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Near-infrared Irradiation and Graphene Oxide Film Fabricated on Dentin Surface Exhibit Photothermal and Antibacterial Effects

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Abstract

Background and objectives: Graphene oxide (GO) is a monolayer sheet of carbon with a thickness of 1 nm or less. Recent studies have revealed that GO exerts antibacterial properties, absorbs near-infrared (NIR) irradiation and generates heat. In this study, we fabricated a GO film on a human dentin block and investigated the photothermal and antibacterial effects of GO and NIR irradiation against *Streptococcus mutans*.

Methods: The dentin block was immersed in GO dispersion (concentration: 0, 1 and 10 µg/mL). GO-coated dentin blocks were observed using scanning electron microscopy (SEM) and characterized using the dentinal tubule sealing score. The temperature increase of the GO-coated dentin surface following NIR irradiation was examined by thermography. Furthermore, antibacterial effects of the combination of GO film and NIR irradiation against *S. mutans* were evaluated by SEM observation, turbidity measurement, colony formation assessment and live/dead staining.

Results: A thin GO film with a thickness of a few nanometers was successfully formed on the dentin surface. The dentinal tubule sealing score increased in a GO concentration-dependent manner. Even after ultrasonic cleaning, GO residue was frequently observed on the dentin surface. When the GO-coated dentin block was irradiated with NIR light, the temperature of the dentin block surface increased in a GO concentration- and time-dependent manner. In antibacterial assessments, turbidity and colony formation were suppressed by GO and NIR irradiation. In addition, dead bacteria were detected by live/dead staining.

Conclusion: A stable GO film was successfully formed on the dentin surface by immersion in GO dispersion. Photothermal and antibacterial effects were remarkably exhibited by GO and NIR irradiation.

Keywords: Antibacterial property; Dentin; Graphene oxide; Nanocarbon; Near-infrared light; Photothermal therapy; *Streptococcus mutans*.

Introduction

Graphene oxide (GO) exhibits a monolayer sheet of carbon with a thickness of 1 nm or less, which partly contains sp³ hybridized carbon atoms and oxygen functional groups, such as epoxy, carboxy and hydroxy groups. The presence of oxygen functional groups causes high hydrophilicity and dispersibility of GO. Recently, investigators have reported the antimicrobial properties of GO. Krishnamoorthy et al. showed that GO exerted antibacterial activity against *Pseudomonas aeruginosa* and *Streptococcus iniae* [1]. Gurunathan et al. demonstrated that GO and reduced GO dose-dependently inhibited the growth of *P. aeruginosa* and suggested that oxidative stress of reactive oxygen species (ROS) attacked bacterial cells [2]. Hu et al. reported that oxidized graphene sheets showed antibacterial activities, however, the cytotoxicity of GO sheets was slight [3]. Hence, GO may be relevant for the treatment of infectious diseases in various therapeutic fields.

Photothermal therapy (PTT) comprises light irradiation of a photosensitizer, which subsequently damages bacterial cells or cancer cells by heat generation via photoexcitation [4,5]. PTT has been focused on in the field of drug delivery to locally provide biological substances to targets [6,7]. Various photosensitizers, such as gold nanoparticles [8,9] and nanocarbons [10,11], have been widely investigated. In particular, GO may be a strong candidate for PTT. Robinson et al. reported that GO could generate fever after irradiation of near-infrared (NIR) light and successfully kill cancer cells [12]. In addition, chemo-photothermal therapy using GO loading of anticancer drugs was investigated [13]. Since long wavelengths showed low phototoxicity compared with short wavelengths such as UV light [14], we anticipate the application of NIR

light is suitable for PTT using GO. We speculate that GO should adhere to the tooth surface and exhibit photothermal effects via NIR light irradiation. PTT using GO would likely exert strong bactericidal action against cariogenic bacterial cells. However, these hypotheses have not been investigated thus far. Accordingly, in this study, we evaluated whether GO films stably coated the surface of root dentin. In addition, we assessed the antibacterial effects of GO film irradiated by NIR light against an oral bacterium, *Streptococcus mutans*.

Materials and Methods

Preparation of dentin blocks

Dentin blocks were prepared from extracted vital third molars of patients (20–40 years of age) at Hokkaido University Hospital. The

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use of human teeth in this study was approved by the Institutional Review Board of Hokkaido University Hospital for Clinical Research (approval no. 12-46). The tooth root was cut with a sterilized diamond disc (87xFSI, Horico, Berlin, Germany) and sandpaper (#240 and #600) to prepare dentin blocks of 5 mm×5 mm size with 1-mm thickness. After ultrasonic cleaning twice with distilled water (DW) for 5 minutes, dentin blocks were sonicated with 3% ethylenediaminetetraacetic acid (EDTA) (Smearclean, Nippon Shika Yakuhin Co., Ltd., Shimonoseki, Japan) for 60 seconds. After ultrasonic cleaning with DW, dentin blocks were obtained for evaluation.

Fabrication and evaluation of GO film on dentin blocks

GO dispersion (nanoGRAX®, Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan) was produced by oxidation and chemical separation of graphite (Hummers-Offeman method [15]) (Figure 1A). Each dentin block was immersed in diluted GO dispersion (concentration: 0, 1 and 10 µg/mL) for 30 seconds and then immersed in DW for 5 minutes. To investigate GO film fabrication, some GO-coated dentin blocks were washed with ultrasonic waves (28 kHz, NS-100, Nippon Rikagaku Kikai Co., Ltd, Tokyo, Japan) for 20 seconds. Subsequently, dentin blocks were dehydrated in a graded ethanol series and then dried overnight. Dentin blocks were then coated with Pt-Pd and observed by scanning electron microscopy (SEM; S-4000, Hitachi Ltd., Tokyo, Japan) at 10 kV. Three area units (90 µm×120 µm) of SEM images were selected and then the ability of the GO film to cover dentinal tubules was scored from 1 to 4 as described previously with some modification [16-18] (Table 1).

Assessment of surface temperature of dentin blocks after NIR irradiation

NIR irradiation of GO-coated dentin blocks was carried out using an NIR irradiation device (wavelength 800–1000 nm; LA-100 IR, Hayashi Watch-Works Co., Ltd., Tokyo, Japan). The irradiation distance was set to 1, 2 and 3 cm, and the irradiation time was set to 0, 10, 20 and 30 seconds. After NIR irradiation and a 5-second pause, the surface temperature was measured with thermography (FLIRix, FLIR Systems, Inc., Wilsonville, OR, USA). Furthermore, the temperature of the GO-coated dentin surface was measured after repeated NIR irradiation. The irradiation distance and time were set to 1 cm and 10 seconds, respectively. After NIR irradiation and a 5-second pause, the surface temperature was determined with thermography. After 30 seconds for cooling of the dentin block, NIR irradiation and subsequent temperature measurement were performed in the same manner. The same procedure was repeated again. The surface morphologies of dentin

blocks receiving single and 3-times irradiation were then observed by SEM.

Antibacterial effects of GO film and NIR irradiation

A suspension of facultative anaerobic bacteria, *S. mutans* ATCC 35668, was cultured and kept frozen until analysis. Bacterial stocks were incubated in brain heart infusion broth (BHI; Pearlcore®, Eiken Chemical, Co., Ltd., Tokyo, Japan) supplemented with 0.1% antibiotic (gramicidin D and bacitracin, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1% sucrose (Wako Pure Chemical Industries, Ltd.). GO-coated dentin blocks were placed into micro plates and then the suspension of *S. mutans* (final concentration: 1.5×10^6 colony-forming units (CFU)/mL) was dispensed. After anaerobic incubation at 37°C for 5 hours, dentin blocks were irradiated by NIR light for 30 seconds. Control specimens received no irradiation. Dentin blocks were then cultured again for 5 hours and the surface of the dentin blocks was observed by SEM. Some specimens were cultured for 24 hours after NIR irradiation to measure turbidity using a turbidimeter (CO7500 Colourwave, Funakoshi Co., Ltd, Tokyo, Japan) at 590 nm. In addition, *S. mutans* suspensions cultured for 12 hours after NIR irradiation were diluted 10-fold in fresh BHI broth, spread onto BHI agar plates (Eiken Chemical Co., Ltd.) and incubated at 37 °C for 48 hours to determine *S. mutans* colony counts.

Dentin blocks not incubated or incubated for 5 hours after NIR irradiation (10 or 30 seconds) were stained by the Live/Dead® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Dentin blocks were observed using fluorescence microscopy (Biorevo BZ-9000, Keyence Co., Osaka, Japan). In the incubated group, dead bacteria (red) and live bacteria (green) were counted in three area units (40 µm × 50 µm per unit) and the percentage of dead bacteria was calculated.

Statistical analysis

Statistical analysis was performed by Scheffe's test. P-values <0.05 were considered statistically significant. All statistical procedures were performed using a software package (Statistical Package for the Social Sciences 11.0, IBM Corporation, Armonk, NY, USA).

Results

Evaluation of GO film fabrication on dentin blocks

The color of the dentin blocks did not change after GO application (Figures 1B and 1C). In SEM observation, dentin block surfaces showed open dentinal tubules after EDTA treatment (Figure 2A). After immersion in GO solution, a thin wrinkled film of nanoscale thickness was observed on the dentin surface that sealed dentinal tubules (Figures 2B–2D). The dentinal tubule sealing score [mean ± standard deviation (SD)] was 1.02 ± 0.03 , 3.02 ± 0.03 and 3.78 ± 0.18 after GO coating using 0 (control), 1 and 10 µg/mL GO solution, respectively (Table 2). GO coating increased the sealing score and the sealing ability of 10 µg/mL GO was significantly greater than that of 1 µg/mL GO ($p < 0.05$). After ultrasonic cleaning, a part of the GO film disappeared and open dentinal tubules were frequently observed (Figures 2F and 2G). However, residual GO film was observed on the intertubular dentin. After ultrasonic washing, the dentinal tubule sealing score (mean ± SD) was 1.76 ± 0.17 and 2.10 ± 0.36 for 1 and 10 µg/mL GO, respectively (Table 2). High concentrations of GO possessed greater coatability compared with low concentrations of GO (Figures 1A–2D).

Assessment of the temperature of the dentin surface after NIR irradiation

The temperature of the GO-coated dentin surface increased

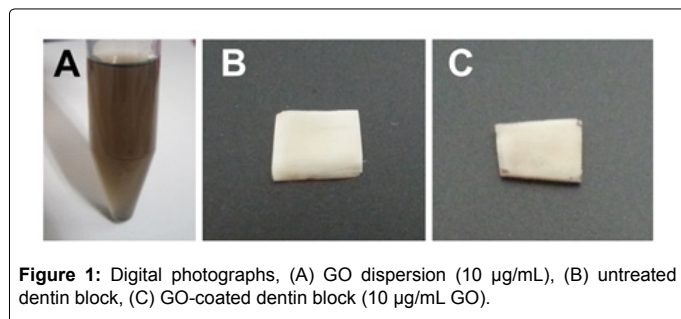


Figure 1: Digital photographs, (A) GO dispersion (10 µg/mL), (B) untreated dentin block, (C) GO-coated dentin block (10 µg/mL GO).

Score	Definition
1	Dentinal tubules were clearly opened.
2	Less than 50% of dentinal tubules were closed.
3	More than 50% of dentinal tubules were closed.
4	Dentinal tubules were completely closed.

Table 1: Scoring system to evaluate the creation of GO film.

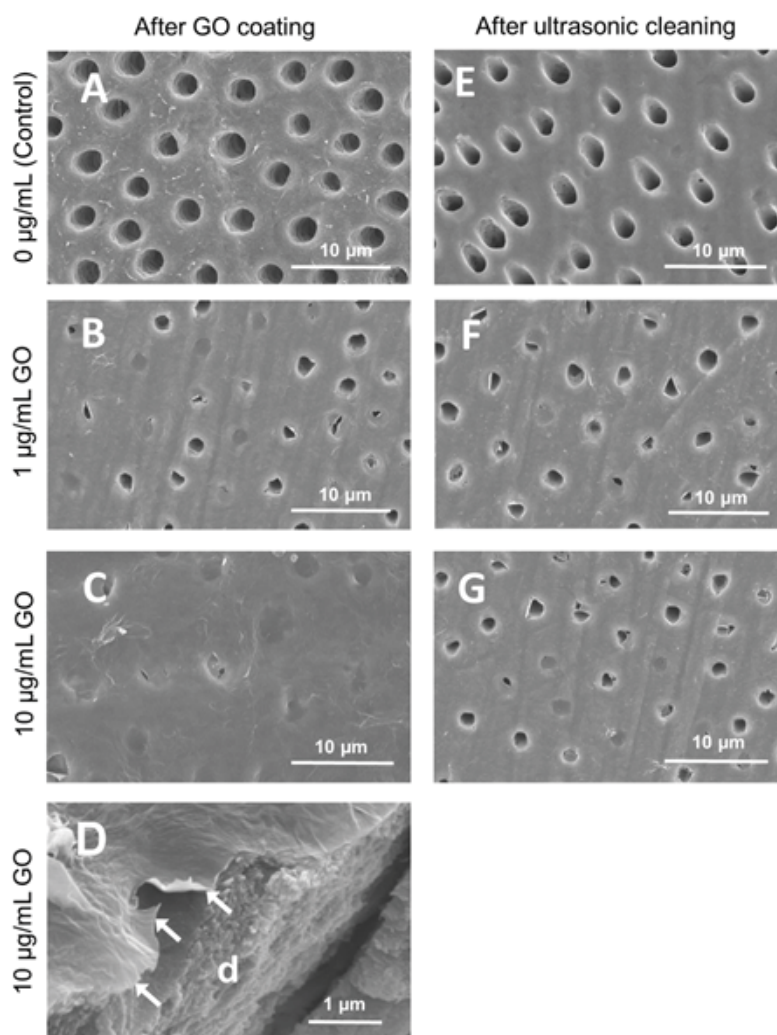


Figure 2: SEM images of the dentin block surface, (A–C) images of the dentin block surface after immersion in 0, 1 and 10 µg/mL GO dispersion, respectively, (D) cross-sectional image of a dentin block (d) coated in 10 µg/mL GO. The GO thin film (arrows) covers the dentinal tubules, (E–G) images of GO-coated dentin blocks after ultrasonic cleaning.

Parameters		0 µg/mL GO	1 µg/mL GO	1 µg/mL GO	10 µg/mL GO	10 µg/mL GO
		(control)		(after UC)		(after UC)
Score (mean ± SD)		1.02 ± 0.03	3.02 ± 0.03	1.76 ± 0.17	3.78 ± 0.18	2.10 ± 0.36
Mean difference		0.03	0.04	0.21	0.23	0.45
P-value	vs. 0 µg/mL GO (control)	NA	<i>p</i> <0.001*	<i>p</i> =0.002*	<i>p</i> <0.001*	<i>p</i> <0.001*
	vs. 1 µg/mL GO	<i>p</i> <0.001*	NA	<i>p</i> <0.001*	<i>p</i> =0.002*	<i>p</i> <0.001*
	vs. 1 µg/mL GO (after UC)	<i>p</i> =0.002*	<i>p</i> <0.001*	NA	<i>p</i> <0.001*	<i>p</i> =0.231
	vs. 10 µg/mL GO	<i>p</i> <0.001*	<i>p</i> =0.002*	<i>p</i> <0.001*	NA	<i>p</i> <0.001*
	vs. 10 µg/mL GO (after UC)	<i>p</i> <0.001*	<i>p</i> <0.001*	<i>p</i> =0.231	<i>p</i> <0.001*	NA

Note: GO- Graphene Oxide; UC- ultrasonic cleaning; SD- standard deviation; NA- not applicable; **p*<0.05

Table 2: Mean dentinal tubule sealing scores (*n*=4).

after NIR irradiation. The dentin surface temperature increased in a GO concentration- and time-dependent manner. In addition, NIR irradiation decreased the surface temperature in a distance-dependent manner. NIR irradiation at a distance of 1 cm significantly increased the surface temperature of GO-coated dentin blocks compared with untreated dentin blocks (*p*<0.05). In contrast, irradiation at distances of 2 and 3 cm tended to decrease the degree of temperature rise. For NIR

irradiation of 30 seconds at a distance of 1 cm, the surface temperature was 39.9, 47.1 and 58.5°C on 0 (control), 1 and 10 µg/mL GO-coated dentin blocks, respectively (Figure 3A).

We next confirmed the stability of the GO film after repeated NIR irradiation of GO-coated dentin blocks (*n*=4). The dentin surface temperature (mean ± SD) was 49.7 ± 0.50, 50.3 ± 0.49 and 50.9 ± 0.70°C

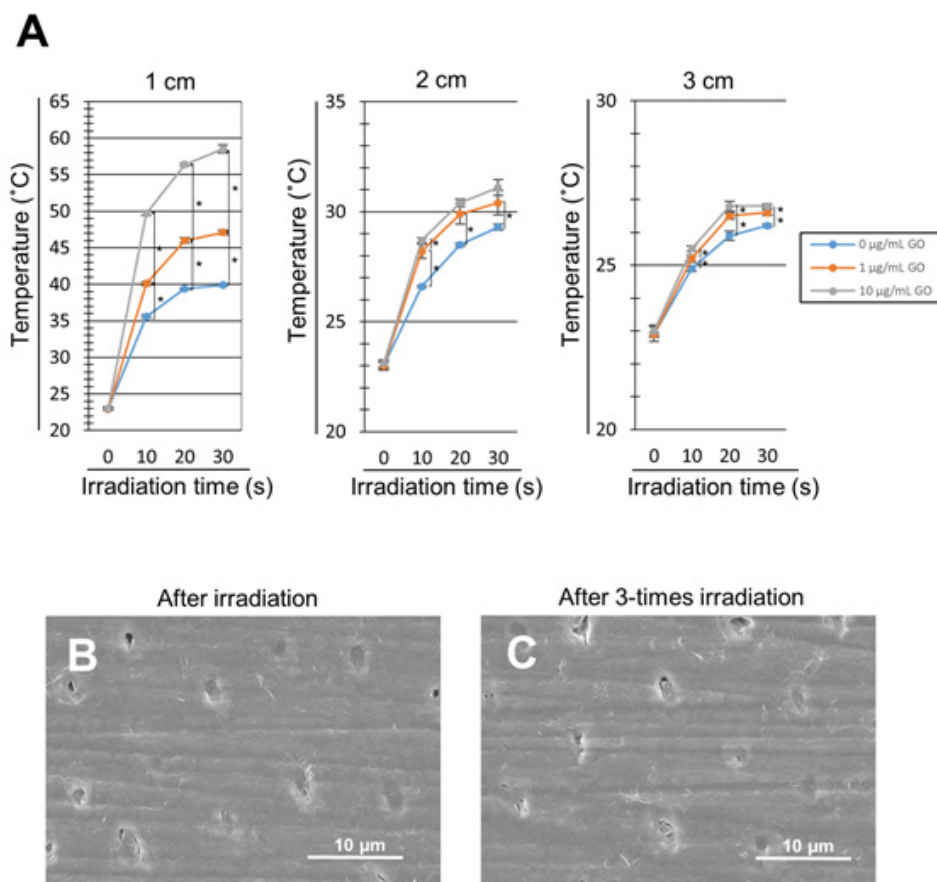


Figure 3: Assessment of the temperature of the dentin surface after NIR irradiation. (A) Measurement of the temperature of the GO-coated dentin surface ($n=4$, mean \pm SD). * $p<0.05$. (B,C) SEM image of a GO-coated dentin block after one and three rounds of NIR irradiation.

after single, 2-times and 3-times irradiation, respectively. No significant difference was found between the three groups. SEM images showed GO films exhibited no morphological change. Dentinal tubules were completely sealed by GO film even after repetitive NIR irradiation (Figures 3A-3C).

Antibacterial evaluation of GO film and NIR irradiation

In SEM observation, bacterial aggregation on dentin block surfaces was remarkable in control specimens receiving no GO or NIR irradiation (Figure 4A). In contrast, slight *S. mutans* growth appeared on GO-coated dentin blocks (Figures 4B and 4C). Furthermore, GO and NIR light application strongly suppressed bacterial growth and colonization (Figure 4C).

Assessment of *S. mutans* turbidity and colony count was carried out to determine the antibacterial capability of GO film and NIR irradiation. Under no irradiation, the optical density (mean \pm SD) was 0.22 ± 0.01 , 0.22 ± 0.01 and 0.19 ± 0.01 in 0, 1 and 10 $\mu\text{g/mL}$ GO-coated dentin, respectively. The optical density of 10 $\mu\text{g/mL}$ GO-coated dentin samples was significantly decreased compared with untreated dentin samples ($p<0.05$) (Figure 4D). When NIR irradiation was applied, the optical density (mean \pm SD) was 0.23 ± 0.01 , 0.11 ± 0.02 and 0.02 ± 0.02 in 0, 1 and 10 $\mu\text{g/mL}$ GO-coated dentin, respectively. The optical density of GO-coated samples was significantly lower than that of untreated samples ($p<0.05$). The number of *S. mutans* colonies of 10

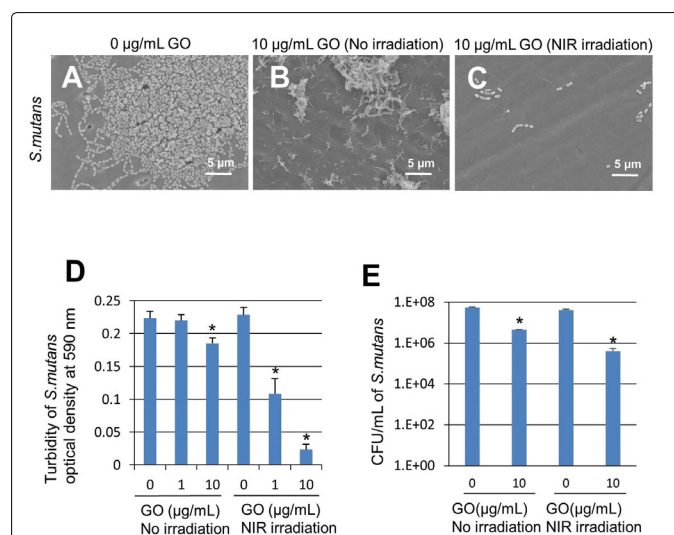


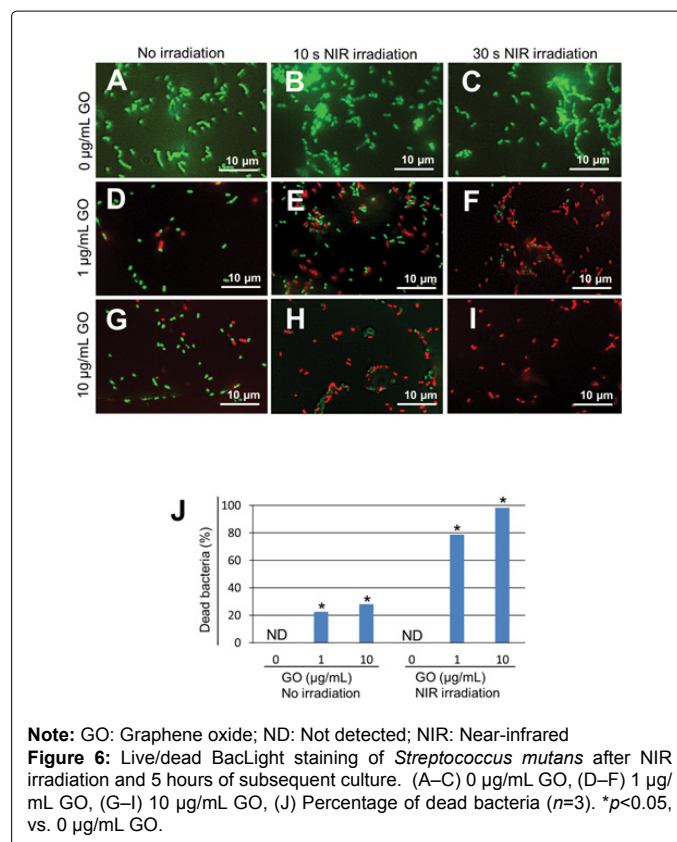
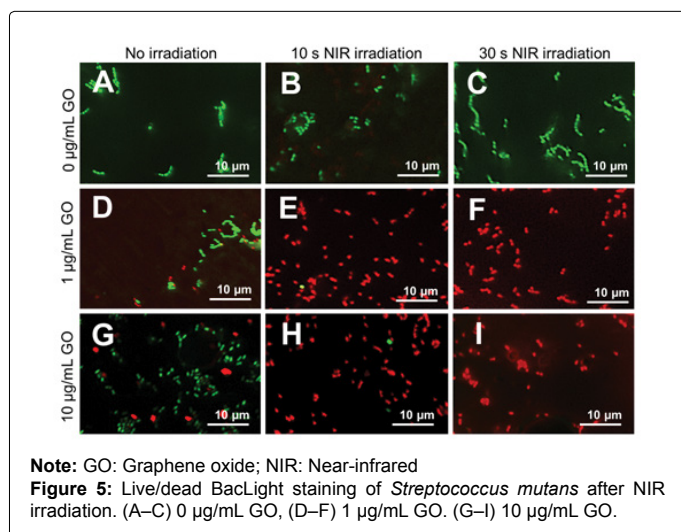
Figure 4: Antibacterial evaluation of GO film and NIR irradiation. (A–C) SEM images of the GO-coated dentin block surface after *Streptococcus mutans* seeding and NIR irradiation, (A) 0 $\mu\text{g/mL}$ GO, (B) 10 $\mu\text{g/mL}$ GO and no irradiation, (C) 10 $\mu\text{g/mL}$ GO and NIR irradiation, (D) Turbidity of *S. mutans* ($n=6$, mean \pm SD). * $p<0.05$, vs. 0 $\mu\text{g/mL}$ GO, (E) CFU/mL of *S. mutans* ($n=2$, mean \pm SD). * $p<0.05$, vs. 0 $\mu\text{g/mL}$ GO (No irradiation).

µg/mL GO-coated dentin samples was reduced compared with that of untreated dentin samples. In addition, the combination of GO and NIR irradiation strongly inhibited the formation of bacterial colonies (Figures 4E).

To assess the bactericidal effects of GO film and NIR irradiation, live/dead staining of *S. mutans* was carried out. GO coating and NIR irradiation increased the number of dead bacteria after 0 and 5 hours of incubation (Figures 5A–I and 6A–I). In the assessment of the percentage of dead bacteria after incubation for 5 hours, no dead bacteria were found in untreated dentin, regardless of application of NIR irradiation. When NIR irradiation was not applied, the mean percentage of dead bacteria was 22.6% and 28.4% in 1 and 10 µg/mL GO-coated dentin, respectively. In contrast, with NIR irradiation for 30 seconds, the mean percentage of dead bacteria was 78.9% and 98.3% in 1 µg/mL and 10 µg/mL GO-coated dentin, respectively (Figures 5A–6J).

Discussion

SEM observation of GO-coated dentin revealed that the wrinkled film structure sealed dentinal tubules on dentin block surfaces. The cross-sectional SEM image of GO-coated dentin revealed a very thin film on the dentin. In contrast, control specimens showed that dentinal tubules were fully opened. Thus, dispersed GO easily aggregated to form thin GO films on the dentin, consequently sealing dentinal tubules. Regarding the concentration of the GO dispersion, a high concentration of GO (10 µg/mL) remarkably covered the dentin surface compared with a low concentration of GO (1 µg/mL), suggesting that GO coating covered dentinal tubules in a concentration-dependent manner. Ultrasonic cleaning of GO-coated dentin resulted in partial removal of the GO film to open dentinal tubules. However, as compared with the control, GO-coated dentin showed a high sealing ratio of dentinal tubules and GO film strongly remained on the intertubular dentin after ultrasonic cleaning. Therefore, GO film can stably cover the surface of root dentin. Some investigators showed that GO possesses protein absorption capability [19,20]. GO has a large amount of hydrophilic oxygen functional groups on its surface because of oxidation processes [21]. GO-dentin binding force may be enhanced by covalent bonding between epoxy groups of GO and dentin protein exposed by EDTA treatment [22]. Due to these attractive forces, GO film would readily form on the dentin surface by only the immersion process. The formation of a stable GO film on the root surface would be beneficial for dental GO therapy against root caries and periodontal diseases.



The temperature of GO-coated dentin surfaces time-dependently increased via NIR light irradiation, however, dentin without GO coating exhibited no temperature rise after NIR irradiation. It was suggested that both application of GO film and NIR irradiation increased the surface temperature of dentin. Robinson et al. detected the absorption spectrum of GO and revealed the NIR absorption ability of GO [12]. Li et al. showed that the temperatures of a solution containing GO was increased by NIR irradiation [23]. Taken together, GO film formed on dentin possesses NIR absorption ability and releases thermal energy to generate heat after NIR irradiation. In the present study, the morphology of GO film remained unchanged after repetitive (3 rounds) NIR irradiation (Figures 3B and 3C). It is considered that the film stably covered the dentin even with heat generation.

Live/dead staining showed that dead bacteria were significantly observed on GO-coated dentin that received subsequent NIR irradiation. In addition, *S. mutans* turbidity and colony formation were significantly reduced by GO and NIR irradiation. It was suggested that the GO-NIR system provided thermal damage to *S. mutans* and consequently facilitated bacterial sterilization. Ma et al. demonstrated that the growth of mesophilic bacteria, such as *S. mutans*, occurs at 30–47°C [24]. In the present study, the surface temperature of GO-coated dentin via NIR irradiation was higher than 50°C. Therefore, *S. mutans* would likely receive profound damage, inhibiting bacterial proliferation. The GO-NIR system conclusively exhibited great antibacterial effects against *S. mutans*.

Although NIR irradiation was not performed, *S. mutans* was sparsely observed on dentin blocks immersed in 10 µg/mL GO dispersion compared with biofilms grown on control specimens in SEM images. In addition, GO application with no irradiation reduced *S. mutans*

turbidity and colony formation compared with the control. These findings suggested that GO-coated dentin independently exhibited antibacterial effects. The antimicrobial properties of GO against various bacteria, such as *P. aeruginosa*, *Escherichia coli* and *S. iniae*, have been investigated [1,2]. Some investigators concluded that the antimicrobial properties of GO were exerted by ROS production, which caused bacterial cell damage [2]. It was considered that GO should gradually and continuously produce ROS because of GO reduction and release the oxygen functional groups by temporal degradation. Accordingly, GO films on the tooth surface would exert a long-term antibacterial effect.

Conclusion

We investigated the possibility of GO film coverage on the surface of root dentin and the effectiveness of photothermal effects by NIR irradiation on oral bacterium *S. mutans*. The stable, thin GO film formed on the dentin surface to cover dentinal tubules in a concentration-dependent manner. Furthermore, the combination of GO film and NIR irradiation remarkably exhibited photothermal and bactericidal effects. The GO-NIR system is anticipated to be an effective photothermal antibacterial therapy against oral infections.

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