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Published in:
Journal of Conservative Dentistry

DOI:
10.4103/JCD.JCD_274_20

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

ISSN: 0972-0707

Journal of Conservative Dentistry

Volume 23 Issue 1 January-February 2020

Official Publication of
Indian Association of Conservative Dentistry and Endodontics
Online full text at
www.jcd.org.in

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Original Article

The effect of adding graphene oxide nanoplatelets to Portland cement: Potential for dental applications

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Abstract

Background: The potential of graphene-based materials to improve the physiomechanical properties of Portland cement-based materials without compromising biocompatibility is of interest to dental researchers and remains to be discovered.

Aim: This study investigated the effects of adding graphene oxide nanoplatelets (GONPs) on the surface microhardness and biocompatibility of Portland cement.

Materials and Methods: Three prototype Portland cement powder formulations were prepared by adding 0, 1, and 3 wt % GONPs in powder form to Portland cement. Prototype cement specimens were in the form of disks, with a diameter of 10 mm and a thickness of 2 mm. In experiment 1, surface microhardness was measured using the through indenter viewing hardness tester, 20 surface hardness values were obtained from all specimens. In experiment 2, Balb/C 3T3 fibroblasts were cultured with the material disks and the viability of cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Statistical Analysis: The data were analyzed using the analysis of variance followed by Dunnett test ($\alpha = 0.05$) or Tukey test ($\alpha = 0.05$).

Results: In response to material disks, the addition of 1 wt % GONPs had a proliferative effect on cells at day 3 and day 7 with a significant difference from the control. The addition of 3 wt % GONPs showed a remarkable increase in surface microhardness; however, it exhibited initial cytotoxicity.

Conclusions: The addition of 1 wt % GONPs to Portland cement improved surface microhardness without compromising biocompatibility; therefore, it has a greater potential for dental applications. The results of this work give other researchers leads in future assessments of this prototype material.

Keywords: Biocompatibility; fibroblasts; graphene; hardness; nanoplatelets

INTRODUCTION

Portland cement-based materials have been extensively and successfully used in dentistry for a wide range of purposes: treatment of root defects, direct pulp capping, and root-end filling, to name but a few. To date, there is no material that has proven to possess outstanding biocompatibility, bioactivity, and optimal physicomechanical properties to be used in the broad range of applications in dentistry. Although some newer Portland cement-based formulations look promising, they fall short in certain aspects limiting their use to just a few clinically relevant situations.[1] Therefore, the development of new materials and formulations that can improve these aspects is crucial and has numerous positive implications in dentistry.

It has been reported in the literature that attempts of adding nanomaterials to calcium-based formulations have

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Date of submission : 19.06.2020
Review completed : 04.07.2020
Date of acceptance : 14.07.2020
Published : 10.10.2020

Access this article online

Quick Response Code:

Website: www.jcd.org.in

DOI: 10.4103/JCD.JCD_274_20

How to cite this article: Qutieshat AS, Al-Hiyasat AS, Islam MR. The effect of adding graphene oxide nanoplatelets to Portland cement: Potential for dental applications. J Conserv Dent 2020;23:15-20.

resulted in improved physiomechanical and chemical properties.[2] These attempts were, however, mostly limited to typical use (i.e., engineering and construction) that is not specific to dentistry. For example, a prototype Portland cement supplemented with nano-silica exhibited higher compressive strength and shortened setting time.[3] Similarly, hydroxyapatite showed higher flexural strength and fracture toughness compared to its pure form when zirconia and alumina were added.[4] Physiomechanical improvements were also reported with the addition of carbon nanotubes to Portland cement-like formulations.[5-7]

Graphene sheets, carbon nanotubes, and nanoplatelets have attracted considerable interest as nanoreinforcements for cement composites owing to their favorable mechanical properties. Graphene is a novel nanomaterial that is considered one of the stiffest materials; its atomic thickness, large surface area, high elastic modulus, and flexural strength make it ideal for many potential reinforcement applications.[8]

Graphene sheets in their original condition magnified osteogenic differentiation of periodontal ligament stem cells,[9] while its oxide substrate promoted the expression of odontogenic and osteogenic-related genes in dental pulp stem cells.[10] Moreover, adding graphene oxide to conventional Portland cement enhanced its compressive, flexural, and tensile strength.[11] Interestingly, the addition of graphene oxide nanosheets regulate the microstructure of hardened cement paste by enhancing its strength and toughness in a concentration-dependent manner.[12]

The addition of graphene nanosheets to hydroxyapatite composites maintained the biocompatibility and improved the hardness of the material.[13] Similarly, the addition of graphene oxide nanosheets improved the mechanical properties of calcium-based cements and enhanced its apatite mineralization ability.[14]

Despite the improvements provided by different forms of nanomaterials such as graphene in calcium-based cements outside the domain of dentistry, the potential of graphene-based materials to improve the physiomechanical properties of dental materials without compromising the materials' biocompatibility remains to be discovered. The objective of this work is to evaluate the effects of adding graphene oxide nanoplatelets (GONPs) on the surface microhardness and biocompatibility of lab-prepared Portland cement formulations.

MATERIALS AND METHODS

Preparation of material specimens

Material disks were aseptically prepared inside the laminar flow hood (ESCO, Changi, Singapore). Three prototype Portland cement powder formulations were prepared by adding 0, 1 and 3 wt % GONPs in powder form (Graphitene, batch# HTS-300-02, Scunthorpe, North Lincolnshire, UK) to Portland cement (ACWCI, MSDS# 1651, Jordan). Subsequently, each formulation (1.0 g) was mixed with sterile distilled water (0.4 ml) and placed into autoclaved plexiglass rings used as molds, with an inner diameter of 10 mm, and a thickness of 2 mm to produce material specimens in the form of disks. Excess material was removed using a sterile blade, and materials were left to set for 24 h at 37°C and 95% humidity. Twelve material disks were prepared for each composition, eight of which were used for cell viability evaluation, three for microscopic observation, and the remaining disk was reserved for microhardness testing.

Microhardness testing

Surface microhardness was measured quantitatively and qualitatively using the through indenter viewing (TIV) hardness tester (GE Measurement and Control, Groby, UK) under a 9.8 N load. The charged-coupled device (CCD) camera integrated into the probe uses special optics to generate the high-quality images of the Vickers diamond penetrating into the surface. The camera was used to view through the diamond during the indentation process as it happened (TIV).

Reproducible orientation of specimens was achieved by fabricating a custom-made plastic jig into which the specimen disk was firmly placed. This was designed using computer-aided drafting software (Solid Works Simulation software package 2018) and produced via a three-dimensional printer (acrylonitrile butadiene styrene filament). This jig base was 5 mm thick and had a 2 mm deep circular slot to accommodate the specimen. The surface hardness at 20 sites per specimen was measured. The site of each indent was confirmed by visualization through the TIV, and the corresponding images were obtained for the qualitative analysis.

Cell culture

Balb/C 3T3 fibroblasts Cells (Clone A31, European collection of cell culture, Salisburg, Wilts, UK) were routinely maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum, and antibiotic/antimycotic mixture (GIBCO Invitrogen Life Technologies, Grans Island, NY) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. They were routinely passaged by trypsinization.

Cellular response to the material disks

The measurement of cell proliferation in the presence of the test materials was performed using 12-well plates. Balb/C-3T3 fibroblasts were seeded at a concentration of 5×10^5 cells/ml into the 12-well plate, and then, one disk of material was placed in each well for an incubation period of 1 day, 3 days and 7 days at 37°C. Wells containing

Teflon disks served as controls. Eight replicates were made for each group of specimens. Following exposure of the cells to the materials, the effect on cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co, St. Louis, MO).

Microscopy

To further investigate the biocompatibility of material formulations, we used a different cell line, WPS9 (human peri-radicular fibroblasts). WPS9 cells were cultured with the material disks and observed at day 1, 3, and 7. By the end of each period, cells were washed gently with phosphate-buffered saline, covered again with fresh medium. Photomicrographs were taken of close and remote areas around the material specimen (adjacent to disk, away from disk, and adjacent to the edge of the culture dish) using an Olympus IX70 microscope at 4X objective lens.

Statistical analysis

Absorbance values obtained for each well represent the amount of MTT reduction, which is proportional to the number of viable cells. To assess the percentage of viable cells, the absorbance values were related to those of the control. This was achieved by setting the mean absorbance of the control at 100%.

Formula 1: Percentage of viable cells calculation

Percentage of viable cells = (absorbance value/mean absorbance of the control) × 100.

Graph Pad Prism version 8.2.0 (Graph Pad Software, Inc.) was used for the statistical analysis. The effects of the materials on cells were compared to that of the control using the percent viability values as indicators of cell numbers. Hardness and biocompatibility data were analyzed using the analysis of variance (ANOVA) followed by Dunnett's test ($\alpha = 0.05$) or Tukey's test ($\alpha = 0.05$).

RESULTS

Experiment 1: Surface microhardness

Surface microhardness mean values are shown in Figure 1a. The mean microhardness increased with increasing GOPNs content, 44.9 ± 4.9 HV at 0%, 122.2 ± 11.5 HV at 1% and 227.6 ± 37.9 HV at 3%. The addition of GONPs was associated with surface microhardness increases of 2.7- and 5-fold, respectively. Statistical analysis (ANOVA) showed highly significant effects between the groups ($P < 0.0001$) on surface microhardness. Follow-up comparison by Tukey test ($\alpha = 0.05$) showed that the three different formulations were significantly different from each other ($P < 0.0001$).

The corresponding CCD images for Vickers diamond indentations made by the hardness tester upon the three

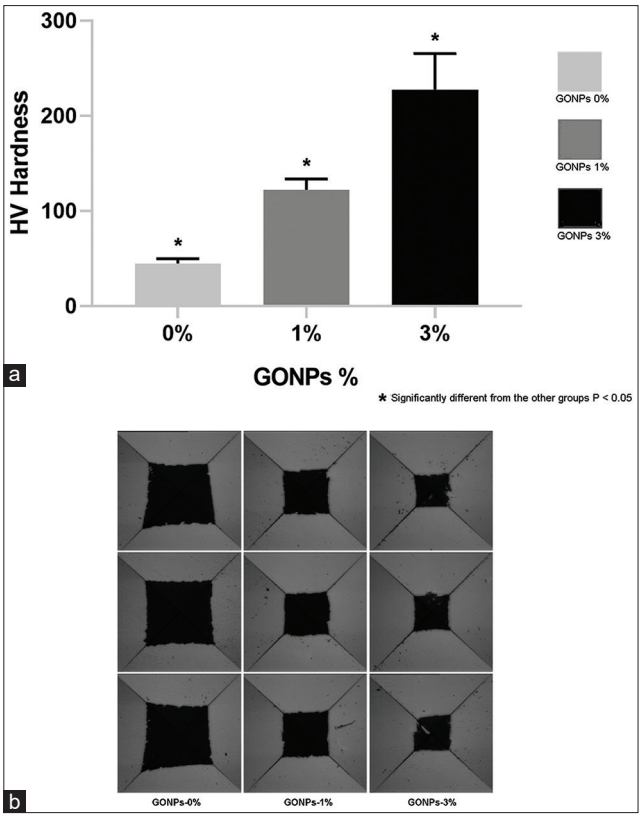

Figure 1: (a) Mean hardness values of the three Portland cement formulations. Bars carrying the asterisk sign (*) are significantly different from other groups at $P < 0.05$. (b) Vickers diamond indentations into the surfaces of the three Portland cement formulations

formulations are shown in Figure 1b. The images show that the indentation size decreases in the ascending order of GONPs-0%, GONPs-1%, and GONPs-3%.

Experiment 2: Biocompatibility

Relative cell viability results over 1, 3, and 7 days are shown in Figure 2a. In the 1-day group, cells exposed to the three formulations showed a reduction in viability. Statistical analysis (ANOVA) showed highly significant effects between the groups ($P < 0.0001$) on cell viability. Follow-up comparison by Dunnett's test ($\alpha = 0.05$) showed that only the GONPs-containing formulations were significantly different from the control ($P < 0.0001$).

In the 3-day group, the increase in cell proliferation was obvious with GONPs-1% (i.e., + 28%). GONPs-3% bounced back to control level, whereas the GONPs-0% showed a substantial reduction in cell viability (i.e.,-32%). Statistical analysis (ANOVA) showed highly significant effects between the groups ($P < 0.0001$) on cell viability. Follow-up comparison by Dunnett's test ($\alpha = 0.05$) showed that the changes, whether positive or negative, were significantly different relative to the control ($P < 0.0001$).

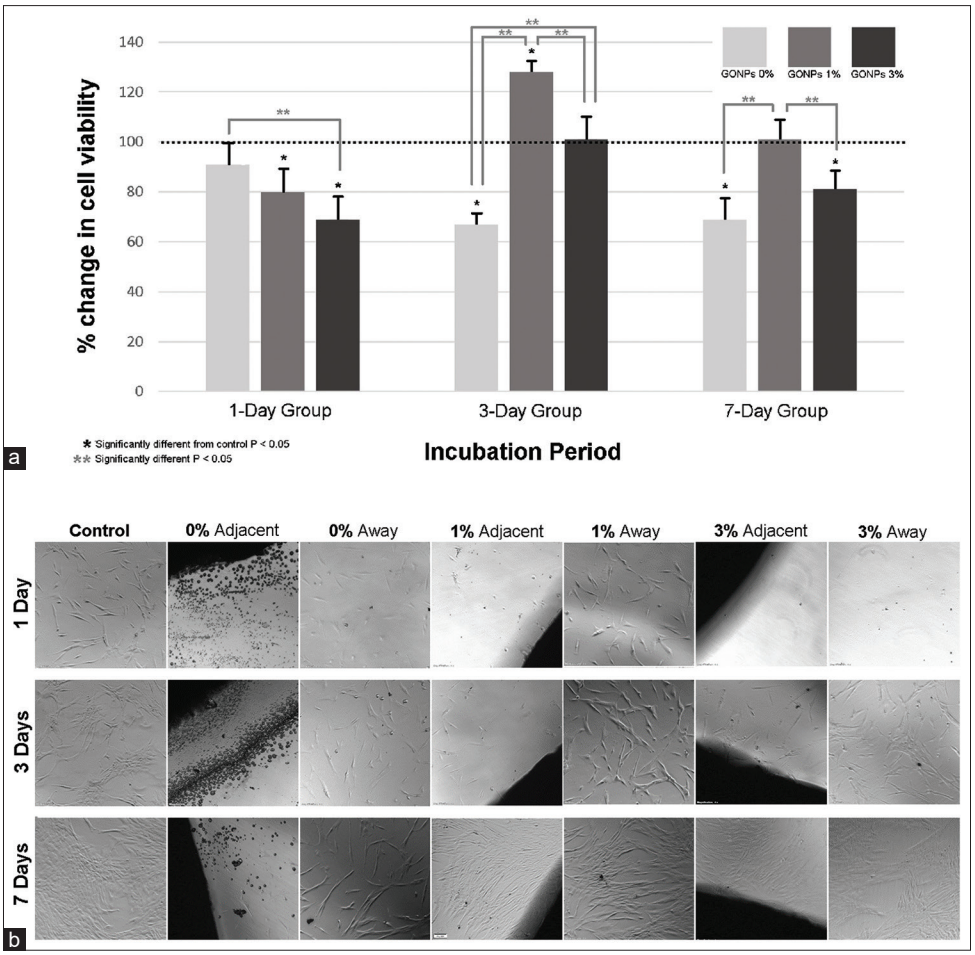

Figure 2: (a) Percentage of cell viability (mean ± standard deviation) relative to the control (100% viability) over 1, 3 and 7 days upon exposure to material disks. Bars carrying the asterisk sign (*) are significantly different from the control at $P < 0.05$. Bars carrying the double-asterisk sign (**) are significantly different from each other at $P < 0.05$. (b) Photomicrographs were taken of close and remote areas around the material specimen (adjacent to disk, away from disk at day 1, 3 and 7

In the 7-day group, only the GONPs-1% maintained favorable biocompatibility, whereas GONPs-0% and GONPs-3% showed reductions in cell viability. Statistical analysis (ANOVA) showed highly significant effects between the groups ($P < 0.0001$) on cell viability. Follow-up comparison by Dunnett's test ($\alpha = 0.05$) showed that the reductions in viability were significantly different relative to the control ($P < 0.0001$ and $P = 0.0002$, respectively).

Overall, there was a reduction in cell viability after 1 day for all the materials tested. Cell viability was found to improve over the longer time periods (i.e., 3 and 7 days) with the GONPs-containing formulations which was not the case with the GONPs-free formulation. Statistical analysis (ANOVA) followed by Tukey test showed that a significant difference was found between GONPs-0% and GONPs-3% ($P < 0.0001$) after 1 day, between all three formulations after 3 days ($P < 0.0001$), and between GONPs-1% and the other two formulations ($P < 0.0001$) after 7 days.

Microscopic observation [Figure 2b] showed that human peri-radicular fibroblasts grew better with GONPs-containing formulations as compared with the GONPs-free formulation. This is even more clear over longer periods of time (i.e., 3 and 7 days). Interestingly, cells in GONPs-1% and GONPs-3% cultures exhibited spindle shape and elongated morphology both adjacent to and away from the material disk. While in the GONPs-0% culture, the area adjacent to the material disk showed no or minimal cell growth.

DISCUSSION

Graphene and its derivatives are increasingly applied in the biomedical field due to their unique features, that is, high mechanical properties, large surface area, and the ability to be combined with several substrates and biomaterials.[15,16] Graphene derivatives possess great potential to enhance the physicomechanical and biological properties when combined with other biomaterials.[10,17-19]

Based on the results of this study, the addition of GONPs increased the surface microhardness of Portland cement and exerted a proliferative effect on Balb/C 3T3 fibroblasts. The positive effect on cell proliferation was more evident over longer periods of time. Clearly, the formulation that included 1% GONPs exhibited the best cellular response and increased the surface microhardness by 2.7-fold. The main problem of the GONPs-3% formulation was that, despite showing a remarkable 5-fold increase in surface microhardness and a positive effect on cell proliferation after 3 and 7 days, it exerted an initial cytotoxic effect on cell proliferation (i.e., day 1).

Initial cytotoxicity of Portland cement-based materials such as mineral trioxide aggregate and lab-prepared Portland cement formulations has been well-reported in the literature.[20] This cytotoxic effect might be attributed to the initial high surface pH of the cements that gradually decreases over time, as the material sets.

The strong bioactivity of GONPs-containing calcium-based formulations reported in this study might be attributed to the lower crystallinity of the modified material, higher mineral deposition, and the increased rate of calcium and phosphate ion release.[21]

Corresponding results were reported in a study that assessed the addition of graphene nanosheets to commercially available calcium-based cements.[2] However, we believe that nano-scale modifications and/or reinforcements are preferably employed while the core material (i.e., calcium-based material) is in the raw state, rather than a finished product that has already been reinforced with biomolecules and fillers.[1,22] Other studies that investigated the effect of GONPs in different contexts showed that GONPs improved the mechanical properties of different biomaterials and at the same time maintained their favorable biological properties.[23] It was also found that the addition of GONPs reshapes the microstructure of Portland cement-based materials, with stronger bonds being formed between GONPs and the calcium-silicate-hydrate gel.[12,24] As seen from Figure 1a, among the samples with higher GONPs content, the standard deviation values are higher. This might indicate that the distribution of GONPs within the same specimen is variable and thus contribute to higher hardness values in the areas that are seeded with higher GONPs content.

CONCLUSIONS

Portland cement reinforced with GONPs showed excellent biocompatibility. Addition of GONPs improved surface microhardness of the Portland cement due to its outstanding physical and mechanical properties. The present results demonstrated enhanced biocompatibility of GONPs-1%, compared to GONPs-3% and GONPs-free formulations, showing excellent cellular viability and density. Increased biocompatibility of GONPs-containing formulations was attributed to the possible role of GONPs in reshaping the microstructural composition of Portland cement. The use of GONPs-based Portland cement formulations might be considered as potential substrates for future generation dental materials.

The findings in this study led to the conclusion that although the addition of both 1% and 3% of GONPs to Portland cement improved surface microhardness, the 1% formulation exhibited better bioactivity compared to other formulations. In this respect, GONPs-1% has a greater potential for dental applications compared to the other formulations because it greatly improved surface microhardness without compromising biocompatibility. These findings can also help narrow down the range of possible GONPs wt % for the determination of the optimal GONPs-Portland cement formulation (i.e., 1% $\leq$GONPs% $<$3%). This does not necessarily indicate that the GONPs-3% is not suitable for further development, but it certainly demonstrates the potential behind this promising nano-reinforcement method.

This study, we believe, can provide a tentative basis for further, more rigorous investigations. Nevertheless, we do feel that the elucidation of our results has permitted a better picture that is important enough to be presented now rather than be delayed in order to give other researchers leads in future assessments of this prototype material. Further work is needed and planned based on the findings of this study.

Financial support and sponsorship

This research is financially supported by a grant from Jordan University of Science and Technology, Jordan (Faculty Member Research Grant, FMRG Research grant number 20190203).

Conflicts of interest

There are no conflicts of interest.

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