Research Article

The Effect of Irradiation with a 405nm Blue-Violet Laser on the Bacterial Adhesion on the Osteosynthetic Biomaterials

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Delayed postoperative infection is known as a major complication after bone surgeries using osteosynthetic biomaterial such as titanium (Ti) and biodegradable organic materials. However, the precise cause of this type of infection is still unclear and no effective prevention has been established. The purpose of this study is to investigate the effect of irradiation with a 405 nm blue-violet laser on the bacteria adhered on the Ti and hydroxyapatite-poly-L-lactic acid- (HA-PLLA) based material surfaces and to verify the possibility of its clinical application to prevent the delayed postoperative infection after bone surgeries using osteosynthetic biomaterial. The suspension of Staphylococcus aureus FDA 209P was delivered onto the surface of disks composed of Ti or HA-PLLA. Bacterial adhesion on each disk was observed using a scanning electron microscope (SEM). After thorough washing with distilled water, the growth of bacteria attached to the material surfaces was examined with an alamar blue-based redox indicator. Moreover, a bactericidal effect of 405 nm blue-violet laser irradiation on residual bacteria on both materials was investigated using colony-forming assay. As a result, there was no significant difference in the bacterial adhesion between Ti and HA-PLLA materials. In contrast, 45 J/cm² of irradiation with 405 nm blue-violet laser inhibited the bacterial growth at approximately 93% on Ti disks and at approximately 99% on HA-PLLA disks. This study clearly demonstrated the possibility that the irradiation with a 405 nm blue-violet laser is useful as an alternative management strategy for the prevention of delayed postoperative infection after bone surgeries using osteosynthetic biomaterials.

1. Introduction

Several types of biomaterials including titanium (Ti) and biodegradable organic materials such as poly-L-lactic acid (PLLA) are used for osteosynthesis in the fields of orthopedics and cranio-maxillofacial surgery [1, 2]. Ti is a traditional material and most commonly used for osteosynthesis because of its high biocompatibility and superior physical properties [3, 4]. In contrast, PLLA is one of representative biodegradable materials, which is hydrolyzed by moisture in the environment, resulting in a decrease in the molecular weight and then ultimate decomposition of the material into carbon dioxide and water. For this property, PLLA has been used as a source reagent of biodegradable osteosynthetic materials since the 1990s [5, 6]. In 2006, new biomaterial for osteosynthesis was developed, which was composed of incalescent/unsintered hydroxyapatite (u-HA) particles and PLLA, and has been commonly used due to (1) its better osteoconductivity [7–10], (2) no need for secondary surgery to remove the materials [11, 12], (3) no restriction to bone growth in young patients [13–15], and (4) no elution of metal ions that could act as allergens [16–19].

Delayed postoperative infection, one of major and serious complications after bone surgeries using osteosynthetic materials, often follows a protracted course as a persistent
complication [20–23], resulting in the eventual removal of materials followed by the failure of bone tissue regeneration/wound healing [22, 24–26]. Particularly for bioreabsorbable materials, a removal surgery due to postoperative infection completely negates the beneficial property of the materials. Therefore, it is very important to prevent the delayed postoperative infection after bone surgeries using osteosynthetic materials. However, the precise cause of this type of infection is still unclear, and effective prevention has not yet been established.

Although a variety of bacteria are known to cause the postoperative infections, *Staphylococcus aureus* is the most responsible one in surgical site infections. In addition, methicillin-resistant *S. aureus* is one of the offending microorganisms of lethal infections due to its resistant characteristics to β-lactam antibiotics. One of the alternative therapies to antibiotics, the bactericidal effect of blue light against *S. aureus*, has been verified in previous studies. Guffy and Wilborn reported that the irradiation with a 405 nm blue-violet laser had a bactericidal effect against *S. aureus* and *Pseudomonas aeruginosa* [27]. Other studies also reported that visible light is effective against *Porphyromonas gingivalis, Fusobacterium nucleatum, Staphylococcus aureus, Streptococcus mutans,* and *Escherichia coli* [28, 29]. The results of our previous study also confirmed that the irradiation with a 405 nm blue-violet laser had a bactericidal effect against *P. gingivalis* which is a major periodontopathogen microorganism as well as *Prevotella intermedia*, and even against *Candida albicans* which is a major responsible fungus causing candidiasis [30]. These results demonstrate the possibility that the irradiation with a 405 nm blue-violet laser is capable of eliminating a variety of microorganisms adhered to the surface of osteosynthetic materials and preventing effectively the delayed postoperative infection.

Consequently, in the present study, we attempted to explore the possibility that the irradiation with 405 nm blue-violet laser had a bactericidal effect on *S. aureus* adhered to the surface of Ti and HA-containing PLLA (HA-PLLA) materials and that the irradiation could be effective and useful for the prevention of postoperative infections after bone surgeries using osteosynthetic materials.

2. Materials and Methods

2.1. Preparation of Samples. The Ti sample disks (10 mm in diameter, 2 mm in thickness; Daido Bunseki Research Inc., Nagoya, Japan) were made of commercially available pure Ti (ISO 5832/2, Grade 4A). The surface of the disks were barrel-polished and anodized after acid treatment to remove the oxide layer with a diluted mixture of nitric acid and hydrofluoric acid, which is the same processing procedure used in clinical practice. The disks were then ultrasonically degreased in ethanol and then deionized water (DW) for 10 min each. The HA-PLLA sample disks (10 mm in diameter, 2 mm in thickness) which was composed of a mix of PLLA and particulate bioreabsorbable u-HA (Super Fixsorb®) were supplied by Takiron Co. Ltd., Osaka, Japan.

The surface topographies (i.e., contact angle and surface roughness) of the two materials were examined. Contact angles were measured by the sessile drop method using a measurement device (Kyowa Interface Science Co. Ltd. DM-300). Surface roughness average (Ra) was measured using the SURFCOM 550A surface roughness tester (Tokyo Seimitsu Co., Ltd.) with a 2 μm radius tip at three separate points on each disk. In addition, the diffuse transmittance of disks was measured at normal incidence (0°) and the reflectance of disks was measured at the angle of incidence of 8° in the 200–2500 nm region using a UV-3100PC (Shimadzu Corporation, Kyoto, Japan). The surface texture of disks of each material was observed using a scanning electron microscope (SEM; JSM 5600LV, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 10–15 kV.

2.2. Observation of Bacterial Adhesion with SEM. *S. aureus* FDA 209P was precultured aerobically in 3 mL of brain heart infusion (BHI) medium (Becton Dickinson and Company, Sparks, MD, USA) supplemented with 0.5% yeast extract, 0.05% L-cysteine, and 0.025% resazurin overnight at 37°C. Thereafter, 100 μL of culture solution was inoculated to 10 mL of BHI and cultured aerobically for 5 h with agitation at 37°C.

Each disk was sterilized and placed into a well of a 48-well microtiter plate. Then, 100 μL of bacterial suspension adjusted to 1 × 10⁶ colony-forming units (CFU)/mL and 1 mL of BHI medium were added to each well and cultured aerobically at 37°C for 3, 6, 24, or 48 h. Three disks were prepared for each condition of each material. The disks were rinsed twice with DW to remove nonadherent bacteria, further washed in a vortex with DW for 30 s to dislodge bacteria adhered poorly to the material surfaces, and fixed in 2.5% glutaraldehyde in phosphate-buffered saline for 12 h at 4°C. The disks were dehydrated using a series of graded concentrations of ethanol (50%, 60%, 70%, 80%, 90%, and absolute alcohol), freeze-dried using an ID-2 freeze dryer (EIKO Engineering K.K. Ltd., Tokyo, Japan), and coated with gold using the SC-701AT Quick Auto Coater (Sanyu Denshi, Tokyo, Japan). Four areas were arbitrarily selected and observed with a SEM at an accelerating voltage of 10–15 kV.

2.3. Observation of the Growth of Residual Bacteria with Redox Indicator and SEM. For monitoring the growth of residual bacteria on the material surfaces, a modified redox indicator assay (alamarBlue®; Bio-Rad Laboratories, Hercules, CA, USA) was performed. The redox indicator is a nonfluorescent blue oxidation-reduction pigment which is transformed into red pigment when reduced by bacterial proliferation. The absorbance of this red pigment was measured using a multidetection reader (LabSystems Multiskan®, MultisSoft, Helsinki, Finland) at a wavelength of 570 nm.

After incubation in bacterial solution for 3 h at 37°C, the disks of the Ti and HA-PLLA materials were rinsed twice with DW to remove nonadherent bacteria and further washed in a vortex with DW for 30 s to dislodge bacteria adhered poorly to the material surfaces. Aside from this, some disks that were only rinsed twice with DW for 30 s were prepared as a control group. Thereafter, the disks of each material were transferred into 5 mL of BHI medium containing 500 μL of redox indicator (as 10% of the sample volume)
and incubated for 120 min at 37°C. The absorbance was measured using an automated multidetection reader at a wavelength of 570 nm. To examine the growth of residual bacteria, some other disks of each material were cultured for an additional 24 h and observed with SEM at an accelerating voltage of 10–15 kV.

2.4. Effect of Irradiation with a 405 nm Blue-Violet Laser on Bacteria Adhered on Material Surfaces. One hundred µL of bacterial suspensions at 1 × 10^8 CFU/mL and 1 mL of BHI medium were inoculated onto the surfaces of the Ti and HA-PLLA disks in 24-well culture plates for 3 hours at 37°C. After incubation, each disk was washed with DW and excess water on the surface was absorbed. Thereafter, the disks were irradiated with 405 nm of blue-violet laser in a moisture chamber at 100% relative humidity, under constant output power of 0.2 W (176 mW/cm²) for various irradiation times of 0 s (as a control), 180 s (27 J/cm²), and 300 s (45 J/cm²) using a laser-emitting device equipped with bundling of 20 fibers coupled with 405 nm laser diodes (Ushio Inc., Tokyo, Japan). The 405 nm single-wavelength emission of the laser from the present device was verified and confirmed using a spectrophotometer (USB2000 Miniature Fiber Optic Spectrometer and OceanView Spectroscopy Software, Ocean Optics Inc., FL, USA) (data not shown). The laser output was maintained at a stable wattage, which was measured before every irradiation by a laser power meter and a sensor (Orion/TH P/N 1Z01801: 188784, 3A-P-SH-V1 P/N 1Z02622: 187487; Ophir Optronics Solutions Ltd., Jerusalem, Israel). After irradiation, the samples were stamped on tryptic soy agar plates. After incubation at 37°C for 12 hours, the numbers of bacterial colonies on the plates were counted.

To eliminate possible thermal effects on the material surfaces due to the absorption of irradiation, changes in surface temperature during irradiation were measured with a thermocouple and monitoring device (TC620 NTC Thermistor; Wavelength Electronics Inc., Bozeman, MT, USA).

2.5. Statistical Analysis. For multiple-group comparisons, data were analyzed by one-way analysis of variance. The significance of individual differences was evaluated using the Mann-Whitney U test. A probability value (p) of <0.05 was considered statistically significant.

3. Results

3.1. Morphological Characteristics of the Material Surfaces. The differences in the morphological characteristics were noted between Ti and HA-PLLA. The Ra values of Ti and HA-PLLA were 0.30 ± 0.05 and 0.45 ± 0.005, and the contact angles of each material were 67.4 ± 5.28 and 88.8 ± 1.04, respectively (Table 1). Transmittance and reflectance of each material in the wavelength of 405 nm are presented in Table 2. SEM images also showed obvious differences in surface topology between the two materials. The surface of Ti was relatively smooth and micropores were found in some places, while that of HA-PLLA was comparatively rougher with interspersed white particulates that seemed to be HA crystals (Figure 1).

3.2. Bacterial Adhesion. Although there were differences in morphological characteristics/surface topology between Ti and HA-PLLA, no differences were noted in the bacterial adhesion between the two materials. At 3 hours after inoculation of the bacterial suspension, only a few bacteria were noted to adhere on the surface of both materials (Figures 2(a) and 2(b)). After incubation for 24 hours, small colonies or the bacterial clumps consisting of a few bacteria were observed on HA-PLLA compared with Ti in both groups (Figures 2(c) and 2(d)). After incubation for 48 hours, much more amount of bacteria and larger colonies were formed on the entire surface of both materials (Figures 2(e) and 2(f)).

3.3. Growth of Residual Bacteria after Washing. The growth of residual bacteria on the surface of each material was examined with the redox indicator, alamarBlue® reagent. No obvious difference in bacterial growth was noted until 2–3 hours after 30 s washing with DW between the vortex group and the control group in both materials. In contrast, at 4–5 hours after 30 s washing with DW, more bacterial growth was observed on HA-PLLA compared with Ti in both groups (Figure 3). SEM observation showed the bacterial growth and colony formation at 24 hours after vortex washing on both materials (Figures 4(a) and 4(d)). These results indicated that some few bacteria remained on the surface of both materials even after thorough washing and were capable of growing with time.

3.4. Effect of Irradiation with a 405 nm Blue-Violet Laser on Bacteria Adhered to Material Surfaces. The effects of irradiation with a 405 nm blue-violet laser on bacteria adhered to the surfaces of both materials were analyzed by counting the numbers of colonies formed on tryptic soy agar plates. Obvious differences in the amount of viable bacteria were observed in the control group (without irradiation) between both materials (Figures 5(a) and 5(b)). In the Ti group, irradiation for 180 s (Figure 5(c)) inhibited the colony formation at approximately 76%, while irradiation for 300 s (Figure 5(e)) inhibited at approximately 93%. In the
HA-PLLA group, irradiation for 180 s (Figure 5(d)) and 300 s (Figure 5(f)) inhibited the colony formation at approximately 90% and 99%, respectively. Interestingly, the 405 nm blue-violet laser exerted a more bactericidal effect on HA-PLLA than Ti. There was no significant difference in the control group (without irradiation) between both materials (Figure 6). Irradiation for 300 s caused an increase in surface temperature of HA-PLLA from 20°C to 23°C and from 20°C to 30°C of the Ti (data not shown).

### 4. Discussion

Delayed postoperative infection after bone surgeries using osteosynthetic materials composed of Ti or bioresorbable materials is one of the thorny problems that remain to be solved. However, the precise cause of this type of infection is still unclear and effective prevention measures have not yet been established. The findings of the present study confirmed that *S. aureus*, which is the most responsible bacterium for postoperative infection, could adhere easily to the surface of either material composed of Ti or HA-PLLA and continue to survive. It was also confirmed that once the surface of osteosynthetic material becomes contaminated, a certain amount of the bacteria could remain lodged on the surface of material even after vigorous washing, which then led to colony formation on the material surface. Even with scrupulous attention during surgery, it is difficult to prevent entirely any contaminations by some few bacteria and to remove all contaminating bacteria by washing with physiological saline. This fact indicates the possibility that some cases of inflammation after surgery using osteosynthetic materials could be due to the growth of trace amounts of bacteria which attached to the material surface in surgery and were introduced into the body. Further studies are necessary, however, to elucidate the precise mechanisms of the establishment of postoperative infection after bone surgeries using osteosynthetic biomaterials.

In fact, there are a considerable number of reported cases of delayed infection or inflammatory abscess formation after surgical treatment using bioresorbable osteosynthetic materials such as HA-PLLA [31–33]. So far, such delayed postoperative infections have conventionally been considered to result from the inflammation associated with the decomposition of the material, that is, a foreign body reaction or defective absorption of the crystal component [26, 34, 35]. However, the results of the present study indicate another possibility that delayed postoperative infections are due to delayed onset of inflammation caused by a small number of contaminating microorganisms adhered to the surfaces of biomaterials. This concept could be expected to give some new suggestion to solve this clinical problem.

Judging from the results of the present study, in order to prevent postoperative infection after implantation of osteosynthetic materials, it seems useful to reduce the amount of microorganisms introduced into the living body just before wound closure. So, we examined the usefulness of 405 nm blue-violet laser irradiation, which has been shown to have bactericidal effects, in sterilization of the microorganisms adhered to osteosynthetic biomaterials. Guffey and Wilborn reported that the irradiation with a 405 nm blue-violet laser at 15 J/cm² resulted in a 90–95% mortality rate to a solution of *S. aureus* [27], while Maclean et al. reported a 100% mortality rate with a 405 nm blue-violet laser at 36 J/cm² [36]. Several other studies showed that titanium oxide could exert an antimicrobial effect when irradiated with ultraviolet or short-wavelength visible light [37–39]. In the present study as in previous studies, the irradiation with a 405 nm blue-violet laser exerted a bactericidal effect on *S. aureus* attached to the surface of biomaterials composed of Ti. This bactericidal effect of the 405 nm blue-violet laser was observed to rise with increasing irradiation energy, in accordance with the findings of previous studies [30, 36, 40]. Imamura et al. confirmed the significant bactericidal effect on *P. gingivalis* with irradiation at 0.2 W for 300 s [30]. When exposed to irradiation at 0.2 W for 300 s, at an energy density of 45 J/cm² of disk, the bactericidal effect was approximately 93% on Ti and 99% on HA-PLLA, which is considered to be sufficient to sterilize the residual amount of *S. aureus* on biomaterials after washing. Moreover, the irradiation time of 300 s is considered to be within the acceptable range in practice and the potential burden to the patients would be minimal, even when the irradiation is performed during surgery.
Interestingly, although there was no significant difference in the bacterial adhesion and the growth rate between two materials, more bactericidal effect was observed on HA-PLLA than Ti. Although the real reasons or precise mechanisms of the difference in bactericidal effect between two materials are still unclear, several possibilities might be involved. One of the possibilities is the difference in color and reflectance between the two materials. Since HA-PLLA has a higher reflectance than Ti as the properties of the material surface, more reflection of 405 nm blue-violet laser light does occur on the surface. Because of this, the bacteria adhered to the surface of HA-PLLA could be more irradiated with laser light even in the same irradiation time compared with Ti, and the bactericidal effect would be more enhanced. Another possibility is the influence of morphological characteristics of materials. The calculated surface roughness (Ra) and maximum height (Rz) was larger in Ti compared with HA-PLLA. In addition, SEM showed some micropores scattered on Ti. These differences might affect the efficiency of irradiation to bacterial cells lodged on materials; that is, some bacteria adhered on shady areas of the surface of Ti might be much less irradiated compared with those on HA-PLLA. More interestingly, a slightly more bacterial growth was observed on the surface of HA-PLLA compared with Ti at

Figure 2: Bacterial adhesion of on the surface of Ti (a, c, e) and HA-PLLA (b, d, f) at 3 h (a, b), 24 h (c, d), and 48 h (e, f) after the inoculation of bacterial suspension. SEM images show that more and larger bacterial colonies were formed with the passage of incubation time. No obvious differences were noted in the bacterial adhesion between the two materials.
Figure 3: The growth of residual bacteria on Ti and HA-PLLA. No significant difference was detected between vortex and control groups in both materials until 2–3 hours after 30 s washing with DW. In contrast, at 4–5 hours of incubation after 30 s washing with DW, more bacterial growth was observed on HA-PLLA compared with Ti in both groups. The error bars represent the standard deviation. Values are mean ± SD from 3 samples per group. *p < 0.05. Circle shows HA-PLLA and square shows Ti as control.

Figure 4: The SEM images of the surface of Ti (a, c) and HA-PLLA (b, d) at 24 hours of incubation after vortex washing. The images show that some few bacteria remained on the surface of both materials even after thorough washing and were capable of growing with time.

4–5 hours of culture after vortex washing, despite of no significant differences in bacterial adhesion between both materials. This phenomenon might come from a modest antimicrobial activity of photocatalytic titanium oxide layer covering the surface of Ti, because the investigation was not performed in complete darkness.

In clinical application of the sanitization or disinfection method, their influence on neighboring tissue must be carefully considered in regards to the potential damage of living cells and tissues. Judging from the fact that the wavelength of the 405 nm blue-violet laser used in this study is within the range of visible light and the
irradiation time is at most 300 s, this irradiation is considered to be harmless and noninjurious to neighboring tissues. In addition, monitoring the thermal effect of irradiation indicated that the surface temperature of each material increased to at most 30°C in Ti even after the irradiation for 300 s, which suggests that this procedure is harmless to neighboring tissues and also influential in bacterial growth. For these reasons, the irradiation with a 405 nm blue-violet laser is considered to be a clinically applicable and promising measure to prevent the delayed postoperative infection after bone surgeries using osteosynthetic materials.

**Figure 5:** Photographs of tryptic soy agar plates in the stamp assay. The effects of irradiation with a 405 nm blue-violet laser on bacteria adhered to the surface of Ti (a, c, e) and HA-PLLA (b, d, f) were analyzed by counting the numbers of colonies formed on tryptic soy agar plates. Compared with the nonirradiated control plates (a, b), colony formation was inhibited by the irradiation for 180 s (c, d) and 300 s (e, f) in both materials.
This study is also the first to examine the bactericidal effect of 405 nm blue-violet laser light on bacteria adhered to surfaces of osteosynthetic biomaterials and to confirm the usefulness of irradiation for a relatively short time with clinical application in mind. Although the mechanisms underlying the bactericidal effect of 405 nm blue-violet laser light are still under investigation, porphyrin, a metabolite present in bacteria, might be involved. Endogenously produced porphyrins could act as photosensitizers and generate reactive oxygen/free radicals under light irradiation, leading to the reduction in bacterial growth and viability, and eventual cell death [41, 42]. Further investigations on intracellular photosensitizers including porphyrins will be capable of resolving this issue in the near future.

5. Conclusion

The present study clearly demonstrated that the irradiation with a 405 nm blue-violet laser effectively reduces the number of microorganisms adhered to osteosynthetic biomaterials and introduced into the living body in bone surgeries. These findings strongly suggest the potential usefulness of the 405 nm blue-violet laser irradiation as an alternative strategy for prevention of postoperative infection after implantation of biomaterials.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The funders had no role in the study design, date collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that there is no other conflict of interest regarding the publication of this article.

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Supplementary Materials

Supplementary Figure 1: spectrophotometry confirming the single-wavelength emission of the laser at a wavelength of 405 nm. (Supplementary Materials)

References


