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ORIGINAL RESEARCH

COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF GRAPHENE OXIDE AND REDUCED GRAPHENE OXIDE NANOPARTICLES AGAINST COMMON ORAL PATHOGENS:IN VITRO STUDY

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ABSTRACT

Background: The escalating threat of antimicrobial resistance (AMR) has driven the search for alternative antibacterial agents. Graphene-based nanomaterials, particularly graphene oxide (GO) and reduced graphene oxide (rGO), have emerged as promising candidates due to their unique physicochemical properties and potential antimicrobial mechanisms.

Objective: This study aims to evaluate and compare the antimicrobial efficacy of GO and rGO nanoparticles against common oral pathogens—*Streptococcus mutans*, *Enterococcus faecalis*, and *Candida albicans*.

Materials and Methods: The antimicrobial activity of GO and rGO was assessed via agar well diffusion and time-kill kinetics. GO and rGO were tested at concentrations of 25, 50, and 100 µg/mL, with standard antibiotics as controls. Inhibition zones were measured, and statistical significance was evaluated using one-way ANOVA and Tukey's post hoc test

Results: GO demonstrated superior, dose-dependent antimicrobial activity compared to reduced GO, particularly against *Candida albicans* (18.48 ± 0.06 mm at 100 µg/mL), followed by *S. mutans* and *E. faecalis*. rGO showed limited efficacy in inhibition zone assays, although time-kill kinetics revealed its bactericidal potential, especially against *E. faecalis*.

Conclusion: GO exhibited stronger antimicrobial performance than rGO, likely due to its higher oxygen content and associated reactive oxygen species (ROS) generation. While rGO was less effective in static assays, its performance in time-kill kinetics suggests potential in dynamic or prolonged-contact applications. Graphene-based nanomaterials show promise for dental antimicrobial use, but require further optimization and in vivo validation

Keywords: Graphene oxide, Reduced graphene oxide, Antimicrobial efficacy, Oral pathogens, Nanoparticles

1. INTRODUCTION

The surge in bacterial infections has become a major threat to global health, with significant impacts on public well-being and millions of individuals worldwide. Antibacterial agents are essential for protecting human health from these severe and potentially life-threatening diseases. Nonetheless, the inappropriate and excessive use of conventional antibiotics has led to the emergence of antibiotic-resistant bacteria, rendering many infections difficult, if not impossible, to treat effectively[1]. This phenomenon, known as antimicrobial resistance (AMR), has emerged as one of the most critical and pressing public health challenges of the 21st century[2]. AMR refers to the capacity of microorganisms—including bacteria, viruses, fungi, and parasites—to resist the effects of drugs that were previously effective in eradicating

or controlling infections[3].

The antimicrobial effectiveness of dental products is crucial for fighting oral pathogens, preventing dental caries, gingivitis, and periodontal diseases, while also reducing plaque buildup and controlling bacterial growth to promote overall oral health[4].Various antibacterial materials, including metal ions and oxides, antibiotics, quaternary ammonium compounds, and antimicrobial peptides, have been utilized in efforts to combat bacterial infections. However, each of these materials has its own set of limitations and challenges. Metal ions and oxides are known for their broad-spectrum antimicrobial activity against a range of pathogens, including fungi, viruses, and bacteria. Despite their effectiveness, they can exhibit toxicity towards certain mammalian cells. Prolonged use of antibiotics and quaternary ammonium compounds often leads to the development of resistant strains of bacteria. Furthermore, the production and use of pure antimicrobial peptides as antibacterial agents are often hindered by their high costs. To overcome these challenges, researchers have increasingly turned to alternative antibacterial materials such as metal nanoparticles (NPs), carbon nanotubes, metal oxide NPs, and graphene-based materials[1].

Nanomaterials, defined by dimensions ranging from 1 to 100 nm, display distinct physical, chemical, and biological properties that differentiate them from their bulk counterparts[5]. Nanoparticles (NPs) are insoluble particles smaller than 100 nm, and the science of manipulating them is known as nanotechnology. Due to their small size and ability to penetrate biological systems easily, they are widely used in medicine, drug delivery, and dentistry. Their applications are supported by properties such as antimicrobial activity, friction reduction, anti-inflammatory effects, and antioxidant capabilities[6].

Among the myriad of chemical elements, carbon occupies a distinctive and pivotal role. It serves as the fundamental scaffold for biological systems, thanks to its diverse orbital hybridization states which facilitate the formation of various chemical bonds with multiple spatial configurations.

Consequently, carbon exists in several allotropic forms, such as graphite and diamond, and possesses the potential to produce a broad spectrum of nanostructures, including monolayer graphene sheets, single-walled and multi-walled carbon nanotubes, carbon fibers, fullerenes, carbanions, and Nano diamonds[7].

Carbon is a fundamental element with versatile bonding characteristics that enables it to form a variety of allotropes. These carbon-based materials have gained attention for their potential applications due to their remarkable mechanical and biological properties. Carbon-based nanostructures (CNSs), such as fullerenes, carbon nanotubes (CNTs), and various forms of diamond, are especially noteworthy for their wide-ranging applications in fields such as drug delivery, tissue engineering, imaging diagnostics, and cancer therapy[8]. Among the different forms of carbon, graphene is of particular interest because of its exceptional properties[9].In 2004, Geim and colleagues achieved a significant breakthrough by synthesizing a stable monolayer of graphene through mechanical exfoliation techniques. This accomplishment earned Geim and Novoselov the Nobel Prize in Physics in 2010. Since then, graphene has become one of the most extensively studied two-dimensional materials in scientific research. Graphene consists of a single atomic layer of carbon atoms arranged in a hexagonal lattice, with a thickness of only 0.334 nm, making it the thinnest material known. It possesses a range of unique properties, including an extraordinarily high specific surface area (~2600 m^2/g), exceptional electron mobility (200,000 cm^2/Vs), superior thermal conductivity (3000–5000 W/m·K), remarkable optical transparency (97.4%), and outstanding mechanical strength, with a Young's modulus of 1 Tpa[10].Graphene composites are emerging as versatile materials for developing specialized inks with functions like biocompatibility and electrical conductivity, which can enhance 3D and 4D bio printing processes, enabling the creation of dynamic tissue structures that better mimic natural tissue behavior and support regeneration across various types of tissues, including bone and nerve[11]. Graphene oxide (GO) is a derivative of graphene that has been oxidized to introduce various oxygen-containing functional groups, such as carboxylic acids (–COOH), carbonyls (–C=O), and hydroxyls (–OH), on its surface and edges. These functional groups facilitate interactions with biomolecules and contribute to bacterial inactivation through several mechanisms, including membrane stress, oxidative damage, entrapment, and photo-thermal effects. The sharp edges of GO nanosheets can also cause physical damage to bacterial membranes, leading to bacterial death through the leakage of intracellular components[12-14]. Graphene oxide, a single-layer 2D sheet of sp^2-bonded carbon atoms in a honeycomb pattern, is valued for its strong mechanical properties, electrical conductivity, and especially its barrier capabilities. Its simple synthesis process also makes it an effective carrier in nanocomposites[15]. Another noteworthy derivative of graphene is reduced graphene oxide (rGO). This material is produced by removing the oxygenated functional groups from graphene oxide through electrochemical reduction. The resulting rGO films have a significantly lower number of oxygen-containing groups compared to GO[16].

Due to the distinct differences in their physical, chemical, structural, and electronic properties, graphene (Gr), GO, and rGO interact with bacterial cells in varied ways[17].The rising demand for graphene oxide (GO) and reduced graphene oxide (rGO) in drug delivery, biomedical applications, and diagnostics is largely attributed to their significant impact on cytotoxicity, biodistribution, and biotransformation, with 3D scaffolds of these materials enhancing preclinical research due to their reactive oxygen functional groups and electrical insulation properties[18].

This study evaluates and compares the antimicrobial efficacy of graphene oxide (GO) and reduced graphene oxide (rGO) nanoparticle against common oral pathogens, including *Streptococcus mutans*, *Enterococcus faecalis*, and *Candida albicans*.

2. MATERIAL AND METHODS

Antimicrobial Activity Assessment

Preparation of nano particle

Graphene oxide (GO) nanoparticles were synthesized using a modified Hummers' method. Briefly, graphite powder was oxidized using concentrated sulfuric acid (H_2SO_4), sodium nitrate ($NaNO_3$), and potassium permanganate ($KMnO_4$), followed by controlled heating, washing with distilled water, and centrifugation until a neutral pH was reached. The obtained GO was dried at 60 °C. Reduced graphene oxide (rGO) was subsequently synthesized by chemical reduction of the prepared GO using ascorbic acid as the reducing agent under mild heating and continuous stirring. Both GO and rGO powders (0.100 g each) were dispersed in 5 mL of ethanol, and a series of different concentrations were prepared for subsequent antimicrobial analysis.

The antimicrobial properties of graphene oxide (GO) and reduced graphene oxide (rGO) nanoparticles were assessed using the agar well diffusion method. Mueller Hinton agar plates were prepared, sterilized at 121°C for 15–20 minutes, and then poured into sterile Petri dishes to cool to room temperature. A suspension containing *Streptococcus mutans*,Entercoccus faecalis , and *Candida albicans* was spread across the agar surface with sterile cotton swabs. Wells with a 9 mm diameter were formed in the agar plates using a sterile polystyrene tip and filled with various concentrations of GO and rGO (25, 50, and 100 µg/ml). An antibiotic control (Amoxyrite for bacteria and comparison. Plates were incubated at 37°C for 24 hours (48 hours for fungi), and inhibition zones were measured Fluconazole for fungi) was included for in millimeters (mm) for a comprehensive analysis,

Time-Kill Kinetic Analysis

To further investigate bactericidal effects, a time-kill curve assay was conducted. Pathogens were cultured in Mueller Hinton Broth with varying concentrations of GO and rGO (25, 50, and 100 µg/mL). An antibiotic control was used for comparison, and pre-incubation for four hours allowed pathogens to reach a stable growth phase. A 0.5 McFarland standard inoculum was prepared from Mueller Hinton agar cultures grown at 37°C for 18–20 hours. Thirty microliters of inoculum was diluted into 15 mL of antimicrobial-free broth pre-warmed to 37°C, and 90 µL of this mixture was added to each well of a 96-well ELISA plate. Ten microliters of GO and rGO at five concentrations were added, along with an untreated control, providing insights into antimicrobial kinetics and nanoparticle effectiveness against pathogens.

Statistical Analysis

All experimental data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine pairwise differences between groups. A p-value < 0.05 was considered statistically significant. Results are presented as mean ± standard deviation (SD).

3. RESULTS

Graphene oxide (GO) and reduced graphene oxide (rGO) demonstrated varied antimicrobial activities across concentrations and pathogens (Figure 1) .GO exhibited a dose-dependent increase in the zone of inhibition, particularly effective against Candida albicans (18.48 ± 0.06 mm at 100 µg/mL), followed by Streptococcus mutans (11.57 ± 0.05 mm) and Enterococcus faecalis (9.17 ± 0.05 mm).(Table 1)

In contrast, rGO showed limited antimicrobial efficacy with less pronounced differences across concentrations. The highest zone of inhibition was observed against Candida albicans (14.83 ± 0.06 mm at 100 µg/mL), while E. faecalis and S. mutans showed zones below 9.3 mm.(Table2)

Post hoc test revealed significant differences ($p < 0.001$) in antimicrobial activity among concentrations for all organisms tested. GO showed significant differences between all concentrations for each organism. Post hoc tests also confirmed that GO's efficacy, while significant, was consistently lower than the standard antimicrobial agents. (Table 3)

For rGO, while Candida albicans showed statistically significant dose-dependent efficacy, no significant difference was observed between concentrations against S. mutans. The efficacy of rGO was also consistently inferior to the standard controls.(Table 4)

Table 1. Mean Zones of Inhibition Graphene Oxide

Microorganism	Concentration (mg/mL)	N	Mean	SD
E. faecalis	100	4	9.17	0.0520
	25	4	8.88	0.0485
	50	4	9.53	0.0490
	Standard	4	32.08	0.0540
S. mutans	100	4	11.57	0.0530
	25	4	8.67	0.0470
	50	4	9.72	0.0510
	Standard	4	19.38	0.0560
C. albicans	100	4	18.48	0.0590
	25	4	11.07	0.0505
	50	4	11.22	0.0515
	Standard	4	32.48	0.0580

Table 2. Mean Zones of Inhibition reduced graphene oxide

Microorganism	Concentration (mg/mL)	N	Mean	SD
E. faecalis	100	4	8.72	0.0520
	25	4	9.28	0.0495
	50	4	8.67	0.0510
	Standard	4	33.73	0.0550
S. mutans	100	4	8.93	0.0535
	25	4	8.78	0.0480
	50	4	9.03	0.0505
	Standard	4	28.82	0.0570
C. albicans	100	4	14.83	0.0585
	25	4	9.13	0.0515
	50	4	8.63	0.0490
	Standard	4	34.92	0.0600

Table 3. Graphene Oxide (GO) - Post Hoc Comparisons

Microorganism	Comparison	Mean Difference	P Value	Significance
E. faecalis	100 vs 25	0.29	<0.05	Significant
	100 vs 50	-0.36	<0.05	Significant
	100 vs Standard	-22.91	<0.001	Significant
	25 vs 50	-0.65	<0.05	Significant
	25 vs Standard	-23.20	<0.001	Significant
	50 vs Standard	-22.55	<0.001	Significant
S. mutans	100 vs 25	2.90	<0.001	Significant
	100 vs 50	1.85	<0.05	Significant
	100 vs Standard	-7.81	<0.001	Significant
	25 vs 50	-1.05	<0.05	Significant
	25 vs Standard	-10.71	<0.001	Significant
	50 vs Standard	-9.66	<0.001	Significant
C. albicans	100 vs 25	7.41	<0.001	Significant
	100 vs 50	7.26	<0.001	Significant
	100 vs Standard	-14.00	<0.001	Significant
	25 vs 50	-0.15	>0.05	Not Significant
	25 vs Standard	-18.85	<0.001	Significant
	50 vs Standard	-18.70	<0.001	Significant

Table 4. Reduced Graphene Oxide (RGO) - Post Hoc Comparisons

Microorganism	Comparison	Mean Difference	P Value	Significance
E. faecalis	100 vs 25	-0.56	<0.05	Significant
	100 vs 50	0.05	>0.05	Not Significant
	100 vs Standard	-25.01	<0.001	Significant
	25 vs 50	0.61	<0.05	Significant
	25 vs Standard	-25.45	<0.001	Significant
	50 vs Standard	-26.06	<0.001	Significant
S. mutans	100 vs 25	0.15	>0.05	Not Significant
	100 vs 50	-0.10	>0.05	Not Significant
	100 vs Standard	-19.89	<0.001	Significant
	25 vs 50	-0.25	>0.05	Not Significant

The time-kill assay showed that GO had bacteriostatic effects, producing only slight CFU reductions over time. In contrast, rGO displayed a stronger bactericidal trend, especially at higher concentrations. This was most notable in E. faecalis and C. albicans, where CFU counts significantly declined over the 24-hour period, supporting the potential for time-dependent antimicrobial action. (Figure 2).

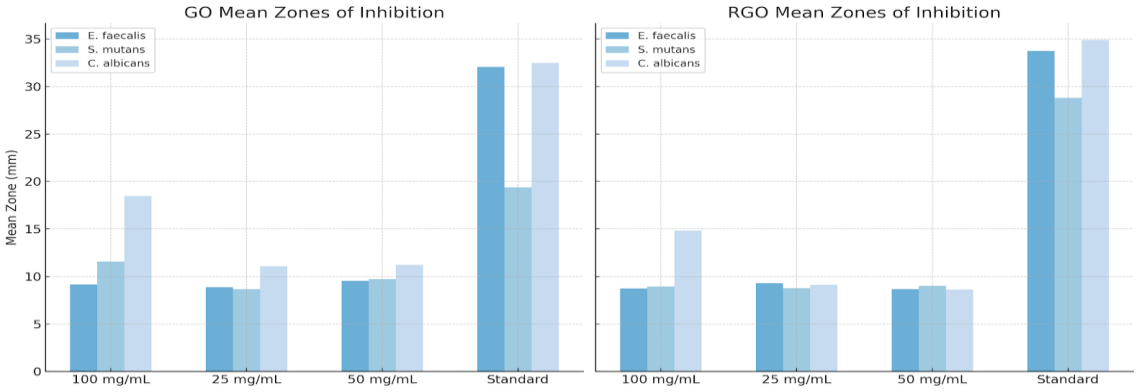

Figure 1. Mean Zones of Inhibition of Graphene Oxide and Reduced Graphene Oxide

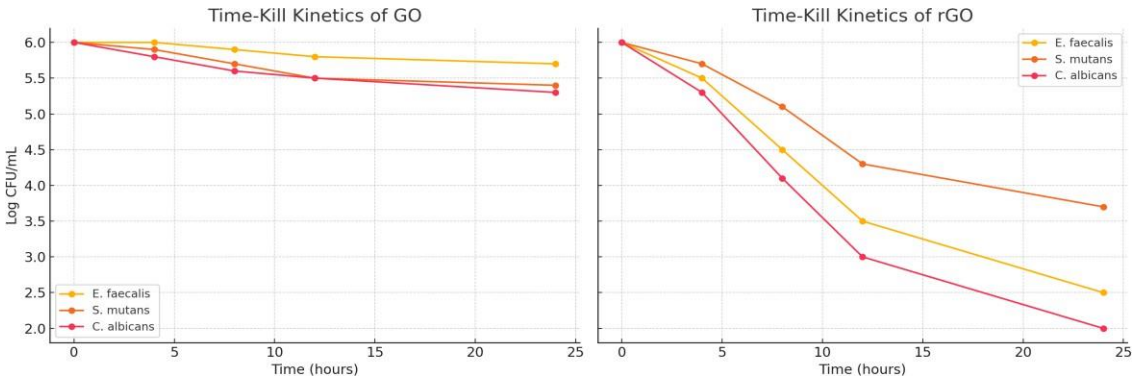

Figure 2. Time-Kill Kinetics of GO and rGO against E. faecalis, S. mutans, and C. albicans over 24 hours.

4. DISCUSSION

The results of the study shows that Graphene oxide demonstrated superior antimicrobial activity, particularly against *Candida albicans*. The zone of inhibition increased in a dose-dependent manner, with the highest concentration (100 µg/mL) producing a zone of 18.48 mm. This enhanced performance of GO can be attributed to its abundant oxygen-containing functional groups, such as hydroxyl and carboxyl groups, which generate reactive oxygen species (ROS) capable of damaging microbial membranes[14]. Furthermore, the time-kill kinetics analysis confirmed that GO has a significant fungicidal effect, reducing the colony-forming units (CFU) of *Candida albicans* over time, particularly at higher concentrations.

On the other hand, reduced graphene oxide showed limited antimicrobial activity compared to GO. The zone of inhibition for rGO remained small for both *Streptococcus mutans* and *Enterococcus faecalis*, with a notable exception at 100 µg/mL against *Candida albicans*, where the zone of inhibition increased to l4.83 mm. The reduced antimicrobial efficacy of rGO can be explained by its lower oxygen content, which reduces the ability to produce ROS and weakens its interaction with bacterial cell membranes [19]. However, despite its weaker performance in the inhibition zone assays, rGO showed stronger bactericidal effects in time-kill kinetics, particularly against *Enterococcus faecalis*. This suggests that rGO may still be effective in dynamic environments where prolonged exposure enhances its bactericidal capabilities.

The observed difference in antimicrobial activity between GO and rGO aligns with existing research. GO's higher oxygen content enhances its ability to generate ROS, causing oxidative damage and physical disruption of bacterial membranes, leading to cell death (Perreault et al.)[20]. These mechanisms explain the greater inhibition zones observed for GO in this study, particularly at higher concentrations. Additionally, the sharp edges of GO nanosheets contribute to membrane piercing, which further enhances its antimicrobial action [12].Reduced graphene oxide's limited efficacy in the inhibition zone assays, particularly against *Streptococcus mutans* and *Enterococcus faecalis*, is consistent with findings that rGO's lower oxygen content diminishes its ROS production, resulting in weaker interactions with bacterial cells (Akhavan et al.).[12]However, the time-kill kinetics data suggest that rGO's antimicrobial action may be more pronounced under prolonged exposure, as seen in its bactericidal effect against *Enterococcus faecalis* at higher concentrations. This indicates that while rGO maynot perform as effectively as GO in static assays, it could still be a viable option in applications where prolonged contact time is feasible.

Despite these promising results, it is important to note that both GO and rGO exhibited lower antimicrobial activity compared to standard antibiotics used as controls in the study. This underscores the challenge of developing nanomaterials that can match or exceed the efficacy of traditional antibiotics. The standard antimicrobial agents showed significantly larger inhibition zones, particularly against *Enterococcus faecalis*, suggesting that further modification or combination of GO and rGO with other antimicrobial agents may be necessary to enhance their effectiveness (Liu et al.)[14]. Combining graphene-based materials with metal nanoparticles or antimicrobial peptides could provide a synergistic effect, improving their bactericidal and fungicidal properties while maintaining their biocompatibility[19].

This study highlights the potential of graphene-based materials, particularly graphene oxide, as antimicrobial agents against oral pathogens. GO demonstrated superior antimicrobial efficacy compared to rGO, particularly against *Candida albicans*, due to its oxygen-rich structure and ability to generate ROS. While rGO showed limited efficacy in inhibition zone assays, its bactericidal properties in time-kill kinetics suggest potential applications in prolonged exposure settings. However, both materials need further enhancement to compete with conventional antibiotics, and future research should explore combining them with other antimicrobial agents or modifying their surface properties to optimize their efficacy.

This study's primary innovation is its direct comparison of graphene oxide (GO) and reduced graphene oxide (rGO) against a diverse range of dental pathogens, enhancing our understanding of their distinct antimicrobial properties. Unlike previous research, which has typically concentrated on either bacterial or fungal pathogens, this study evaluates both simultaneously, offering a more comprehensive assessment. It also emphasizes that while rGO exhibits enhanced properties in other biomedical applications, this does not necessarily equate to superior antimicrobial efficacy against fungal pathogens like *Candida albicans*. Despite these valuable insights, the study has several limitations. The in vitro nature does not replicate the complex interactions found in the oral cavity, where factors like saliva, biofilm formation, and host immune responses are crucial.

Additionally, the study relies solely on inhibition zones to measure antimicrobial activity and did not assess other important aspects such as biofilm prevention, resistance development, and the cytotoxicity which refers to the quality of being damaging to cells[21]. These parameters are crucial for determining their safety in clinical applications.

5. CONCLUSION

Graphene oxide (GO) showed stronger antimicrobial effects than reduced graphene oxide (rGO), especially against *Candida albicans*, due to its oxygen-rich surface and ROS generation. While rGO was less effective in static tests, it demonstrated time-dependent bactericidal activity against *Enterococcus faecalis*. These results support the potential of graphene-based nanomaterials in dental antimicrobial applications, though further in vivo evaluation are needed . Both nanomaterials require further optimization, and their clinical application warrants in vivo validation.

DECLARATIONS

Ethical approval and consent to participate

Not Applicable

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Competing and Conflicting Interests

The authors declare no competing or conflicting interests related to this study.

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