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Improve ICG Based Photodynamic Properties Through Conjugation of ICG Into Nano-Graphene Oxide Against *Enterococcus faecalis*

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Abstract

Background: Nowadays, a new technique such as photodynamic therapy (PDT) is used to achieve effective root canal disinfection and eliminate *Enterococcus faecalis* as the most prevalent species associated with secondary endodontic infections and treatment failures. Employment of an optimized nontoxic photosensitizer (PS) such as indocyanine green (ICG) is a crucial part of this technique; the current study aimed at improving ICG photodynamic properties through conjugation of ICG into nano-graphene oxide (nGO) as a new PS, to evaluate the antimicrobial effects of nGO/ICG against *E. faecalis*.

Methods: The nGO was synthesized based on the modified Hummer method and then, direct loading of ICG onto its surface. The nGO formation was evaluated using the scanning electron microscope (SEM). The antimicrobial effect of nGO/ICG-PDT against *E. faecalis* was assessed by counting colony forming units (CFUs).

Results and Conclusion: The SEM analysis confirmed successful synthesis of nGO. The nGO/ICG-PDT at an incorporated concentration of 400 μg/mL ICG with irradiation at an energy density of 31.2 J/cm^2 showed significant reduction in the number of *E. faecalis* higher than PDT based on ICG (1000 μg/mL) ($P < 0.05$). Since nGO-ICG-PDT showed a significant reduction in the count of *E. faecalis* at low concentration of ICG (400 μg/mL), it could be proposed as a new approach to treat endodontic infections, alone or in combination with conventional root canal treatment.

Keywords: Photodynamic Therapy, Indocyanine Green, Graphene Oxide, *Enterococcus faecalis*

1. Background

Enterococcus faecalis is a facultative anaerobic gram-positive coccus (1) that plays an important role in the failure of endodontic treatment and recurrent/secondary endodontic infection (2, 3). This bacterium is resistant against environmental stresses and common disinfectants including calcium hydroxide, sodium hypochloride and chlorhexidine, and a wide range of antibiotics in such a way that application of these agents may cause a shift in the microbial flora in favor of *E. faecalis* (4, 5). Biofilm formation in the dentinal tubules and lateral canals of teeth is considered as the major virulence factor of *E. faecalis* contributing to endodontic infections (4). Elimination of the intracanal bacterial pathogens with a combination of mechanical instrumentation, chemical agents, and irrigation is the main objective of root canal treatment (6, 7). However, in addition to increased bacterial resistance, complexities of the root canal system make the complete elimination of intracanal bacterial pathogens almost impossible (7-10). Numerous studies showed that the above methods only render 50% -70% of the infected canals free of microorganisms, depending on which method is used (11, 12); therefore, a large number of teeth require retreatment and/or periradicular surgery to successfully treat persistent infections (11). Hence, new disinfection methods and standard endodontic antimicrobial procedures should be developed to increase the success rate of endodontic therapy (11, 13) and preserve, and if possible enhance, the dentin chemical/mechanical stability through selective bactericidal properties (14). Photodynamic therapy (PDT) in combination with organic nanomaterials is a promising method to achieve such goals (8, 11, 15, 16). PDT is a non-thermal photochemical reaction

that includes the simultaneous presence of visible light, oxygen, and a dye or photosensitizer (PS) during which ROS formed through types I or II mechanisms may inactivate sub-cellular components of microbial cells (8, 15, 17). For an effective antimicrobial PDT, the ideal PS should have low levels of dark toxicity and be selected for microbial cells versus mammalian host cells. Moreover, it should have optical window (600 - 900 nm) for sufficient tissue penetration (18). A good candidate is indocyanine green (ICG) as an amphiphilic dye, also known as Cardio-Green®, as the only food and drug administration (FDA)-approved PS (19), with the unique peak absorbance at 780 nm (20, 21). Most PSs such as ICG, have limitations such as little photostability and concentration-related aggregation (21, 22). Therefore, there is a growing interest to develop nanomaterials with higher PDT efficacy considering the improved near infrared (NIR)-absorption, stability, and photo-thermal conversion efficiency (21). Graphene oxide, which is the oxidized form of graphene with the single-layer 2-dimensional structure of carbon nanomaterials, has attracted much attention due to its Increased water solubility, compared with graphene as well as other unique properties such as 1) its large surface area presenting many functional groups and domains that make it ideal to carry photosensitizers and other substances through π-π stacking, 2) biocompatibility, 3) lack of obvious toxicity, 4) photothermal activity, and 5) intrinsic high NIR absorbance (21-23).

The current study used nano-GO (nGO) as a platform to load ICG via the π-π stacking interactions. It was found that this new nanostructure carrier had a good photo and physicochemical stability and can deliver targeted antibacterial PDT. To the best of authors' knowledge, no study evaluated the antibacterial effect of PDT using this group of nanocarriers. It was assumed that ICG modified with this nGO compared with ICG, may have a marked antibacterial effect on *E. faecalis*; therefore, the current study aimed at evaluating the effect of PDT based on ICG loaded on nGO on *E. faecalis* by counting colony forming units (CFUs).

2. Methods

2.1. Synthesis of nGO

The synthesis of graphene was carried out using the Hummer method (24). Graphite flakes (Qingdao Tianhe Graphite Co. Ltd., Quingdao, china) were oxidized by dissolving a combination of powerful reagents; i e, potassium persulfate (K2S2O8), and phosphorus pentoxide (P2O5) in sulfuric acid (H_2SO_4, 99%); all were purchased from Sigma-Aldrich (St Louis, MO, USA). After that, dispersed GO in toluene was treated with 3-aminopropyltriethoxysilane (APTES) in toluene under nitrogen atmosphere for 24 hours at 110°C. Following the completion of the reaction, the residual APTES was completely removed through repetitive toluene washing. The product was left to dry overnight in a vacuum drying oven (Thermo Napco vacuum Oven Model 5831; MA, USA) under vacuum conditions at 60°C (25). After that, nGO was transferred to the solution. Then, the precipitates were washed with distilled water and ethanol (99%) at 20°C and dried in a vacuum desiccator at room temperature (26).

2.2. ICG loading of nGO (nGO/ICG)

Loading of ICG (Surva, USA) onto nGO was performed as follows: first, 0.605 mg/mL ICG was added to 5 mL nGO aqueous suspension (final concentration: 4 mg/mL) and stirred. The product was centrifuged at 6000 rpm for 10 minutes at 10°C to remove unbound ICG molecules; the fabricated nGO/ICG solution was stored at 4°C in the dark for further use (19).

2.3. Confirmation of Synthesized nGO-ICG

Scanning electron microscopy (SEM-EDX Philips) was used to achieve higher resolution 3D topographic images of nGO/ICG and its nanosized sheets to confirm the synthesis of nGO.

2.4. Bacterial Strain and Culture Conditions

The current study used *E. faecalis* ATCC 29212. The bacteria were aerobically cultured in the fresh brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) at 37°C until the logarithmic growth phase (4 - 5 hours culture). The cell density was adjusted in a spectrophotometer to a final concentration of 1.0×10^{-6} CFU/mL (optical density [OD] 600 nm: 0.2) (3).

2.5. Preparation Photosensitizer for ICG-PDT

A stock solution of ICG was prepared at 4 mg/mL in sterile distilled water, filter-sterilized by passing through a 0.22 μm pore sized filter immediately, and then stored in a dark chamber before use.

2.6. Light Source

A 808-nm diode laser system (DX82, Konftec, Taiwan) with an output power of 250 mW was used for 60 seconds in the continuous mode as the light source for aPDT experiments. The laser probe was fixed 1 mm above the well. The energy density was 31.2 J/cm^2. The output power of the laser was checked with an optical power meter (Laser check, Coherent, USA) (27).

2.7. Study Design

The nGO/ICG-PDT and ICG-PDT effects on viability of *E. faecalis* with different ICG concentrations were investigated in the 5 following test groups (A-E):

A. ICG group (incubation only with 1000 μg/mL of ICG)

B. ICG + laser group (irradiated at an energy density of 31.2 J/cm^2 in the presence of 1000 μg/mL of ICG)

C. The nGO/ICG group (incubation with nGO/ICG incorporated concentration of 400 μg/mL of ICG)

D. The nGO/ICG + laser group (irradiated at an energy density of 31.2 J/cm2 in the presence of nGO/ICG incorporated concentration of 400 μg/mL of ICG)

E. Control group (only bacterial suspension)

2.8. Antimicrobial photodynamic therapy (aPDT)

2.8.1. Colony Count Assessment

Antibacterial effect of ICG and ICG loaded in functionalized GO against *E. faecalis* was evaluated by broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (28), as described previously (3). In panel A, the wells of a 96-well round-bottomed sterile polystyrene microplate (TPP; Trasadin-gen, Switzerland) were filled with BHI broth (columns 1 - 3), then 100 μL of the ICG (1000 μg/mL) was added. In panel B, nGO/ICG (with 400 μg/mL ICG concentration) was made based on the above-mentioned procedure, 100 μL of the nGO/ICG was added to the column 1. Then, wells were inoculated with 100 μL of fresh *E. faecalis* cultures (1.0 $\times$ 10^6 CFU/mL). Both panels had 1 column as the positive (bacterial growth) control and 1 column as the sterility control (without bacterial inoculation). Before irradiation, the wells were incubated in darkness for 5 minutes at room temperature. After incubation of the PDT group, wells were irradiated using the diode laser 808 nm with output power of 250 mW for 1 minute in continuous mode. The laser light was applied to 1 mm above the well with energy density of 31.2 J/cm^2. Then, a serial dilution of each well was prepared in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 2.7 mM KCl and 137 mM-NaCl at pH 7.4). Samples (10 μL) from each dilution were placed on BHI agar and spread over the entire agar surface with a sterile spreader (1:10 dilution).

A 10 μL from the positive control well was transferred and spread onto a non-selective, enriched nutrient agar (e g, blood agar plates) to check the purity. To ensure that the incolum contained 2 - 8 $\times$ 10^5 CFU/mL (the acceptable range), 10 μL of the positive growth control was diluted with 10 mL saline (1:1000 dilution) and mixed, then 100 μL of the suspension was placed in a non-selective agar medium (e g, trypticase soy agar) and spread over the entire agar surface with a sterile spreader (1:10 dilution). The colony count plates and pure plates were incubated for 24 hours at 37°C. The calculation of CFU/mL of test wells was done based on the Miles and Misra Method (29).

2.9. Statistical Analysis

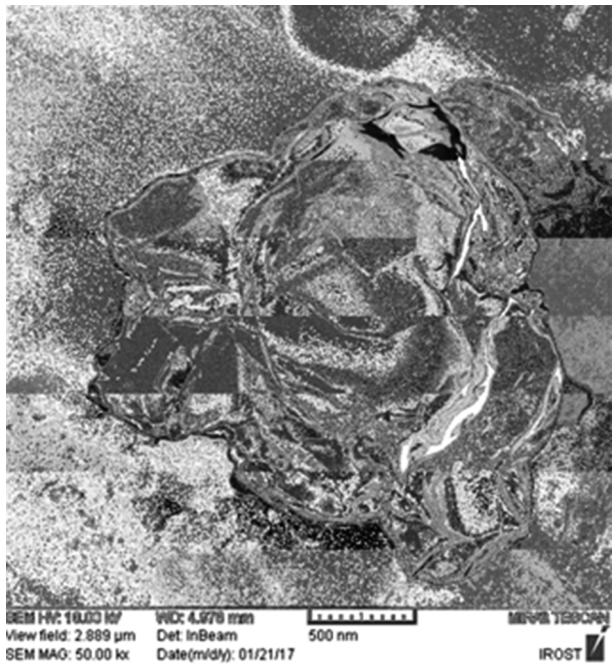
The results of the colony counts measurements were statistically analyzed using the 2-way analysis of variance (ANOVA) followed by Tukey test. All experiments were performed in at least triplicate, and results were reported as mean $\pm$ standard deviation (SD). The significance level was $P < 0.05$ (P3).

3. Results

3.1. SEM analysis

Figure 1 shows the SEM image of nGO. The surface morphology and structure of graphene oxide nanosheets were observed by SEM. SEM images of the nGO had well defined and interlinked 3-dimensional graphene nanosheets.

Figure 1. SEM Image of NGO



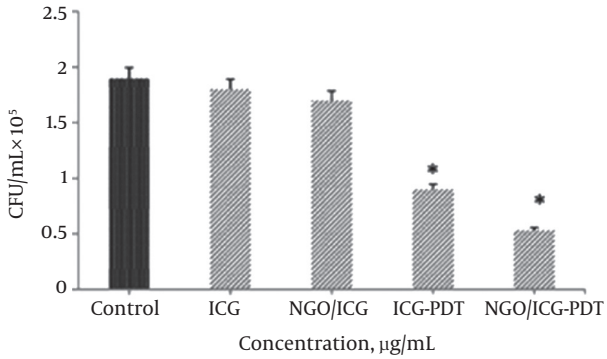
The SEM image implies that the sheets were smooth with small wrinkles at the edges.

3.2. CFU Count

Significantly reduced cell viability of *E. faecalis* was characterized by directly comparing the antimicrobial PDT effect based on nGO/ICG and ICG with the control group

(untreated bacteria) ($P < 0.05$; Figure 2). There was no significant reduction in the count of bacteria in nGO-ICG and ICG alone ($P > 0.05$). A remarkable difference was observed between the values of the nGO/ICG-PDT group at an incorporated final concentration of 400 μg/mL of ICG and ICG-PDT at a higher final concentration of ICG (1000 μg/mL) ($P < 0.05$).

Figure 2. Effects of nGO/ICG-PDT and ICG-PDT on the Reduction of *Enterococcus faecalis* CFU/mL



*Significantly different from that of the control, $P < 0.05$.

4. Discussion

The success of endodontic treatments depends on proper disinfection of the root canal system (7, 15). The available mechanical and chemical methods cannot fully eliminate bacterial pathogens during endodontic treatment (7, 16), which has negative consequences on the treatment outcome (8, 17). *Enterococcus faecalis* is the most common bacterial pathogen in secondary endodontic infections and periapical biofilms, and is frequently found in canals requiring retreatment (7, 13). This microorganism can survive for a long-time without nutrients and is capable of invading the dentinal tubules, resulting in its resistance against irrigating agents and intracanal agents, including calcium hydroxide (7, 11, 13). Therefore, many in vitro and in vivo studies aimed at eliminating *E. faecalis* from the root canal system (10, 30). A number of studies evaluated root canal disinfection with different chemical disinfectants and their effects on *E. faecalis* elimination (7, 31). Direct application of NaOCl or antibiotic treatment may be potentially harmful to the host. For example, NaOCl is highly toxic and malodorous with a bad taste, and/or antibiotic treatment may increase bacterial resistance (7, 31). On the other hand, intracanal irrigants are only effective when they are in direct contact with the surface and cannot penetrate deep due to anatomical barriers (12). Therefore, PDT is suggested as a new method that is simple, painless, and more cost-effective than high power lasers. It is a standard treatment option to eliminate microorganisms involved in periodontal and endodontic infection (7, 11, 13). Studies show that an effective PDT requires the modification of its parameters including PS and irradiation parameters (14). On the other hand, ICG was proposed as PS for its NIR wavelength absorbance at about 800 nm, considering the optical efficacy of laser at NIR wavelength (810 nm) to reduce the *E. faecalis* count in the RCS (32). In addition, this wavelength has a higher depth of penetration compared with red lasers used to produce toluidine blue and methylene blue (22), and is therefore, more effective to eliminate root canal pathogens. Since evidence suggested that the ICG had limitations to eliminate microorganisms residing in the root canal, the system application is limited (7); therefore, it was decided to investigate this issue after addressing and modifying its limitations. Many studies suggested the use of nanomaterials to enhance the efficacy of PDT and overcome the limitations of photosensitizers used (21), because it is reported that nanoparticles, alone or in combination with PDT, help to disinfect the root canals. Functionalization of nanomaterials with PSs can give them unique physical and chemical properties such as the affinity and selection of certain cells, increased cell surface absorption of PS, and interaction between the cell and material due to surface charge, more stability of PS molecules, and prevention of physical quenching resulting in PS aggregation and controlled ROS release (33). Therefore, many studies made attempts to incorporate ICG into different nanocarriers including PLGA and liposome (19). Herein, a facile method was employed to synthetize nGO and conjugate ICG onto its surfaces with high efficiency in loading ICG to overcome facing challenges. Since loading efficacy of ICG in modified nano-graphene oxide is markedly higher in comparison with those of PLGA and other ICG encapsulating nanoparticles such as liposome, which is reported to have a low stability and loading capacity below 10% (19, 26). Another advantage of nGO is not using toxic metals in its synthesis, which makes it mass production easy and cost-effective in addition to its biocompatibility (20). It is shown that incorporating ICG into nGO increases its photo and physicochemical stability through rich π-π stacking interactions and improves its biocompatibility (19). The nGO thus synthesized by the modified Hummer method in the current study showed the simple and convenient method of synthesis (34). The SEM image (Figure 1) showed that the nanosheets of GO were smooth with small wrinkles at the edges, also the exfoliation of GO nanosheets confirmed that the nanosheets were fully exfoliated (34). These observations were concomitant with those of the studies by Nanda et al. and Chen

et al. (19, 35). Thus, the synthesized GO showed many interesting and unique properties that can be applied in a variety of applications. Therefore, it was expected that a PDT based on ICG incorporated nGO had a high performance effect against *E. faecalis*. In the current study, the results of the conventional PDT (ICG at 1000 μg/mL + irradiation) showed its weaker efficacy compared to that of modified PDT (nGO/ICG incorporated concentration of 400 μg/mL of ICG + irradiation), and nGO/ICG resulted in better canal disinfection and more reduction in the bacterial population at one-fifth of the common and clinically accepted concentration, which confirmed the study hypothesis. To the best of authors' knowledge, it was the first report providing evidence that nGO/ICG-PDT was against the cell viability of *E. faecalis*. Therefore, it was not possible to compare the obtained data with those of other studies. The current study findings showed that nGO/ICG could effectively reduce the number of *E. faecalis* CFUs, and may be a potential adjunct to standard endodontic treatment and root canal disinfection.

5. Conclusions

The PDT based on ICG, at the concentration loaded in nGO, showed a significant reduction in the number of *E. faecalis* at a lower concentration of ICG. In conclusion, the application of nGO as a new drug delivery system, in addition to the anti-bacterial property, offers other benefits such as cost beneficial outcomes due to using the lower dye concentration (less toxicity), and less tooth discoloration. The nGO/ICG-PDT could be proposed as a new approach to treat endodontic infections, alone or in combination with conventional root canal treatments.

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