



Cytotoxic Effect of Addition of Different Concentrations of Nanohydroxyapatite to Resin Modified and Conventional Glass Ionomer Cements on L929 Murine Fibroblasts

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Article Info

Article type:
Original Article

Article History:

Received: 14 Nov 2020
Accepted: 23 Apr 2021
Published: 17 May 2021

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ABSTRACT

Objectives: In this study we assessed the cytotoxic effect of nanohydroxyapatite (NHA) incorporated into resin modified and conventional glass ionomer cements (RMGICs and CGICs) on L929 murine fibroblasts.

Materials and Methods: In this in vitro study, 0wt%, 1wt%, 2wt%, 5wt%, 7wt% and 10wt% concentrations of NHA were added to Fuji II LC RMGIC and Fuji IX CGIC powders. Eighteen samples (5×3mm) were fabricated from each type of glass ionomer, in six experimental groups (n=3): CG₀, CG₁, CG₂, CG₅, CG₇, CG₁₀, RMG₀, RMG₁, RMG₂, RMG₅, RMG₇, and RMG₁₀. Samples were incubated for 72h. The overlaying solution was removed and added to L929 fibroblasts. The methyl thiazolyl tetrazolium bromide (MTT) assay was performed at 24, 48 and 72h. The wavelength was read by a spectrophotometer. Data were analyzed by ANOVA and Tukey's test.

Results: There was no significant difference in cytotoxicity of the two types of glass ionomers, with and without NHA, except for CG₀ and RMG₀ groups after 72h. RMG₀ group was significantly more cytotoxic than the CG₀ group (P<0.05). In CG groups during the first 24h, the cytotoxicity of CG₅ and CG₇ groups was significantly higher than that of CG₁; while, there was no significant difference between the RMG groups. Cytotoxicity significantly decreased in all groups after 24h (P<0.05).

Conclusion: Incorporation of NHA into Fuji II LC RMGIC and Fuji IX CGIC did not affect their biocompatibility and therefore its addition to these materials can provide favorable biological properties, especially considering its beneficial effects on the other properties of GICs.

Keywords: Hydroxyapatites; Glass Ionomer Cements; Fibroblasts; Toxicity Tests

- **Cite this article as:** Golkar P, Ranjbar Omrani L, Zohourinia Sh, Ahmadi E, Asadian F. Cytotoxic Effect of Addition of Different Concentrations of Nanohydroxyapatite to Resin Modified and Conventional Glass Ionomer Cements on L929 Murine Fibroblasts. *Front Dent.* 2021; 18: 17. doi: 10.18502/fid.v18i17.6248.

INTRODUCTION

Conventional glass ionomer cements (CGICs) were first introduced in 1970 to replace

silicate cements in dentistry. These cements have extensive applications in dentistry due to favorable properties such as fluoride release,

antibacterial effect, chemical bond to tooth structure, having a coefficient of thermal expansion similar to that of tooth structure, modulus of elasticity similar to that of dentin, and biocompatibility. However, these cements have some drawbacks as well such as high technical sensitivity, lower esthetics compared with composite resins, and low mechanical properties, which limit their clinical application. Due to the brittleness and low compressive strength, CGICs cannot be used as restorative materials in occlusal load-bearing areas [1,2]. To overcome the limitations of CGICs, resin-modified glass ionomer cements (RMGICs) were developed. RMGICs have lower moisture sensitivity, enhanced mechanical strength, extended working time, and easy clinical handling; however, the reported results were not in acceptable range. Additionally, they were found to be more cytotoxic than CGICs due to the presence of 2-hydroxyethyl methacrylate monomer in their composition [3].

Recently, application of nano-sized bio-material particles was proposed to improve the mechanical properties of glass ionomers, such as addition of nano-TiO₂, nano-hydroxyapatite (NHA), and silver nanoparticles [4-6].

Hydroxyapatite is a naturally occurring mineral, and is the major component of bone and tooth structure [7]. The NHA particles are smaller in size than hydroxyapatite particles and have higher surface charge, which enhance their strength and facilitate their application. In some previous studies, NHA was added to CGICs and RMGICs in 1% to 10% concentrations as a filler with favorable properties and resulted in an increase in flexural strength, compressive strength, wear resistance, antibacterial activity, and bond strength to dentin [5, 8-10].

As a result of high success rate of glass ionomers for root surface restorations, they are the material of choice for these lesions. Since they are in close contact with the gingival tissue, they must be biocompatible [11]. They should not contain toxic or leachable elements since they may be released into the oral environment and result in inflammatory response and tissue damage

[12]. To minimize the risk of adverse local and systemic complications, biocompatibility of dental materials must be assessed in vitro and in vivo prior to their use in the clinical setting in order to ensure their safety [13]. L929 murine fibroblasts are routinely used for cytotoxicity testing of dental materials due to their optimal reproducible growth rates and biological response close to that of human cells [14].

No studies have been conducted on cytotoxic effects of adding NHP to CGICs. Few studies have been conducted on the cytotoxicity of adding NHA to RMGIC. Genaro et al. [3] showed that adding 2%, 5%, and 10% NHA to RMGI decreased its toxic effect on odontoblasts; this reduction increased by increasing the percentage of NHA [3]. Molskin et al. [15] revealed that cytotoxicity of NHA is related to its physicochemical properties. While NHA seems to be a safe material, it may be speculated that the reaction of incorporation of NHA into CGICs and RMGICs may result in formation and release of by-products or components that are cytotoxic [16].

It is crucial to carry out studies that evaluate the biological properties of adding nanoparticles to RMGICs and CGICs aside from other properties. Thus, this study sought to assess the effect of addition of different concentrations of NHA on the cytotoxicity of RMGICs and CGICs. The null hypothesis was that addition of different concentrations of NHA to RMGICs and CGICs would have no significant effect on their cytotoxicity against L929 murine fibroblasts.

MATERIALS AND METHODS

Preparation of samples:

This in vitro, experimental study was conducted on 18 Fuji II LC (GC Corporation, Tokyo, Japan) and 18 Fuji II (GC Corporation, Tokyo, Japan) samples. The samples were divided into six groups of three with 0, 1, 2, 5, 7, and 10wt% NHA.

According to a pilot study, the amount of powder required to produce three RMGI samples with 1wt% NHA was found to be 2 g. The amount of NHA required to obtain 2 g powder was calculated to be 0.02 g. Thus, to

obtain samples with 1wt% NHA, 0.02 g of NHA powder and 1.98 g of RMGIC powder were mixed. The values in other groups were also calculated as such. The amount of powder required to produce three CGIC samples with 1wt% NHA was found to be 1.7 g. The amount of NHA required to obtain 1.7 g powder was calculated to be 0.017 g. The values in other groups were calculated as such. The obtained powders for each concentration of NHA of each glass ionomer were mixed in a mortar and pestle for 2 min to obtain a homogenous mixture of particles. The powders were then kept in dark bottles until the experiment.

To fabricate RMGIC samples, the powder and liquid were mixed in 3.2/1 weight ratio. The mixing time was less than 25 s as recommended by the manufacturer. The powder to liquid ratio was 2.7/1 for the Fuji II CGIC. The pastes were transferred to a stainless-steel mold measuring 5×3 mm. The paste was condensed from one side to prevent void formation. The setting time was 180 s for the CGIC. For the RMGIC, a glass slab was placed over the mold and the cement was light-cured with a curing unit (Blue Phase; Ivoclar Vivadent, Schaan, Liechtenstein) with a light intensity of 600 mW/cm² for 40 s from both sides (20 s from each side). For each percentage of NHA, three samples were fabricated as such. The group definitions and abbreviations are mentioned in Table 1.

Table 1. Group definitions and abbreviations

Type of Glass Ionomer	NHP (%)	Group
Fuji II LC	0	RMG ₀
	1	RMG ₁
	2	RMG ₂
	5	RMG ₅
	7	RMG ₇
	10	RMG ₁₀
Fuji II	0	CG ₀
	1	CG ₁
	2	CG ₂
	5	CG ₅
	7	CG ₇
	10	CG ₁₀

NHP: Nanohydroxyapatite

The negative control group contained L929 fibroblasts and RPMI culture medium. The

positive control group contained L929 fibroblasts and dimethyl sulfoxide culture medium.

Cell culture and passage:

L929 murine fibroblasts were obtained from the National Genetic Bank of Iran and cultured. The fibroblasts were cultured in flasks at 37°C and 5% CO₂. The culture medium contained 10% fetal bovine serum, penicillin-streptomycin, and L-glutamine. The overlaying medium was discarded and the 4th passage cells were detached from the flasks using 0.2% trypsin. After neutralization of trypsin, the cells were collected by centrifugation, and cell suspension was prepared. Next, 100 µL of the cell suspension containing 5×10³ cells was added to each well of a 96-well plate, and the cells were incubated for 24 h.

Experiment:

The prepared disc-shaped samples were subjected to UV radiation for 3 h for sterilization. Next, 1 mL of the cell culture medium was added to each sample and incubated at 37°C for 72 h with 95% oxygen and 5% CO₂ (Innova-Co 170; New Brunswick Scientific, Edison, NJ, USA). Afterwards, the overlaying medium was removed and filtered through a membrane with 0.22 µm pore size. The overlaying medium was discarded, and 100 µL of the extracted medium was added to the cells in a 96-well plate. The methyl thiazolyl tetrazolium (MTT) assay was done at 24, 48, and 72 h after cell incubation. Dimethyl sulfoxide (which is a toxic substance) was added to three wells as the positive control group. In three wells, as the negative control group, only cells and the culture medium were added with no other substance.

MTT assay:

For the MTT assay, 200 µL of the MTT solution (Sigma-Aldrich, St. Louis, MO, USA) and 5 mg/mL concentration of phosphate buffered saline were added to each well and incubated at 37°C, 95% humidity and 5% CO₂ for 4 h, to convert the soluble MTT yellow salt into insoluble purple formazan crystals by the activity of viable cells. Next, the supernatant was discarded and replaced with 100 µL of dimethyl sulfoxide (DMSO, Gibco BRL, Grand

Island, NY, USA). The plate was agitated for 30 min, and the optical density was read by a spectrophotometer (Pharmacia, Biotech, USA) at 570 nm wavelength. At each time point of 24, 48 and 72 h, the cell viability values were recorded for each cement, and the control groups.

Cytotoxicity was rated based on cell viability relative to controls as: non-cytotoxic >90% cell viability, slightly cytotoxic: 60–90% cell viability, moderately cytotoxic: 30–59% cell viability, and severely cytotoxic: <30% cell viability [17].

Statistical analysis:

One-way ANOVA was applied for the comparison of the groups, which showed a significant difference between the groups ($P < 0.05$). Pairwise comparisons of the mean values of the groups were performed using the Tukey's HSD test. Level of significance was set at $P < 0.05$.

RESULTS

Table 2 demonstrates the cell viability values obtained for each cement, and control groups at different times. The results showed that there was a significant difference in cytotoxicity of RMG₀ and CG₀ after 72h, and RMG₀ was more cytotoxic ($P = 0.042$; 91%±0.01 cell viability of CG₀ in comparison with 86%±0.04 cell viability of RMG₀). RMG₀ was slightly cytotoxic and CG₀ was non-

cytotoxic after 72h. Following the addition of NHA, a different trend of cytotoxicity was noted in the two types of cements.

In the CG groups, there was no significant difference in cell viability between CG₀ and other groups ($P > 0.05$). However, in the first 24h, increasing the NHA weight percentage from CG₁ to CG₇, significantly decreased the cell viability ($P < 0.05$) but further addition of NHA up to 10wt% increased the viability of cells, and the cytotoxicity of CG₅ and CG₇ were significantly higher than that of CG₁ ($P < 0.05$). The cytotoxicity decreased from 24h to 48h and 72h. Addition of NHA to RMGIC groups did not affect their cell viability in the first 24h ($P > 0.05$). However, after 48h, the cytotoxicity in all groups decreased; while, the cytotoxicity of RMG₀ was significantly higher than that of RMG₁, RMG₂, and RMG₁₀ groups. The reduction in cytotoxicity continued for up to 72h; the RMG₂ and RMG₁₀ groups were significantly more cytotoxic; however, there was no significant difference between these groups and the RMG₀ group ($P > 0.05$).

DISCUSSION

Nano-materials often measure 100nm or smaller in size and have very high surface energy, which significantly affects their physical and chemical properties, compared with mass materials.

Table 2. Mean±standard deviation of percentage of cell viability in different groups

Fuji II LC		Cell viability					
Groups		RMG ₀	RMG ₁	RMG ₂	RMG ₅	RMG ₇	RMG ₁₀
Time (h)	24	60±0.03	56±0.03	56±0.01	57	55±0.02	63±0.03
	48	77±0.03	90±0.05	88±0.01	79±0.05	83±0.03	89±0.02
	72	86±0.04	91±0.03	81±0.02	81±0.02	92	96±0.02
Fuji II		Cell viability					
Groups		CG ₀	CG ₁	CG ₂	CG ₅	CG ₇	CG ₁₀
Time (h)	24	52±0.31	61±0.02	55	51±0.01	50.6±0.01	55±0.02
	48	82±0.22	86±0.07	85±0.02	83±0.03	80±0.04	94±0.03
	72	91±0.1	92±0.32	90±0.01	85±0.03	91±0.47	99.8±0.05

Also, due to high surface energy, nanoparticles form strong bonds to each other and to other materials; this phenomenon is referred to as agglomeration [18]. To improve the esthetic properties of CGICs, nanotechnology has been used for the fabrication of a new RMGIC namely Ketac nano™ (3M ESPE, St. Paul, MN, USA). In this group of RMGICs known as nanoionomers, a combination of fluoro-aluminosilicate glass and unreacted nano fillers confers unique properties to the cements, which are not present in other CGICs [19]. NHA particles, as a nanofiller with optimal biological properties, have also been added to CGICs, and some of the properties of this new cement have been previously investigated [7]. No dental material has all the ideal characteristics [20]. Dental materials are in contact with the tissues and oral fluids. Thus, selection of a material must be based on its physical and mechanical properties as well as its biocompatibility. Therefore, assessment of cytotoxicity and biocompatibility of materials is as important as their physical and mechanical properties [21]. Evaluation of cytotoxicity of materials *in vitro* is one method to assess their biocompatibility. Assessment of cytotoxicity under controlled and reproducible *in vitro* conditions can help determine the effect of concentration, type and time of exposure to the material on viability of cells. Many different laboratory tests are available to evaluate the cytotoxic activity. The MTT assay is very sensitive and depends on the potency of mitochondrial dehydrogenase enzyme of vital cells to breakdown the yellow tetrazolium ring of MTT. The blue crystals of formazan are impermeable to cell membrane and accumulate within the vital cells. The level of formazan produced is proportional to the number of vital cells [22].

Motskin et al. [15] assessed the correlation of properties of NHA particles with their cytotoxicity and bio-stability and showed that particle load was strongly associated with cytotoxicity, and physical and chemical properties of NHA particles. Shi et al. [23] evaluated the effect of size of NHA particles on proliferation and apoptosis of pre-osteoblast

cells and reported that particles with 20 nm size had the best effect on cell proliferation and inhibition of apoptosis. In our study, according to the information provided by the manufacturer, the particles were smaller than 10 μ m. Size control during the fabrication of NHA particles is difficult, and this may be a possible explanation for the difference in the results of different studies.

The results of this study showed that cell viability in Fuji II cement groups was higher than in Fuji II LC at 72 h; which is in accordance with the results of other studies [24,25]. This result might be due to the release of 2-hydroxyethyl methacrylate resin monomer from Fuji II LC [25].

Assessment of the interaction effect of weight percentage of NHA and type of cement on the cell viability in our study showed that in CG groups, by an increase in weight percentage of NHA from CG₁ to CG₅ and CG₇, the cell viability decreased; however, the cell viability increased again in CG₁₀. There was a significant difference between CG₅ and CG₇ with CG₁ NHA-containing groups. On the other hand, by increasing the weight percentage of NHA particles by up to CG₁₀, a reduction in cytotoxicity was noted. This finding may be due to several reasons. According to a study by Mohammadi Basir et al, [8] in concentrations higher than 5%, nanoparticle agglomerates form within the cement matrix. These agglomerates have the highest frequency in presence of 10wt% NHA. Hydroxyapatite nano-bioceramics are soluble in acidic solutions and after mixing the powder with a poly-acid (glass ionomer cement liquid), calcium ions are released from the surface of NHA particles; consequently, acid-base reactions and cross-linking occur within the cement structure, and subsequently reinforce it [26]. The odds of formation of hydrogen bonds increase as well, which is due to the presence of higher hydroxyl and phosphate ions in the cement matrix [27]. Stronger bonds between the organic and inorganic cement networks increase the mechanical strength of the set cement. Increased chemical reactions may be responsible for higher cytotoxicity of the cement and decreased viability of cells.

On the other hand, it can be speculated that due to ionic interaction of NHA crystals and polyacrylic acids, toxic byproducts or components may be formed and released from the NHA-containing GICs; this statement has been supported by other studies [28,29]. However, according to the results of this study, all groups had moderate cytotoxicity.

There was no significant difference in cytotoxicity between RMGI groups at 24 and 48 h. After 72 h, the cell viability of RMG₂ and RMG₅ was significantly higher than other groups containing NHA; however, their cell viability was not higher than that of RMG₀ group, and all groups had moderate cytotoxicity. Studies showed that adding about the same amount of NHA as in RMG₅ to CGIC and RMGIC groups increased their mechanical properties [6-8, 24]. Mohammadi Basir et al. [8] assessed the mechanical properties of RMGICs containing NHA particles and concluded that flexural strength increased by addition of up to 5wt% NHA; while, further increase in the concentration of NHA decreased the strength. Poorzandpoush et al. [10] assessed the wear resistance of RMGICs containing NHA and concluded that addition of 2wt% and 5wt% NHA increased the wear resistance, and 5wt% yielded the highest wear resistance. They explained that NHA particles are tiny particles containing calcium and phosphorus; when NHA is exposed to polyacrylic acid, the release and cross-linking of ions increase, resulting in higher strength of the cement matrix in 5wt% group. It seems that NHA by up to 5wt% serves as reactive filler and reinforces the cement matrix. The lower cytotoxicity in 2wt% and 5wt% NHA groups after 72 h in this study may also be due to these chemical reactions, and increase in resin crosslinking may reduce the ion release from the RMGI bulk after 72 h [30]. However, long-term storage may show a different trend of cytotoxicity. Genaro et al. [3] stated that adding NHA to RMGI decreased its cytotoxicity, and this reduction increased by increasing the NHA percentage to 10%.

It is obvious that addition of NHA to powder of CGIC changes the chemical reactions that happen during setting. Due to the difference in the composition and setting mechanism of

CGICs and RMGICs, different chemical reactions may occur, and different amounts of materials might release from the cured matrix. As no chemical analysis was done in this study, we could not identify the exact components released. The current study had an in vitro design. Thus, generalization of results to the clinical setting must be done with caution. Future in vivo studies are required to find the most optimal concentration of NHA particles for incorporation into the formulations of cements.

CONCLUSION

Within the limitations of this study, the results showed that addition of NHA to GIC powders by up to 10% did not decrease cell viability.

ACKNOWLEDGMENTS

This study was supported by Dental Research Center, Tehran University of Medical Sciences (grant Number: 93-03-69-26679).

CONFLICT OF INTEREST STATEMENT

None declared.

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