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Original Article

In vitro effect of diode laser against biofilm of *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Objective: The main objective is to investigate the antibacterial effect of diode laser against *Aggregatibacter actinomycetemcomitans* biofilm. **Materials and Methods:** Biofilms of *A. actinomycetemcomitans* plus *Streptococcus sanguinis* grown on bovine root surfaces were treated with an 810-nm diode laser using a noncontact pulsed mode with a pulse interval and pulse length of 20 ms. Four protocols, that is, one episode of 1.5 or 2.5 W for 30 s and three episodes of 1.5 or 2.5 W for 30 s were tested. No treatment and 0.2% chlorhexidine treatment served as negative and positive controls, respectively. Viable bacterial number was determined by colony counting. **Results:** Treatment with chlorhexidine and all laser protocols except that using single episode of 1.5 W reduced the number of *A. actinomycetemcomitans* in either single-species or dual-species biofilm compared to negative control. A higher percentage of *A. actinomycetemcomitans* reduction was demonstrated after increasing the power output or repeating the irradiation. **Conclusions:** The laser protocols used in this study could reduce the number of viable bacteria but not eradicate *A. actinomycetemcomitans* biofilm.

Key words: *Aggregatibacter actinomycetemcomitans*, biofilm, diode laser, periodontitis treatment

INTRODUCTION

Mechanical periodontal treatment comprising of scaling and root planing aims to remove bacterial plaque, endotoxins, and calculus from the root surface. This measure, together with oral hygiene control, leads to an improved periodontal condition.[1] However, scaling and root planing are not effective enough in removing such deposits from inaccessible areas[2] as well as some bacteria including *Aggregatibacter actinomycetemcomitans*[3] which can invade into the periodontal tissue.[4]

Diode laser is widely used in the field of dentistry. The ability of diode laser to eliminate pathogens and remove diseased pocket epithelium makes it a potential adjunct to conventional mechanical treatment.[5,6] At present, the antimicrobial property of adjunctive diode laser has been studied with an inconclusive result.[5,7-10]

In addition to its efficacy, the safety of diode laser is of great importance. To minimize the tissue damage, high laser energy should be avoided. The irradiation should also be split into multiple short episodes instead of using a single episode of long duration.[11] Thus, this study aimed to test the antibacterial property of an 810-nm diode laser using different irradiation protocols against biofilm of *A. actinomycetemcomitans*.

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MATERIALS AND METHODS

The biofilm was developed as previously described[12,13] with slight modifications.

Bacterial strains and culture conditions

To prepare bacterial inoculums, *A. actinomycetemcomitans* ATCC 29523 or *Streptococcus sanguinis* ATCC 10556 was grown in Brain Heart Infusion agar (BHI agar; Difco, USA) in a 5% CO_2 incubator at 37°C. Then, 3–5 colonies of each bacterial species were inoculated in 5 mL of BHI broth (Difco) in a 5% CO_2 incubator at 37°C for 18–24 h. Bacterial suspension was adjusted to 1×10^8 colony-forming units/mL (CFU/mL) using spectrophotometer (Biochrom, UK) at an OD_{600}. The suspensions were used as inoculums in the biofilm development.

Tooth specimen preparation

Extracted single-rooted bovine teeth were purchased and kept at −80°C until used. The teeth without gross damage or caries lesions were selected. Residual tissue around the root surface was removed. Each tooth was cut and the crown and apical-third portion of the root were discarded. The cervical to middle-third portion of the root was polished with 800-grit sandpaper. The polished surface was left exposed, while the other surfaces of tooth specimen were embedded in silicone putty material (Coltoflax®, USA) to a size of 10 mm × 10 mm × 5 mm. After that, the specimens were coated with nail varnish, leaving a window of 4 mm × 4 mm in the polished surface for the bacterial biofilm to form. All specimens were sterilized by ethylene oxide.

Biofilm development

Each sterile tooth specimen was placed in an individual well of a 24-well polystyrene plate (Corning, USA). For the formation of single-species biofilm, 1.5 mL of *A. actinomycetemcomitans* suspension was added into each well. For the formation of dual-species biofilm, 750 µL of *S. sanguinis* and 750 µL of *A. actinomycetemcomitans* suspension were mixed and transferred into each well. The plate was then incubated at 37°C in a 5% CO_2 incubator shaker for 48 h. Before the treatment, all specimens were washed four times with sterile normal saline solution (NSS) to remove loosely attached bacteria.

Treatment protocols

The experiment comprised four laser irradiation groups, a chlorhexidine (CHX) group and a no treatment group. Each experimental group was carried out in triplicate and repeated three times on different occasions.

Laser irradiation

Laser treatment was carried out using a diode laser (LaserSmile™, BIOLASE, Germany) set to a noncontact pulsed mode at a wavelength of 810 nm with a pulse interval of 20 ms and a pulse length of 20 ms. The laser was delivered through an optical fiber with a diameter of 400 µm. The tip of the fiber was moved, at 0.5–1 mm away from the biofilm surface, with a sweep motion. There were four different laser protocols:

- Protocol A: A single episode of irradiation using a power output of 1.5 W for 30 s (140.625 J/cm²)
- Protocol B: Three episodes of irradiation using a power output of 1.5 W for 30 s (421.875 J/cm²). Each episode was separated by a 30 s pause
- Protocol C: A single episode of irradiation using a power output of 2.5 W for 30 s (234.375 J/cm²)
- Protocol D: Three episodes of irradiation using a power output of 2.5 W for 30 s (703.125 J/cm²). Each episode was separated by a 30 s pause.

After irradiation, each specimen was removed from the silicone block and transferred to a 15 mL microcentrifuge tube containing 2 mL of sterile NSS. The tubes were vigorously vortexed for 3 min. Tenfold serial dilution of bacterial suspension was performed, and it was then plated out on BHI agar. The number of bacterial colonies was counted and calculated as CFU/mL.

Chlorhexidine treatment

Each specimen was removed from the silicone block and transferred to a 1.5 mL microcentrifuge tube containing 1 mL of 0.2% chlorhexidine digluconate (CHX) solution, and then incubated for 30 s. After incubation, the specimens were washed four times with NSS to remove trace amounts of CHX. The specimens were then prepared for colony counting as previously described.

Scanning electron microscopic study

Each specimen was transferred to desiccators until dried. The specimen was coated with a thin layer of gold alloy (100–300 Å) using a sputter coater and viewed under scanning electron microscope (JSM-6610 LV, JEOL, Tokyo) with an accelerating voltage of 20 kV.

Statistical analysis

The CFU/mL data were log-transformed. To identify differences in the log-transformed data between

groups, Kruskal–Wallis nonparametric one-way analysis of variance was performed. A Mann–Whitney U-test was applied for comparison of differences between two groups. Statistical comparisons were performed using SPSS software version 19 (SPSS, IL, USA). For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Bacterial elimination following the treatments

As shown in Table 1, log CFU/mL of *A. actinomycetemcomitans* in the single-species and dual-species biofilms decreased after laser or CHX treatments. All treatment groups except laser Protocol A significantly reduced the number of bacteria compared to the no treatment group [Table 2]. A higher percentage of *A. actinomycetemcomitans* reduction was seen after an increment of the power output (Protocol C), after repeating the irradiation (Protocol B) or both (Protocol D) in either single-species or dual-species biofilm. The amount of *A. actinomycetemcomitans* after treatment with laser Protocol D was reduced by approximately, 2 log CFU/mL in the single-species biofilm and by 1 log CFU/mL in the dual-species biofilm. However, CHX treatment exhibited >3 log CFU/mL reduction.

Biofilm morphology from scanning electron microscopy

In a 48 h-old, single-species biofilm, numerous coccobacilli were found on the bovine root surface [Figure 1a]. In a 48 h-old, dual-species biofilm, the aggregation of cocci and coccobacilli were found. Some formed a cluster of bacteria and some formed layers on top of each other [Figure 1b].

After laser treatment, the mass of extracellular matrix covering bacteria was found on the root

Table 1: Mean±standard deviation of log colony-forming units/mL and percentage of bacterial reduction following treatments

Group	Mean±SD of log CFU/mL (percentage reduction[#])		
	A. actinomycetemcomitans in SSB (%)	*A. actinomycetemcomitans* in DSB (%)	Total bacteria (%)
No treatment	7.66±0.51	7.16±0.19	7.49±0.33
A	7.57±0.40 (41.739)	6.89±0.46 (23.387)	7.37±0.42 (9.820)
B	6.12±0.26 (98.212)	6.08±0.27 (91.210)	6.50±0.34 (89.524)
C	6.53±0.41 (94.411)	6.40±0.32 (80.645)	6.73±0.26 (84.288)
D	5.80±0.36 (99.000)	5.94±0.51 (90.645)	6.22±0.50 (93.425)
CHX	4.40±0.82 (99.902)	1.94±0.48 (99.999)	2.92±0.71 (99.994)

[#]Percentage reduction between no treatment and treatment groups. Groups - A: 1.5 W; 30 s × one episode, B: 1.5 W; 30 s × three episodes, C: 2.5 W; 30 s × one episode, D: 2.5 W; 30 s × three episodes. CHX: 0.2% chlorhexidine digluconate 30 s, *A. actinomycetemcomitans*: *Aggregatibacter actinomycetemcomitans*, DSB: Dual-species biofilm, SSB: Single-species biofilm, CFU: Colony-forming units, SD: Standard deviation

Table 2: *P* values for the differences in the log colony-forming units/mL of *Aggregatibacter actinomycetemcomitans* and total bacteria following treatments

	Group	A	B	C	D	CHX
A. actinomycetemcomitans in SSB	No treatment	0.82	<0.001*	<0.001*	<0.001*	<0.001*
	A		<0.001*	<0.001*	<0.001*	<0.001*
	B			0.031*	0.063	<0.001*
	C				0.002*	<0.001*
	D					<0.001*
A. actinomycetemcomitans in DSB	No treatment	0.486	0.029*	0.029*	0.029*	0.029*
	A		0.029*	0.114	0.057	0.029*
	B			0.2	0.686	0.029*
	C				0.2	0.029*
	D					0.029*
Total bacteria (DSB)	No treatment	0.433	<0.001*	<0.001*	<0.001*	<0.001*
	A		<0.001*	0.001*	<0.001*	<0.001*
	B			0.136	0.297	<0.001*
	C				0.024*	<0.001*
	D					<0.001*

*Statistically significant difference using Mann-Whitney U-test. Groups - A: 1.5 W; 30 s × one episode, B: 1.5 W; 30 s × three episodes, C: 2.5 W; 30 s × one episode, D: 2.5 W; 30 s × three episodes. CHX: 0.2% chlorhexidine digluconate 30 s, *A. actinomycetemcomitans*: *Aggregatibacter actinomycetemcomitans*, DSB: Dual-species biofilm, SSB: Single-species biofilm

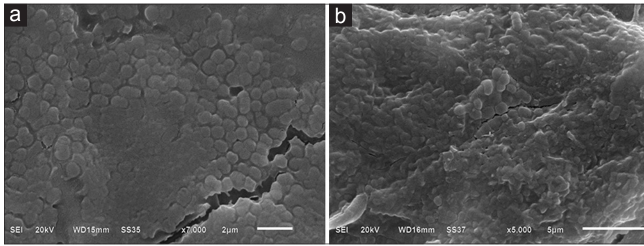

Figure 1: Scanning electron micrographs of (a) single-species biofilm (original magnification × 7000) and (b) dual-species biofilm (×5000) on bovine root surface

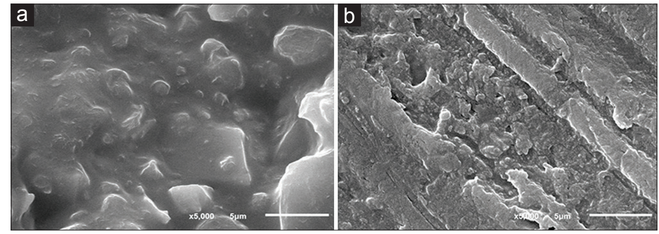

Figure 2: Scanning electron micrographs of (a) single-species biofilm and (b) dual-species biofilm (×5000) after laser treatment using Protocol D

surface, while the morphology of a few bacteria with irregular shape was observed in the dual-species biofilm. Few microbes remained on the root surface [Figure 2a and b].

DISCUSSION

Mechanical debridement is a standard method for periodontal therapy because it is a cause-related approach. This mode of treatment results in a significant reduction of several periodontal pathogens including *Porphyromonas gingivalis* and *Tannerella forsythia.* However, the level of *A. actinomycetemcomitans* does not significantly decrease after therapy.[3] *A. actinomycetemcomitans* is known to involve in the inflammation-driven alveolar bone loss through regulation of chemokine signaling in several cell types.[14] Its presence is considered a risk marker for the progression of attachment loss.[15] Since the elimination of bacteria is important to delay subgingival recolonization, many approaches including the use of antibiotics[16] or antimicrobial photodynamic therapy (PDT)[17,18] have been introduced. However, these bacteria can resist such therapeutic procedures as shown by the reduced antibiotic susceptibility.[16] Regarding PDT, not all studies could decrease bacteria by at least 3 log CFU/mL (99.9%).[17]

When reviewing the antimicrobial effect of diode laser, conflicting results were obtained, partly because of differences in the irradiation parameters used.[7-10] For example, Moritz *et al*.[8] found that three episodes of irradiation (at 1 week, 2 months, and 4 months after scaling and root planing) with an 805-nm diode laser, set at 2.5 W with a pulse duration of 10 ms and a pulse rate of 50 Hz, led to a significant bacterial reduction at 6 months over H_2O_2 rinsing of the periodontal pockets. In contrast, Song *et al*.[10] reported that two episodes of irradiation with an 810-nm diode laser using a power of 0.8 W in a continuous-wave mode for 30 s could kill only 17%–54% of the multispecies biofilm. Kreisler *et al*.[7] investigated the antimicrobial effect of an 809-nm diode laser against *S. sanguinis* on dental implant surfaces using a power output between 0.5 W and 2.5 W and found that bacteria could be reduced by approximately 50% at 0.5 W to 99% at 2.5 W.

In terms of thermal damage, the critical temperature that can induce heat injury varies between tissues. Kreisler *et al*.[19] evaluated the morphologic alterations of the root surface after a noncontact 809-nm GaAlAs laser irradiation. They found no alterations in lasing dry or saline-moistened root specimens. However, blood-coated specimens showed partial or total carbonization after irradiation with ≥1.5 W in a continuous mode for 10–30 s. Kreisler *et al*.[11] also investigated intrapulpal temperature change during root surface irradiation with an 809-nm GaAlAs laser. With a remaining dentin thickness of 1 mm, only irradiation at 0.5 W for a maximum of 10 s did not increase an intrapulpal temperature beyond a critical threshold. When irradiation was stopped for 30–40 s, the temperature returned to baseline. These authors suggested that irradiation should be interrupted to allow the hard tissue and pulp to cool down.

According to the manufacturer's recommendation, the diode laser parameters for treating periodontal pockets are 1.5 W in a pulsed mode with a pulse interval of 20 ms and a pulse length of 20 ms. The recommended duration is 30–240 s depending on the severity of inflammation. Thus, these parameters were chosen in this study. In addition, we questioned whether the sterilization effect would be increased if the irradiation was delivered three times with a 30 s pause between each episode. The reason for this was to increase the laser energy while allowing the elevated temperature to return to baseline.[11]

The bactericidal mechanism of a diode laser is primarily based on the photothermal effect. Pirnat *et al*.[18] indicated that bacterial cell death is induced by short-term localized heating of bacterial microenvironment to a lethal temperature. In this study, the reduction of *A. actinomycetemcomitans* after laser treatment of 1.5 W

for 30 s was not significantly different compared to negative control. However, laser irradiation of 1.5 W for 30 s, when performed three times, significantly decreased *A. actinomycetemcomitans* compared to the groups receiving a single episode of irradiation. The killing effect of diode laser could result from heat denaturation of macromolecules. Another possible killing mechanism might result from the endogenous photosensitizers of *A. actinomycetemcomitans* which can be light-activated and generate singlet oxygen.[20] In this study, no attempt was made to find the mechanism of bacterial killing.

When the power output was increased to 2.5 W, a lower number of bacteria was found. Furthermore, the log CFU/mL of *A. actinomycetemcomitans* in the single-species biofilm was significantly different after irradiation at 2.5 W for three times, compared to that irradiated with a single episode of 2.5 W (P = 0.002). However, a borderline nonsignificant difference (P = 0.057) in the log CFU/mL of *A. actinomycetemcomitans* in the dual-species biofilm was found between the group receiving 1.5 W for 30 s and that receiving three episodes of 2.5 W for 30 s, which might be explained by a small number of samples. Thus, diode laser could decrease the number of *A. actinomycetemcomitans* in the biofilm form. However, if a minimum of 99.9% bacterial reduction must be achieved to claim an antibacterial effect, no laser protocols used in this study showed antibacterial activity against biofilm of *A. actinomycetemcomitans*.

A dual-species biofilm of *A. actinomycetemcomitans* and *S. sanguinis* was used in this study to observe the effect of the diode laser against mixed-species bacterial biofilm. *S. sanguinis* is the most prominent bacterial species among the primary colonizers of dental plaque.[21] Therefore, *S. sanguinis* acts as a bridge between the tooth surface and *A. actinomycetemcomitans*. In this study, the percentage of bacterial reduction after laser irradiation was less in the dual-species biofilm than in the single-species biofilm, suggesting more resistance of dual-species biofilm to laser therapy. This can be explained by a more complex set of polysaccharides or a thicker biofilm produced by more species of bacteria compared to biofilm of a single species of bacteria.

PDT has been used for an antimicrobial purpose in dentistry.[22] However, the results vary depending on the type and concentration of photosensitizer as well as the incubation time for the photosensitizer to reach its target.[17] Mattiello *et al*.[23] studied an *in vitro* photodynamic effect using 0.01% toluidine blue-O for 5 min and an AlGaInP diode laser for 3 min on *A. actinomycetemcomitans* and *S. sanguinis* inoculums and found a 61.53% reduction of the CFU counts for *A. actinomycetemcomitans*. Compared to that study, our results showed a greater reduction of CFU count for *A. actinomycetemcomitans* after irradiation using one episode of 2.5 W for 30 s or three episodes of 1.5 W for 30 s.

In this study, the *in vitro* laser application could reduce clinical variations such as pocket depth or presence of bleeding. However, the use of a single-species or dual-species biofilm was considered a limitation because it could not mimic the polymicrobial biofilm found in the oral cavity.

CONCLUSIONS

The laser protocols used in this study could reduce the number of viable bacteria but could not eradicate the biofilm of *A. actinomycetemcomitans*.

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Conflicts of interest
There are no conflicts of interest.

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