

Comparative Evaluation of Osteogenic Potential of Conventional Glass-ionomer Cement with Chitosan-modified Glass-ionomer and Bioactive Glass-modified Glass-ionomer Cement – An *In vitro* Study

Abstract

Aim: The aim of this study was to compare the osteogenic potential of conventional glass-ionomer cement (GIC) with chitosan-modified GIC (CH-GIC) and bioactive glass-modified GIC (BAG-GIC) as a function of time in varying proportions. **Materials and Methods:** CH-GIC was prepared by adding 10 v/v% (Group II) and 50 v/v% (Group III) CH to the commercial liquid of GIC. BAG-GIC was prepared by the addition of 10 wt% (Group IV) and 30 wt% (Group V) of BAG to the GIC powder. Conventional GIC was kept as Group I. Nine round-shaped samples measuring 2 mm thick and 5 mm in diameter were prepared for every experimental material. Human osteosarcoma cells were cultured and cell proliferation was assessed at 24, 48, and 72 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and cell differentiation was assessed at 7, 14, and 21 days using alkaline phosphatase (ALP) assay. All experiments were done in triplicate. The data obtained were analyzed using one-way analysis of variance and Tukey honestly significant difference *post hoc* multiple comparisons at 0.05 level significance. **Results:** Cell culture studies showed a significant increase in proliferative activity and ALP activity in Group II, III, IV, and V than Group I at all-time intervals ($P < 0.05$). There was no statistically significant difference in osteogenic potential between CH-GIC and BAG-GIC groups. **Conclusion:** The osteogenic potential was significantly higher in CH-GIC and BAG-GIC compared to conventional GIC.

Keywords: Bioactive glass, chitosan, glass-ionomer cement, osteogenic potential

Introduction

Glass-ionomer cements (GIC) possess an edge over other dental materials by its unique property of chemical bonding to the tooth structure, biocompatibility, and fluoride release.^[1] However, it lacks strength, toughness, and it is susceptible to leaching of ions on premature exposure to moisture.^[2] The potential to release incorporated therapeutic agents, absence of toxic monomers, and absence of heat release during setting have pushed the scope of GIC usage beyond dentistry to the biomedical application as bone cement in orthopedic surgery.^[3]

Chitosan (CH) and bioactive glass (BAG) are widely used in bone tissue engineering in recent years. CH being an inexpensive natural biopolymer, with antifungal and antibacterial properties,^[4,5] has been used for wound healing^[6] and periodontal therapy.^[7] BAG has antimicrobial and

anti-inflammatory effects and it displays osteoconductive properties which may assist the repair of bony defects.^[8]

Yli-Urpo *et al.* reported that the addition of BAG to GIC compromised the compressive strength but increased the surface hardness, and more calcium was detected in the BAG-containing materials than in the conventional GIC.^[9,10] A new polyacid to improve the mechanical properties of GI and BAG was formulated by Xie *et al.*^[11] Petri *et al.* reported that the addition of 0.0044wt% of CH improved the flexural resistance, but CH contents higher than 0.022wt% led to poor performance.^[12] Karthick *et al.* suggested that the addition of CH improved the microshear bond strength of the conventional GIC.^[13] The addition of nanochitosan to GIC was found to improve fluoride release as well as the mechanical properties.^[14] However, the synergistic bioactive potential of these materials can be studied for its application in those areas

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How to cite this article: Ranjani MS, Kavitha M, Venkatesh S. Comparative evaluation of osteogenic potential of conventional glass-ionomer cement with chitosan-modified glass-ionomer and bioactive glass-modified glass-ionomer cement – An *In vitro* study. *Contemp Clin Dent* 2021;12:32-6.

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Submitted : 21-Dec-2019

Revised : 16-Apr-2020

Accepted : 23-May-2020

Published : 20-Mar-2021

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Access this article online

Website:

www.contempclindent.org

DOI: 10.4103/ccd.ccd_474_19

Quick Response Code:



that do not demand good mechanical performance such as a root-end filling material or as bone cement.

The aim of this study was to compare the osteogenic potential of conventional GIC with CH-GIC and bioactive glass-modified GIC (BAG-GIC) as a function of time in varying proportions.

Materials and Methods

Preparation of chitosan-modified glass-ionomer cements

1.8 ml of glacial acetic acid is made up to 100 ml with distilled water in a 100 ml standard flask. Twenty milligram of CH (Sigma-Aldrich, USA) was weighed and dissolved in 0.3N acetic acid and made up to 100 ml with the same acetic acid in a 100 ml standard flask to get 0.2 mg/ml CH solution. 0.1 ml of 0.2 mg/ml of CH solution is added to 0.9 ml of conventional GIC (GC Fuji II, GC Corporation, Tokyo, Japan) liquid to get 10 v/v% CH-GIC. 100 mg of CH was weighed and dissolved in 0.3 N acetic acid and made up to 100 ml with the same acetic acid in a 100 ml standard flask to get 1 mg/ml CH solution. 0.5 ml of 1 mg/ml of CH solution is added to 0.5 ml of conventional GIC liquid to get 50 v/v% CH-GIC.

Preparation of bioactive glass-modified glass-ionomer cements

10 wt% BAG (Vivoxid Ltd, Finland) was measured and added to 90 wt% GIC powder (GC Fuji II, GC Corporation, Tokyo, Japan). 30 wt% of BAG was added to 70 wt% GIC powder. The mixtures were then dispensed in 20 ml plastic test tubes, sealed, and placed in a tube roller mixer for 10 min to get uniform filler particle distribution.

The experimental groups considered were as follows:

- Group I – Conventional GIC
- Group II – GIC+10 v/v% CH
- Group III-GIC+50 v/v% CH
- Group IV-GIC+10 wt% BAG
- Group V-GIC+30 wt% BAG
- Control.

Culture of human osteosarcoma cells

Human osteosarcoma (SaOS-2) cell line was procured from the National Center for cell sciences, Pune, India. The cells were grown in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Upon reaching confluence, as observed in the inverted phase contrast microscope, the cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) solution and used for subculture.

Passaging the cells

The medium from the culture flask was aspirated. The flask was rinsed with 2 ml of phosphate-buffered saline (PBS) and aspirated quickly. One milliliter of trypsin-

EDTA solution was added to the flask and swirled gently to cover the entire area for 10 s and aspirated quickly. Then, the flask was incubated at 37°C for 10 min. The detached cells were then resuspended in 10 ml of 10% FBS–DMEM, gently mixed well by pipetting up and down. From the cell suspension, a drop was placed to the edge of the coverslip of Neubauer hemocytometer and the drop was let to run under the coverslip by capillary action. Then, the cells from the E1, E2, E3, E4, and E5 squares were counted under the microscope. The number of cells was calculated using the formula:

$$\begin{aligned} \text{Number of cells} &= \text{Number of cells counted} \times 50,000 \\ &= X \text{ cells/ml.} \end{aligned}$$

Preparation of test samples

Round-shaped samples measuring 2 mm thick and 5 mm in diameter were prepared using a Teflon mold. The Teflon mold was placed on a glass plate and the experimental materials were mixed and packed into the Teflon mold. Another glass plate was placed on the top and the mold was gently compressed between glass plates until the experimental materials were set. The samples were then carefully removed from the mold.

As all the experiments were done in triplicates for three different time intervals to guarantee the reproducibility, the following number of samples was prepared.

- Nine samples for every experimental material were prepared for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and
- Nine samples for every experimental material were prepared for alkaline phosphatase (ALP) assay.

Cell proliferation assessment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The SaOS-2 cells were plated in 24-well plates at a concentration of 3×10^4 cells/well. Twenty-four hour after plating, cells were washed twice with 100 μ l of PBS and starved by incubating the cells with 0.1% bovine serum albumin for 12 h at 37°C in CO₂ incubator. The test samples were rinsed three times with PBS and Alpha-MEM (Minimum Essential Medium) and were placed into the wells of 24-well microtiter plates for 24 and 48 and 72 h and incubated at 37°C. All experiments were done in triplicate. At the end of the treatment, the medium from control and test material treated cells were discarded and 100 μ l of MTT containing DMEM medium was added to each well. The cells were then incubated for 3 h. The MTT containing medium was then discarded and the cells were washed with PBS (200 μ l). The crystals were then dissolved by adding 1 ml of dimethyl sulfoxide. The color developed is directly proportional to the number of live cells. The intensity of purple color was immediately measured in an enzyme-linked immunosorbent assay reader at 545 nm.

Cell Differentiation assessment using alkaline phosphatase assay

2×10^4 SaOS-2 cells were seeded on test samples under culture conditions in osteogenic medium and the level of ALP activity was determined at days 7, 14, and 21. The cells were detached from discs using trypsin/EDTA and centrifuged for 5 min at 1000 rpm after being washed twice with PBS. Cell lysate was obtained, and ALP activity was determined using p-nitrophenyl phosphate (pNPP) as the substrate. All experiments were done in triplicate. Upon dephosphorylated by ALP, pNPP turned yellow and its color change was directly proportional to ALP. The reaction was stopped by the addition of 1 N sodium hydroxide to reaction mixture. This colorimetric assay was finished by detecting the absorbance at 405 nm optical density value using Autoanalyzer. The ALP activity was expressed as micromoles of p-nitrophenol formed/min/microgram of protein. The ALP activity was calculated using the formula:

$$\frac{\text{OD of unknown}}{\text{OD of known}} \times \text{SC} \times \text{TCF} \times \frac{1}{\mu\text{g protein}}$$

= μ moles of p-nitrophenol formed per min per μg protein

* SC-Standard concentration

* TCF-Time correction factor

In case if alignment changes - to avoid confusion - attached a jpeg image for the same formula mentioned above for your kind reference.

Statistical analysis

The raw data obtained were analyzed using analysis of variance to examine the effect of materials and time points and the interaction of these two factors on cell proliferation and ALP activity. The Tukey honestly significant difference test was used for comparison among groups at 0.05 level significance.

Results

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

To assess immediate and late toxic effects of the test materials on cell viability, the MTT assay was carried out at 24, 48, and 72 h. At the end of 24 h, there was increased proliferative activity than control in all groups except Group I (conventional GIC). After 48 h and 72 h, Group I was not significantly different from control. There was a statistically significant increase in proliferative activity in Groups II, III, IV, and V than Group I (conventional GIC) at 24, 48, and 72 h ($P < 0.05$) [Table 1]. There was no statistically significant difference between Group II, Group III, and Group V. At 24 and 48 h, Group IV showed significantly less proliferative activity than Groups II, III, and V ($P < 0.05$) [Figure 1].

Table 1: One-way ANOVA for MTT assay

Duration	Groups	Mean	SD	F	Significant
24 h	Group I	0.82700	0.032187	186.813	0.000
	Group II	2.77433	0.233204		
	Group III	2.80700	0.033422		
	Group IV	1.69733	0.057204		
	Group V	2.79867	0.039323		
	Control	1.57700	0.077621		
48 h	Total	2.08022	0.790549	143.197	0.000
	Group I	0.83000	0.022869		
	Group II	2.83000	0.171852		
	Group III	2.88533	0.119039		
	Group IV	1.84633	0.216632		
	Group V	2.88133	0.165618		
72 h	Control	0.89867	0.057839	56.156	0.000
	Total	2.02861	0.932860		
	Group I	0.85367	0.111935		
	Group II	2.89667	0.213294		
	Group III	2.92667	0.113931		
	Group IV	2.27800	0.469920		
	Group V	2.91467	0.170016	2.12389	0.965627
	Control	0.87367	0.064143		
	Total	2.12389	0.965627		

SD: Standard deviation; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

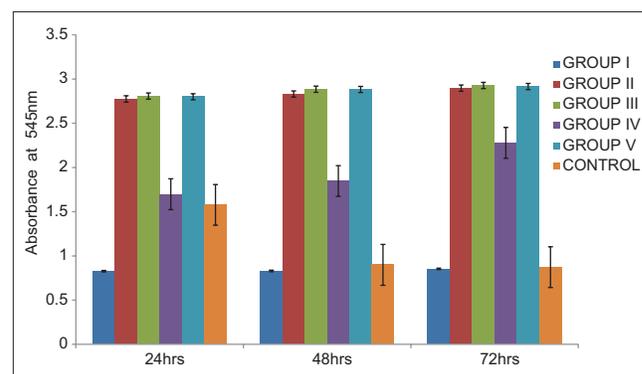


Figure 1: Comparison of optical density values for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Alkaline phosphatase assay

ALP activity was tested at 7, 14, and 21 days to assess long-term bioactivity of the test materials.

Throughout the observation period, Group II, Group III, Group IV, and Group V showed higher ALP activity than Group I (conventional GIC) and it was statistically significant ($P < 0.05$) [Table 2]. There was no statistically significant difference in ALP activity between Group II, Group III, and Group IV and V. At the end of 21 days, Group II, Group III, Group IV, and Group V showed significantly greater ALP activity than control ($P < 0.05$) [Figure 2].

Discussion

GIC is a versatile material which is being subjected to

Table 2: One-way ANOVA for alkaline phosphatase assay

Duration	Groups	Mean	SD	F	Significant
7 days	Group I	268.700	36.6083	9.751	0.001
	Group II	443.600	52.8071		
	Group III	448.600	50.4107		
	Group IV	422.533	32.2598		
	Group V	445.000	33.4591		
	Control	392.900	5.8592		
	Total	403.556	72.6631		
14 days	Group I	455.37	49.14	15.378	0.000
	Group II	318.77	24.94		
	Group III	542.47	48.69		
	Group IV	543.53	53.81		
	Group V	527.07	30.28		
	Control	543.47	15.11		
	Total	488.44	90.73		
21 days	Group I	402.933	25.0847	26.297	0.000
	Group II	574.433	23.1641		
	Group III	582.900	15.1700		
	Group IV	564.367	26.0983		
	Group V	576.833	10.6444		
	Control	477.567	38.8805		
	Total	529.839	72.1643		

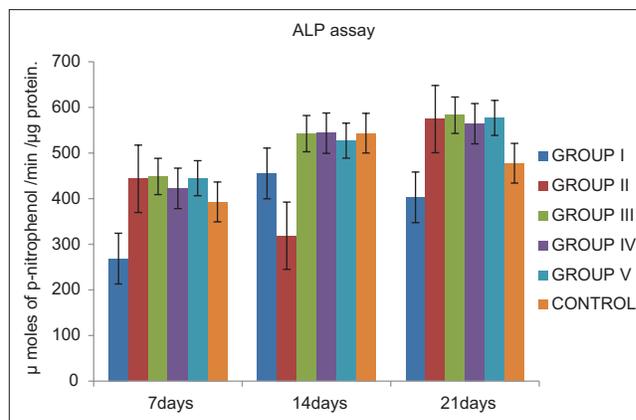
SD: Standard deviation

various modifications to improve its properties. Currently, researches are oriented toward the development of biomaterials having therapeutic functions in addition to its inherent properties.

Yli-Urpo *et al.* researched on addition of 10 wt% and 30 wt% BAG to conventional GIC and found the combination to be bioactive with compressive strength being compromised on increase in the amount of BAG.^[9,10] Petri *et al.* reported that the addition of CH in lesser concentration to GIC improved the flexural strength, but an increase in CH concentration was found to compromise the material properties.^[12] Karthick *et al.* reported increased microshear bond strength with 10 v/v% and 50 v/v% CH-GIC when compared to conventional GIC.^[13] Similar results of increased microshear bond strength were reported by Debnath *et al.*, with 10 v/v% CH-GIC. It was also suggested that 10 v/v% CH-GIC resulted in improved antibacterial property against *Streptococcus mutans*.^[15] Therefore, the study intended to determine the osteogenic potential of GIC by addition of bioactive materials in both lower and higher concentrations.

The results showed that conventional GIC exhibited less proliferative activity than other experimental groups in first 24 h. This could be explained by the fact that the *in vitro* toxicity of GICs was due to a complex mechanism based on both ion release, in particular aluminum and fluoride ions, and pH effects.^[16-18]

The increased cell proliferative activity in Group II (GIC + 10 v/v% CH) and Group III (GIC + 50 v/v% CH) was in

**Figure 2: Comparison of alkaline phosphatase activity expressed as μ moles of p-nitrophenol formed/min/μg protein**

accordance with the previous studies.^[19,20] Shi *et al.* showed CH-coated iron oxide nanoparticles enhanced osteoblast proliferation, decreased cell membrane damage, and promoted cell differentiation, as indicated by an increase in ALP and extracellular calcium deposition.^[20] Mathews *et al.* stated that CH upregulated genes associated with calcium binding and mineralization.^[21]

The higher proliferative activity of Group IV (GIC +10wt% BAG) and GroupV (GIC + 30wt % BAG) than Group I (conventional GIC) could be explained by the fact that BAG has the ability to stimulate cell cycling and subsequently enhance osteoblastic turnover of human primary osteoblasts.^[22,23] At the end of 24 h, the proliferative activity of Group IV was not significantly different from control. However, at 48 and 72 h, Group IV showed higher proliferative activity. The probable reason could be less amount of BAG (10 wt %) added to GIC. With time, the reactivity of BAG increased resulting in release of calcium and phosphate ions on the surface that lead to increased osteoblastic proliferation. De Caluwé *et al.* reported that BAG-GIC improved the bioactivity of the GIC by the formation of an apatite layer, but the strength was compromised with increase in the proportion of BAG and concluded that BAG-GIC with 10 mol% Al³⁺ yielded better physiochemical properties when added in ≤20 wt% to a GIC.^[24]

Within the limitations of this study, both CH- and BAG-modified GIC exhibited excellent osteogenic potential with no statistically significant difference between the two materials. Further studies shall be performed employing CH and BAG into GIC to establish its usage as root-end filling material or as a bone cement with excellent healing properties.

Conclusion

From the present study, it can be concluded that the addition of CH and BAG into GIC resulted in a significant increase

in osteogenic effect when compared to conventional GIC. Therefore, CH-GIC and BAG-GIC shall be suggested as a promising bioactive material with added therapeutic advantage.

Acknowledgment

The authors would wish to thank Department of Endocrinology, Dr. ALM P.G. Institute of Basic Medical Sciences, University of Madras, Sekkizhar Campus, Taramani, Chennai, Tamil Nadu, India.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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