiO_2-HA exhibited lower cytotoxic response as compared to cGIC at 200 mg/ml which was statistically significant ($p<0.05$).

Conclusion

Based on the results of MTT assay, both GIC nanoZrO_2-SiO_2-HA and cGIC showed cell viability more than 50% at all concentrations of the material extracts except 200 mg/ml. Therefore, both GIC nanoZrO_2-SiO_2-HA demonstrated cytotoxicity only at 200 mg/ml concentration. However, a statistically significant difference in cell viability was seen at both 100 and 200 mg/ml concentrations for both the incubation periods. Hence, it is can be concluded that the GIC nanoZrO_2-SiO_2-HA has a cytotoxic response similar to cGIC.

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Author contributions

All authors contributed towards data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

This work does not involve any ethical issues.

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