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Original Research

An In Vitro Study to Evaluate the Cytotoxicity of Different Concentrations of Nano-Graphene Oxide Incorporated into Polymethylmethacrylate

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ABSTRACT:

Objectives: This study evaluated the cytotoxicity of graphene oxide incorporated into polymethylmethacrylate resins at three different concentrations for dental applications. **Material and Methods:** Nano-graphene oxide was incorporated into heat-cured poly methyl methacrylate resin at 0.5%, 1%, and 2% (w/w) concentrations. Cytotoxicity was assessed using MTT assay with NIH/3T3 fibroblasts. Specimens were incubated for 24 hours, followed by MTT reagent exposure and optical density measurement at 450 nm. Cell viability percentages were calculated relative to pure polymethylmethacrylate controls. Statistical analysis was performed using one-way ANOVA with significance at $p<0.05$. **Results:** Significant differences in cell survival were observed among groups ($p<0.0001$). The 2% graphene oxide group showed significantly reduced viability (75.10±2.95%) compared to control (99.99±0.01%). The 1% graphene oxide group demonstrated moderate cytotoxicity (79.18±2.06%), while the 0.5% group maintained high viability (93.47±2.53%) comparable to control. **Conclusions:** Incorporation of 0.5% graphene oxide with polymethylmethacrylate appears safe, while higher concentrations may lead to cytotoxicity.

Keywords- Graphene oxide; PMMA; cytotoxicity

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INTRODUCTION

Graphene oxide is an analogue compound of graphene made up of sp3 – bonded carbon atoms connected with oxygen functional groups, which possess several extraordinary chemical, physical, optical, electrical and mechanical properties [1-5]. Graphene oxide has shown good potential in many research fields, including biomedical and dental applications for its outstanding properties. The demand for research into the biomedical application of graphene oxide and its derivatives is due to many fascinating properties such as its high specific surface area 2630m2 /g, mechanical strength young's modulus of 1100GPa, scalable production, low cost, and easy biological/chemical functionalization. [6,7]

In recent years, many studies have explored the use of graphene oxide for dental and medical applications such as drug/gene delivery, antibacterial materials, biocompatible scaffolds for cell culture, and as a means to improve the physicochemical properties of dental biomaterials including dental implants and cements [8]. Graphene oxide has also shown to improve mechanical strength when incorporated into dental materials as a filler by generating uniform stress distribution, improved wear resistance, tensile strength, flexural strength and thermal properties.

Polymethyl methacrylate (PMMA) is a polymer having high light transmittance, good chemical, weathering, and corrosion resistance properties which has been a widely used material in prosthodontics as well as in many other biomedical applications [9,10]. It is a transparent plastic which is commonly known as acrylic glass. PMMA has also found a wide range of applications in industrial components such as sensors, actuators, optical fibres, electronic devices, coatings, binders and additives [11-13]. However, PMMA in bulk often lacks thermal stability and mechanical properties like low flexural and tensile

strength for high-tech applications including applications in dentistry.

Therefore, different nanomaterials have been incorporated as nanofiller to improve the performance of this polymer. PMMA, which has fine compatibility and processability with carbon nanofillers, plays an important role in the field of nanocomposites [14]. Carbon nanofillers such as graphene, graphene oxide (GO), and graphite have been used for the fabrication of PMMA nanocomposite in recent years [15]. Graphene has also attracted strong research interest in PMMA nanocomposite. Graphene appears to be a cutting-edge material which tends to improve the electrical conductivity, strength, thermal properties, and other important physical characteristics of polymeric nanocomposites, at very low loading level [16,17].

Development of oxygenated form of graphene, i.e., GO has provided an inexpensive way to develop PMMA-based functional materials with improved properties. The inclusion of graphene oxide into PMMA has been found to enhance the thermal, mechanical, biological as well as electrical properties of this nanocomposite. Graphene oxide incorporated into PMMA as a reinforcement filler has also shown to prevent microbial adhesion and makes the material bacteriostatic [18]. Similarly, natural graphite and graphite flakes have also opened up wide interest in the field of dental materials science.

Kuila et al [19]. fabricated poly (methyl methacrylate)/graphene nanocomposite using in situ emulsion polymerization technique. Thermogravimetric analysis (TGA) showed that the thermal stability of this nanocomposites was increased by 35°C. Dynamic mechanical analysis (DMA) and differential scanning calorimetry (DSC) analyses indicated that graphene is indeed a reinforcing nanofiller in PMMA matrix. Zeng et al [20] prepared PMMA/GO nanocomposites by solution blending method. Natural graphite flakes as well as physically or chemically modified graphite have also been reinforced to form polymer/ graphite composites in several studies. Chen et al. [21] obtained graphite nanosheets by treating expanded graphite in aqueous alcohol solution. In situ polymerization of methyl methacrylate (MMA) monomer can also be used to form the nanocomposite. In this way, two types of nanocomposites can be prepared, one with pristine graphite powder and other with modified graphite nanosheet. Zheng et al. [22] also studied obtaining PMMA/modified graphite composites using a direct solution blending method. Technical implementation of poly (methyl methacrylate)/graphene, poly (methyl methacrylate)/graphene oxide, and poly (methyl methacrylate)/graphite nanocomposite includes making of bone cement, flame retardant material, EMI shielding, sensors, supercapacitor and also, prosthodontic implications such as a denture base material and implant frameworks.Due to the development of various graphene-based materials for dental and medical applications, it is necessary to understand their interaction with the biological systems which may lead to potential local and systemic toxic effects. At different concentrations of graphene oxide, the body's response to the same can be different leading to systemic or local side effects. Also, the chemical interactions between graphene oxide and PMMA can lead to formation of cytotoxic by-products at different concentrations of graphene oxide. Hence the purpose of the present study is to evaluate the biocompatibility and cytotoxicity of graphene oxide incorporated into polymethylmethacrylate for dental applications. Limited availability of literature assessing the cytotoxicity level of nanographene oxide incorporated into polymethylmethacrylate for dental applications necessitates the need for further research on its biocompatibility. The present study is intended to access the cytotoxicity of graphene oxide incorporated into PMMA at different concentrations using cultured fibroblastic cells

MATERIALS AND METHODS

Test samples were prepared using a metallic mould of 60mm length, 20mm breath and 2 mm depth(figure 1). Graphene oxide (Nanomatrix materials, India) was measured and mixed with 0.5%, 1% and 2% poly methyl methacrylate resin (DPI Heat Cure, India) powder respectively and heat cured in the metallic mould by long curing cycle. To standardize the test samples, they were measured for equal mass by volume ratio and were cut into small pieces of dimensions 2mm length, 2 mm breadth and 2mm depth(figure 2). All the specimens were sterilized by exposing them to ultraviolet light for 20 minutes on each side. The samples were manipulated under aseptic conditions to prevent the risk of biological contamination during the cytotoxicity testing. T3T mouse connective tissue fibroblasts (NCCS, Pune) were used to study the cytotoxicity of reduced graphene oxide (Nanomatrix materials) incorporated in polymethylmethacrylate resins in vitro and were kept in a CO2 incubator unit. Their present study consisted of four groups and all the experiments are done in triplicates.

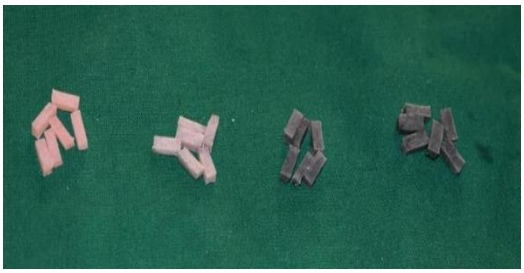
The groups are- 1) The control group consisted of normal untreated mouse fibroblastic cells of the NIH-T3T cell line and unmodified PMMA (control group). 2)Fibroblasts in PMMA with 0.5% reduced graphene oxide (test group 1). 3)Fibroblasts in PMMA with 1% reduced graphene oxide (test group 2). 4)Fibroblasts in PMMA with 2% reduced graphene oxide (test group 3)

All the specimens were immersed in 7 ml of culture medium for about 24 h at 37°C to extract the residual monomer or cytotoxic substances. The culture medium, which contained the material extracts, was sterilized by filtering and then added to the cell cultures containing NIH/T3T cells. These mice's fibroblast cells were cultured at 37°C under a

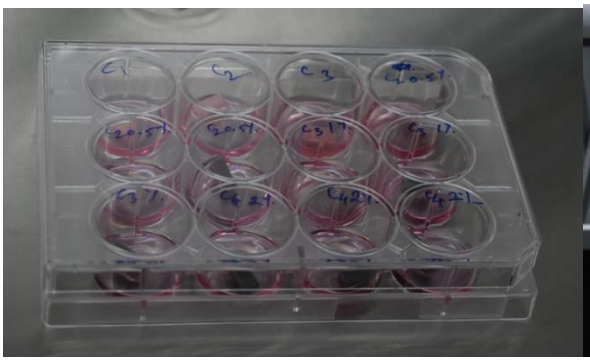
humidified atmosphere of 5% CO2 and 95% air and they were grown in DMEM (Dulbecco's modified eagle medium) and High Glucose medium (HIMEDIA Laboratories, Mumbai). Following which the solution was then supplemented with 10% fetal bovine serum (HIMEDIA Laboratories, Mumbai) and 1% Antibiotic Antimycotic solution (HIMEDIA Laboratories, Mumbai) (figure 3).

Cell survival was determined using a MTT 5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide) assay which is one of the most commonly used test quantitative tests. NIH 3T3 fibroblasts were plated out at a density of 2 x 10^5 per well separately in 6 well plates. They were grown till 60-70% confluence was reached and were verified under a microscope (Motic Image PLUS 2.0). Following this, first the control group and then the test samples were placed in three different six-well plates. This experiment was done in triplicates. After 24 hours of incubation, MTT reagent (200 ml, 5 mg/ml) was added into the fibroblastic cells and kept undisturbed for 2 hours at 37°C. After two hours, MTT solubilizing buffer was added to solubilize the crystals for 1 hour at room temperature. The optical density (OD) value was then measured by Epoch Microplate Reader (Biotek Instruments, Highland Park, VT, USA) at a wavelength of 450nm(figure 4). The experiments were performed at least three times and were calculated using average values of the optical density obtained. Measured absorbance values were directly used for calculating percent of viable cells remaining after the experiment. The cell survival rate of the NIH/T3T fibroblastic cells in each well plate was analysed after the end of the experiment using one way ANOVA test.

(Figure 1)



(Figure 2)



(Figure 3)

(Figure 4)

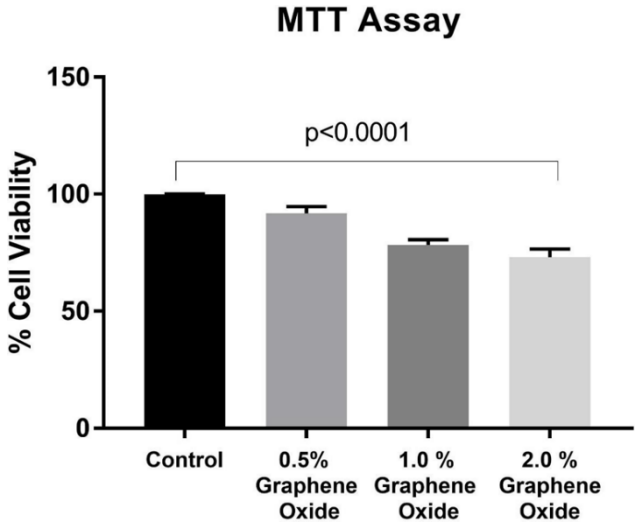
RESULTS

The data obtained during the course of the study was subjected to statistical analysis (One Way ANOVA). Cell survival in each group was compared with that of the untreated control group (Table 1). Data were expressed as a percentage to the control group. The statistical analysis was done using Graph pad prism version 3.02. The results of the MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) was analysed by using one way ANOVA. Differences between mean values were statistically analysed. Significance level was set as $P<0.0001$. The processed data obtained from MTT assay are shown in graphs with a colour scale, making it possible to compare the cell survival rate with different concentrations of graphene oxide in PMMA and PMMA without graphene oxide.

Table 1: cell survival rate (optical density) of different concentrations of graphene oxide compared with that of the control group.

Group	Cell Survival (% of control)	SD(±)
Control (PMMA only)	99.99	0.01
0.5% Graphene Oxide	93.47	2.53
1% Graphene Oxide	79.18	2.06
2% Graphene Oxide	75.10	2.95

According to the results of ANOVA, there were significant differences among the groups in terms of cell survival percentage ($p < 0.0001$). When all the groups were compared, 2% graphene oxide had significantly decreased cell survival rate when compared to the control group. 1% had slightly less cell survival rate than 0.5% graphene oxide and control group. There is an insignificant difference between cell survival rate of 0.5% graphene oxide and control group. The processed data obtained from the MTT assay is shown in a graph with a colour scale (figure 5).



(Figure 5)

DISCUSSION

Polymethyl methacrylate (PMMA) has been clinically widely used in biomedical and dental applications as a biomaterial for removable or implant prosthesis (e.g., denture base resin, provisional restorative materials, maxillofacial prostheses, bone cement). They present good properties, such as low modulus of elasticity, good aesthetics, ease of repair, low cost and have a relatively fast manufacturing process. However, their poor resistance to wear and tear, polymerization shrinkage, lack of strength under fatigue failure, and the microbial adhesion onto PMMA are a major drawback for their long-term use. Over the years, different nano- particles, nano-sheets, nano-fibres or nanotubes have been added to the material to overcome these drawbacks.

Among different nano-materials used to enhance the properties of polymethylmethacrylate resin, graphene oxide has recently been in limelight because of some of its excellent properties. Graphene is a single sheet of one-atom thickness which is arranged in a honeycomb-like lattice where each carbon atom is covalently bonded to three other carbon atoms with sp2 hybridization. The interlayers of the graphene material are re-arranged through weak Van der Waal forces and these forces are responsible for the softness of the material. Graphene exists mainly in three different forms which are graphene sheets, graphene oxide (GO) and reduced graphene oxide (rGO). Graphene oxide properties, such as its biodegradability, strength (Young's modulus of Y ~ 1.0 TPa), antimicrobial-adhesion characteristics, flexibility, and transparency make it a material with potential in prosthodontics.

Studies have proved that graphene oxide incorporated into PMMA can improve its mechanical properties as well as antimicrobial properties which can be very effective with patients who have systemic illness and are resistant to common antibiotics or antifungal drugs. In most of the studies, 0.5% of graphene oxide was used. However, some of the studies have utilized graphene upto 2%.

Literature has also been quoted regarding multiple occurrences of hypersensitivity and incompatibility with such amalgamations of graphene oxide with different materials. Graphene oxide was mixed with PMMA up to 2% only in most of the studies, 0.5% of graphene oxide being the most commonly used combination. However, there is very little evidence of the cytotoxic nature of these types of amalgamations in the literature. Therefore, the purpose of this study is to evaluate the cytotoxicity of graphene oxide powder

at 0.5%,1% and 2% concentrations in PMMA resin using cultured fibroblasts by checking their cell viability, in vitro.

Biocompatibility of potential materials can be adjudicated using different in- vitro tests such as histo-chemical staining of cultured cells, tests for cell growth, LDH (Lactate dehydrogenase) leakage, GSH (Glutathione) content - measured using colorimetric assays, MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay, and colony formation assay. Mutagenicity tests such as Ame's test and HPRT (hypoxanthine-guanine-phosphoribosyl- transferase) test are also conducted using varied cell lines. Testing of dental materials by cell culture methods is relatively simple to perform, reproducible, and can be carefully controlled. The main advantages of cell culture tests are that there are no ethical considerations and their standardization is impeccable. These tests may be more suitable as an alternative to the costly, controversial animal experiments, which may also have several uncontrollable variables. Besides ethical considerations, in vitro cytotoxicity tests undoubtedly have the advantage of easy control of experimental factors that are often problematic when performing experiments in vivo.

In the present study, the cytotoxicity of nano graphene oxide at 0.5%, 1% and 2 % incorporated into PMMA resin as a dental material was evaluated using MTT assay.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay is one of the most popular and standard recognized qualitative test for determination of cytotoxic effects of a given material, among many other cytotoxicity evaluation tests such as cell proliferation assay, cell transformation assay, tests for systemic toxicity and mutagenicity tests.

Even though these tests pose a tiring and time-consuming attempt at counting the number of colonies manually or evaluating them under the microscope, they are still regarded as gold standard tests.

The results demonstrated that the fibroblasts showed no remarkable morphologic alterations. The cell viability observed in the test sample was not increased because of the presence of graphene oxide. But slight decrease in cell viability was noted with increase in graphene oxide concentrations in PMMA resin in the present study. 0.5% graphene oxide incorporation showed more cell survival rate of about 90%. On the other hand, 1% of graphene oxide incorporation showed about 80% of cell survival rate and 2% of graphene oxide incorporation showed about only 75% of cell survival rate, which revealed mild cytotoxic effects. Statistical analysis was done of the values received from the experiment between the test samples and the control group using one way ANOVA. According to the results of ANOVA, there were significant differences between the cell survival rates of these groups with p value <0.0001. 2% graphene oxide has exhibited the highest amount of cytotoxicity followed by 0.1% graphene oxide in PMMA. 0.5% graphene oxide incorporation has shown least amount of cytotoxicity and can be concluded that 0.5% of graphene oxide can safely be incorporated with PMMA to enhance its mechanical and biological properties. Polymethylmethacrylate resins (heat cure) as well as nano graphene oxide at concentrations less than 20g/ml have proven to be non-cytotoxic individually according to another research. However, according to the results of the present study, the interaction between them showed that there is a potential increase in cytotoxicity of the combination with an increase in the percentage of graphene oxide. Therefore, addition of graphene oxide to PMMA should be carefully controlled and arbitrary addition of graphene oxide powder to PMMA to enhance its properties should not be done at the expense of increasing its cytotoxicity. Further scope of the study needs to be carried out on more extensive bases and with different nano-graphene oxide materials marketed by different other companies.

CONCLUSIONS

Within the limitations of this study, it can be concluded that 0.5% of graphene oxide can be incorporated with PMMA safely. But increasing the concentrations of graphene oxide arbitrarily more than 0.5% can be cytotoxic and a prosthodontist should not merely increase the concentration of graphene oxide to enhance its properties at the expense of making the material cytotoxic. However, more studies are required with different tests for checking cytotoxicity and with different forms of graphene oxide manufactured by different other companies to validate the results of the present study.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Conceptualization: Sarma G, Krishnapillai L;
Methodology: Sarma G, Bargale A;
Formal Analysis: Sarma G, Krishnapillai L;
Investigation: Sarma G, Bargale A;
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Writing – Original Draft: Sarma G, Goel A;
Writing – Review & Editing: Sarma G, Goel A;
Supervision: Krishnapillai L.

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