Effects of Erbium:Yttrium–Aluminum–Garnet Laser Irradiation on Bovine Dentin Contaminated by Cariogenic Bacteria

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Abstract

Objective: This study was performed to determine the bactericidal effects of erbium:yttrium–aluminum–garnet (Er:YAG) laser irradiation and the morphological and chemical composition changes in bovine dentin.

Methods: Dentin slabs were prepared from bovine incisors, and then cultured with Streptococcus mutans to produce bacteria-infected dentin samples. The samples were randomly divided into five groups with Er:YAG laser irradiation energy densities of 0, 6.37, 12.73, 19.11, and 25.47 J/cm². After irradiation, samples were stained and observed by confocal laser scanning microscopy. The bactericidal abilities were measured using live/dead staining. The morphology and chemical components were investigated by scanning electron microscopy and energy-dispersive spectrometry.

Results: After irradiation, the elimination of bacteria and the smear layer were significantly better in the high energy density groups (19.11, 25.47 J/cm²) than in the low energy density groups (6.37, 12.73 J/cm²; p < 0.001). On morphological examination, the group with minimum energy density (6.37 J/cm²) showed superficial melting. In the high energy density groups (12.73, 19.11, and 25.47 J/cm²), laser-irradiated dentin showed a clean surface with open orifices. Significant increases were observed in the weight percentages of calcium (from 19.75 – 0.69 to 34.47 – 2.91, p < 0.001) and phosphate (from 8.58 – 0.43 to 15.10 ± 1.81, p < 0.001), whereas significant decreases were observed for oxygen (from 49.84 – 0.69 to 36.39 – 2.86, p < 0.001) and carbon (from 26.06 – 3.58 to 12.80 – 2.26, p < 0.01) with increasing energy density.

Conclusions: This study confirmed that Er:YAG laser irradiation has bactericidal and dentin conditioning effects.

Keywords: laser, caries, dentin, bactericidal effect

Introduction

Dental caries is a widespread chronic disease that results in progressive damage to the teeth caused by bacteria.1 According to the results of the Fourth National Epidemiological Survey of Oral Health in China, the prevalence rates of caries in children aged 5 and 12 years have increased by 5.8–70.9% and by 7.8–34.5%, respectively, compared with 10 years ago.2 Worldwide, dental caries constitutes a huge disease burden on human health.3

In clinical practice, the conventional method of caries removal involves use of a high-speed handpiece. However, this does not guarantee cleaning of the infected dentin and removal of residual bacteria.4 Novel minimally invasive dentistry techniques have been investigated.5 In 1997, the erbium:yttrium–aluminum–garnet (Er:YAG) laser was the first dental laser to be approved by the US Food and Drug Administration for treating dental hard tissue ablation.6 This method has become a feasible substitute for mechanical caries removal using a handpiece in pediatric dentistry,7 as it produces less noise and no vibration thus making it more acceptable for use in young children.8

The Er:YAG laser has an emission wavelength of 2940 nm, which corresponds to the absorption peak of water in process of the wavelength’ interaction with hard tissue.9 Dentin contains 70% inorganic hydroxyapatite crystals, 20% organic matter, and 10% water by weight.10,11 Caries of the dentin involves demineralization followed by bacterial invasion.12 Bacterial acidification not only induces demineralization and exposure of the organic matrix in the dentin

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surface, but also results in activation of dentin-embedded and salivary matrix metalloproteinases and cathepsins. Because carious tissue has a higher water content than healthy tissue, it shows higher Er:YAG laser energy absorption. The Er:YAG laser has potential for the selective removal of carious tissue, and can be used in minimally invasive treatment of dental caries. In addition, Er:YAG laser irradiation has bactericidal effects during cavity preparation, and is strongly absorbed by water inside bacteria, causing physical breakdown of the bacterial cell structure.

Most studies of the bactericidal effects of the Er:YAG laser have focused on the root canal, and tested lasers against Enterococcus faecalis and Escherichia coli. Previous studies have shown that the Er:YAG laser is the most appropriate laser for intracanal debris and smear removal. Nevertheless, there have been few studies on the bactericidal effects of Er:YAG laser irradiation on cariogenic Streptococcus mutans. Er:YAG laser irradiation can be used to modify the chemical compositions of enamel and dentin, which has been investigated as a means of preventing or reducing the tooth demineralization that occurs in dental caries. Er:YAG laser irradiation (1.2 J/cm², 10 Hz) significantly prevents demineralization by decreasing solubility of the enamel. The level of carbonate influences the susceptibility of the enamel to demineralization. However, most studies have focused on the enamel, and the clinical impact and mechanism of action on dentin remain largely unknown. Considering the differences in composition between enamel and dentin, it is necessary to determine the effects of Er:YAG laser irradiation on dentin.

This study examined whether Er:YAG laser irradiation can kill the bacteria within dentin, and lead to changes in the morphology and chemical composition of contaminated bovine dentin.

Materials and Methods

Sample preparation

Incisors from freshly slaughtered cattle without any previous lesions were obtained from a local abattoir. After washing with distilled water, the crown was cut and mid-coronal dentin disks ~1 mm thick (average area, 10 × 10 mm²) were obtained. Twenty-seven dentin specimens were prepared and stored in physiological saline at 4°C until use.

Microbial culture

Before the experiment, dentin disks were ultrasonicated for 30 min and autoclaved at 121°C for 20 min in distilled water. S. mutans (UA 159) was chosen as a standard cariogenic bacterial species. The bacteria were cultivated in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C for 24 h. Then the culture was adjusted to 10⁶ colony-forming unit/mL with an optical density of 0.3 based on preliminary experiments. Dentin slabs were incubated in 1 mL bacterial suspension for 2 days (37°C, 5% CO₂). After coculture, the dentin specimens were transferred to fresh medium and immersed in liquid BHI broth for 5 days. The medium was changed daily.

Laser application

After a 7-day experimental period, the biofilm formed over the slabs was removed with a sterile cotton rod to expose the carious dentin. A commercially available pulsed Er:YAG laser (LiteTouch, Syneron, Israel) was used. The irradiation pattern was designed to simulate clinical conditions with manual operation. The specimens were irradiated from the surface in close contact mode under constant scanning movement (1 mm/sec), with a water spray level of 8. A straight sapphire tip, 1 mm in diameter, was placed at an angle of 15° to the vertical axis of the slab. The laser tip was maintained at a distance of 1 mm from the surface and the irradiation area was 10 mm². Table 1 shows the laser parameters used in each group. Each group consisted of five samples. Group 1 had no laser energy output as a control, but was handled with water and air at the same levels. After irradiation, the samples were immediately placed in phosphate-buffered saline (PBS; Solarbio, Beijing, China) to keep the bacteria alive. To confirm that the slab was not contaminated by other bacteria, two slabs were only exposed to noninoculated BHI broth and saline separately. As expected, no bacterial growth was observed.

Bacteriological evaluation

After irradiation, the dentin slabs were stained with LIVE/DEAD® BacLight Bacterial Viability Kits (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. This kit includes component A (SYTO 9® dye), which stains live bacteria with a green pigment, and component B (propidium iodine dye), which stains dead bacteria with red pigment, thus enabling easy identification of viable bacteria. In this study, 0.5 μL component A and 0.5 μL component B were mixed in 1 mL PBS. The sample surface was dried with sterilized cotton, and then samples were placed in 12-well plates (Corning, Corning, NY), with each well containing 100 μL mixed dye. After incubation at room temperature in the dark for 15 min, the slabs were eluted for 30 min. Images obtained by confocal laser scanning microscopy (CLSM, LSM 5 EXCITER; Carl Zeiss, Oberkochen, Germany). Five random images were taken of each sample at the center of the irradiation area at a 20× magnification. In total, 25 images were taken for each group. Standard images were obtained using 30 sections with a step size of 1 μm, in a format of 1024 × 1024 pixels. The images were fragmented into many layers and converted into TIFF format. These images were exported to ZEN Blue software (ver.1.1.1; Carl Zeiss) to quantify the amounts of live (green) and dead (red) bacteria. Each layer was evaluated.

<table>
<thead>
<tr>
<th>Table 1. Er:YAG Laser Parameters Used in Each Group</th>
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<tr>
<td><strong>Output power</strong></td>
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<td>Group 1</td>
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<td>Group 2</td>
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<td>Group 3</td>
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<td>Group 4</td>
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<td>Group 5</td>
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</table>

Each group consisted of five samples. Group 1: with no laser energy output, but handled with water and air at the same level.
separately and the bacterial viability data were obtained to evaluate the bactericidal effects by measuring the red to green fluorescence area ratio in each slab.

Scanning electron microscopy and energy-dispersive spectrometry observations

After CLSM observation, slabs were preserved in distilled water and sent to the University Analytical Instrumentation Center, Peking University (Beijing, China), for morphological and structural analyses. The protocol for sample preparation was as previously described. The specimens were dehydrated in the molecule pump inside the scanning electron microscope (Bruker Nano, Bremen, Germany). However, the samples were not sputter coated with gold to preserve their innate characteristics. The morphology of the dentin surfaces was observed by scanning electron microscopy (SEM) at an acceleration voltage of 10 kV, under 500, 2000, and 5000 magnifications. Five random images were taken of each sample at the center of the irradiation area. Energy-dispersive spectrometry (EDS; EDS QUANTAX; Bruker, Billerica, MA) analyses were performed in the same area using an X-ray detector system attached to the scanning electron microscope. The carbon, oxygen, calcium, and phosphate contents, as well as the Ca (mol)/P (mol) ratio, and C (mol)/O (mol) ratio were calculated. The stoichiometric Ca/P ratios and C/O ratios were calculated using the following formula, considering the respective atomic masses: Ca (mol)/P (mol) = [Ca (wt%)/40.08 (g/mol)]/[P (wt%)/30.97 (g/mol)]; C (mol)/O (mol) = [C (wt%)/12.00 (g/mol)]/[O (wt%)/15.99 (g/mol)]. A total of 25 images and corresponding data were obtained for each group.

Statistical analyses

Based on CLSM images, the quantities of carbon, oxygen, calcium, and phosphate, as well as the Ca (mol)/P (mol) ratio and the C (mol)/O (mol) ratio according to EDS, were calculated and analyzed. The Kolmogorov–Smirnov test confirmed the normality of the data. One-way analysis of variance was performed to detect significant differences among the groups. Statistical analyses were performed using SPSS software (ver. 20.0; SPSS, Inc., Chicago, IL). In all of the analyses, \( p < 0.05 \) was taken to indicate statistical significance.

Results

Bacteriological evaluation

CLSM observation and live/dead staining allowed us to distinguish live and dead bacteria in dentinal tubules. Green (vital) and red (nonvital) fluorescence were visible within the tubules of infected dentin slabs (Fig. 1). The red to green fluorescence area ratio can be used to measure the bactericidal effect after irradiation. The means and standard deviations of the red to green fluorescence area ratios were determined for each slab and are shown in Table 2. The area ratios significantly increased with increasing energy density (from 0.33 ± 0.08 to 123.48 ± 11.71, \( p < 0.001 \)). In Group 1 without laser energy output, most bacteria were alive and showed green fluorescence (Fig. 1a). In Groups 2 and 3 (6.37 and 12.73 J/cm², respectively), the red to green fluorescence area ratios were increased (from 0.58 ± 0.17 to 1.12 ± 0.31) as red fluorescence area increased (Fig. 1b, c), indicating the increasing bactericidal capability. Groups 4 and 5 (19.11 and 25.47 J/cm², respectively) showed mostly red fluorescence with little green fluorescence (Fig. 1d, e); the red to green fluorescence area ratio was >10, and live bacteria were rarely observed with the extent of bacterial reduction reaching almost 100%.

Morphological analyses

Under SEM observation, normal bovine dentin slabs had a smooth surface with a little smear layer (Fig. 2a). In Group 1, however, the dentin surface was rougher with a heavy smear layer on the slab surface after contamination (Fig. 2b), while the orifice could still be distinguished. In Group 2, superficial melting occurred and partial closure of
dentin tubules was observed (Fig. 2c). Laser-irradiated dentin in Groups 3–5 showed a clean surface and the absence of a smear layer or debris with open orifices. The orifices were widened and peritubular dentin protruded (Fig. 2d–f). No cracks or destructive appearance was observed in any of the groups.

**Chemical composition analyses**

EDS is a semiquantitative method for analyzing elements. Thus, the results are the relative contents of elements. The weight percentages of carbon, oxygen, calcium, and phosphate are presented in Fig. 3. Compared with Group 1, significant increases were observed in the weight percentages of calcium (from 19.75 ± 0.69 to 34.47 ± 2.91, \( p < 0.001 \)) and phosphate (from 8.58 ± 0.43 to 15.10 ± 1.81, \( p < 0.001 \)), whereas significant decreases were observed in oxygen (from 49.84 ± 0.69 to 36.39 ± 2.86, \( p < 0.001 \)) and carbon (from 26.06 ± 3.58 to 12.80 ± 2.26, \( p < 0.01 \)) in Groups 2–5 with increasing energy density. In Groups 2–5, the C (mol)/O (mol) ratio significantly decreased (from 0.70 ± 0.10 to 0.47 ± 0.06, \( p < 0.05 \)) compared with Group 1, whereas there were no differences in the Ca (mol)/P (mol) ratio after irradiation (95% confidence interval: 1.72–1.87, \( p = 0.324 \)).

**Discussion**

The interaction of laser light with dental hard tissues is determined by the parameters of irradiation such as the wavelength, repetition rate, pulse energy, duration of exposure, and optical properties of the tissue.27 The appropriate Er:YAG laser irradiation parameters for achieving antimicrobial effects in carious dentin have not been

<table>
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<th>Group</th>
<th>Ratio (Mean ± Standard Deviation)</th>
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<tr>
<td>1</td>
<td>0.33 ± 0.08(^a)</td>
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<tr>
<td>2</td>
<td>0.58 ± 0.17(^a)</td>
</tr>
<tr>
<td>3</td>
<td>1.12 ± 0.31(^a)</td>
</tr>
<tr>
<td>4</td>
<td>16.78 ± 3.88(^b)</td>
</tr>
<tr>
<td>5</td>
<td>123.48 ± 11.71(^c)</td>
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\(^a\)\(^b\)\(^c\)Data with different lowercase superscript letters indicate a significant difference between randomly two groups (\( p < 0.001 \)).

![FIG. 2. SEM images of dentine surface after Er:YAG laser irradiation (magnification × 2000). (a) The normal bovine dentin slab without bacteria contamination and Er:YAG laser irradiation had a little smear layer on the slab surface. (b) Er:YAG laser irradiation at energy density 0 J/cm\(^2\): a heavy smear layer on the slab surface. (c) Er:YAG laser irradiation at energy density 6.37 J/cm\(^2\): surface melting occurred. (d–f) Er:YAG laser irradiation at energy density: 12.73, 19.11, 25.47 J/cm\(^2\): a clean surface and the absence of a smear layer or debris with open orifices. SEM, scanning electron microscopy.](image-url)
The effects of Er:YAG laser irradiation have been investigated to simulate clinical dentin caries. Bacteria had penetrated into the dentin tubules after coculture for 7 days. 33

The Er:YAG laser emission wavelength is 2940 nm. Therefore, the fundamental chromophores that absorb erbium wavelengths are water and hydroxyapatite, the former of which is present throughout bacterial cells. Therefore, the laser energy damages the cell wall, leading to cell swelling and death. 17, 34 Immediate cell death may not occur, but sublethal damage inhibits cell growth after exposure to laser irradiation.

The morphological changes after Er:YAG laser irradiation depend on the irradiation parameters. 34 Under different energy levels, the Er:YAG laser can be used for the treatment of deep dentin caries for primary teeth and young permanent teeth to preserve pulp vitality. To achieve this goal, the Er:YAG laser irradiation procedure consists of two steps. The first step involves decontamination of the surface with higher energy density. The second step involves melting dentin to decrease postoperative pain. In this study, surface melting occurred with partial bacterial reduction at the minimum energy level (6.37 J/cm²). This suggestion was also supported by the findings of Moosavi et al. 36 Surface melting with tubular blockage can decrease postoperative pain to some degree. 21

Previous studies have confirmed that laser irradiation reduces the degree of dentin dissolution. The studies tested the weight percentages of calcium and phosphate and the Ca/P and C/O ratios to determine the changes in dentine apatite crystals. 37 It has been suggested that changes in the chemical composition after Er:YAG laser irradiation have mechanistic effects. 38 In this study, we found significant increases in the weight percentages of calcium and phosphate, and significant decreases in those of oxygen and carbon. These observations were consistent with the study of Mei et al., 39 who reported that laser irradiation of dentin led to improvements in mechanical properties. The Ca/P ratio of amorphous calcium phosphate is variable and can contribute to the lack of significance of the measured Ca/P ratio. However, it is not clear whether laser irradiation increases the acid resistance of dentin. Manesh et al. reported that laser irradiation was not particularly effective for increasing or decreasing the rate of dentin demineralization, 40, 41 whereas Ceballos et al. 42 reported positive effects on both of these parameters. This discrepancy may have been due to the lower energy in the former study (8 J/cm²), and to differences in the test instruments used between the studies. Therefore, we speculated that the rate of dentin demineralization may be decreased after Er:YAG laser irradiation. For clinical application, this may be useful for management of deep caries. However, additional studies are required as the causative bacteria in carious lesions are not limited to one species, and the different species may have different susceptibilities to laser irradiation. 33, 34 In addition, future studies should be performed with larger sample sizes and with use of human teeth.

Conclusions

In conclusion, the results showed that Er:YAG laser irradiation has bactericidal effects and can cause both morphological and chemical changes in bovine dentin. A relatively weak bactericidal effect and melting of the dentin surface were observed with irradiation at lower energy densities. Superior antimicrobial capacity was observed with higher energy densities, and the dentin surface was clean with open orifices. Significant increases were observed in the weight percentages of both calcium and phosphate with increasing irradiation energy density, indicating that Er:YAG laser irradiation facilitated an asymptomatic postoperative course and prevented recurrence of caries.
Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

References