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Original Article

Antimicrobial effect of photodynamic therapy on *Staphylococcus aureus* biofilms on zirconia disks

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Purpose: *Staphylococcus aureus* can attach to extracellular matrix components and plasma proteins deposited on biomaterial surfaces, ultimately forming biofilms. Therefore, managing *S. aureus* is recommended for preventing and treating peri-implant diseases. This study aimed to evaluate the biofilm-forming ability of *S. aureus* on zirconia surfaces and to assess the efficacy of photodynamic therapy (PDT) in reducing these biofilms. **Materials and Methods:** Biofilm growth analysis showed optimal formation at 48 hours. Therefore, *S. aureus* ATCC 25923 was inoculated onto a sterilized zirconia disk and cultivated for 48 hours to form the biofilm. Thereafter, the biofilms were treated with phosphate-buffered saline (PBS; control), chlorhexidine (CHX), tetracycline (TC), toluidine blue O (TBO), cold diode laser (laser), or PDT (TBO + laser), with each group consisting of seven disks. The bacterial load was quantified using colony-forming unit (CFU) counts, and biofilm viability was evaluated using confocal laser scanning microscopy (CLSM). **Results:** Significant reductions in bacterial counts were observed in the CHX (95.3%), TC (95.0%), and PDT (93.8%) groups compared to the control ($P < 0.001$). CLSM revealed a greater number of dead bacteria in the CHX, TC and PDT groups compared to that in other groups. **Conclusion:** Within its limitations, this study demonstrated that *S. aureus* can form biofilms on zirconia surfaces. PDT showed similar efficacy to conventional antimicrobial treatments such as CHX and TC for reducing *S. aureus* biofilms. **(J Dent Rehabil Appl Sci 2024;40(4):241-8)**

Key words: bioFilm; photodynamic therapy; *Staphylococcus aureus*; zirconia

Introduction

Peri-implant diseases are inflammatory conditions affecting the tissues surrounding dental implants and are categorized into peri-implant mucositis and peri-implantitis, similar to the classification of periodontal diseases into gingivitis and periodontitis.[1] According to previous consensus reports,[2-4] peri-implant mucositis is defined as a reversible inflammatory condition in the soft tissue around the dental implant without progressive marginal bone loss. In contrast, peri-implantitis involves inflammation of the peri-implant mucosa accompanied by continuing bone loss.

These conditions are infectious diseases caused by bacteria[3]; however, the specific microorganisms involved in peri-implant diseases are not yet fully understood. Pathogenic bacteria have been isolated from both healthy and diseased implant sites,[5] sug-

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gesting that bacterial biofilm formation on implant surfaces may be similar to that on natural teeth.[6] However, microorganisms not related to periodontal diseases have been isolated from peri-implant disease sites.[7] Additionally, studies have shown differences in the microbiota between periodontal and peri-implant diseases.[8,9] A recent meta-analysis found no specific microbial species exclusively associated with peri-implantitis.[8] However, the prevalence of *Aggregatibacter actinomycetemcomitans, Prevotella intermedia*, and *Tannerella forsythia* in peri-implantitis was higher than that in periodontitis and around healthy implants. Another study showed that the prevalence of *Fusobacterium* and *Streptococcus* species, *Peptostreptococcus micros*, and *Staphylococcus aureus* on dental implants was higher than that in periodontitis.[9]

S. aureus is commonly found in the human body and is associated with various infections, including skin, respiratory, and systemic infections. In the oral cavity, it is found around both around healthy implants and those with peri-implant disease,[10] and it may play a role in implant failure.[11] *S. aureus* can attach to extracellular matrix components and plasma proteins deposited on biomaterial surfaces, ultimately forming biofilms, with a particular affinity for titanium surfaces.[12] Because biofilm formation is a significant step in the pathogenesis of implant-related infections,[13] managing *S. aureus* is recommended for preventing and treating peri-implant diseases.

Effective plaque control is essential for preventing and managing peri-implantitis; however, no gold standard treatment has been established.[14] Photodynamic therapy (PDT) uses light and a photosensitizer to selectively target abnormal cells or specific bacteria, minimizing damage to the surrounding healthy cells.[15] Light activates the photosensitizer, releasing reactive oxygen species (ROS), such as singlet oxygen and free radicals, which are highly reactive and can damage cells. Currently, several photosensitizers are available, and toluidine blue O (TBO) is widely used. TBO is effective against both gram-positive and gram-negative periodontal pathogens and interacts with lipopolysaccharides, which are key components of the gram-negative bacterial outer membrane.[16,17]

Zirconia is a ceramic material widely used in dentistry due to its excellent mechanical properties, biocompatibility, and aesthetics.[18] It is used for fabrication of dental-implant abutments and fixtures, particularly in the anterior region. Its smooth surface and low plaque affinity reduce the likelihood of plaque deposition compared to that on titanium,[18] potentially contributing to better oral health and a lower risk of peri-implant diseases. However, zirconia is not entirely resistant to plaque formation, and peri-implant diseases can still occur, necessitating plaque removal from zirconia abutments and fixtures.

Although several studies have evaluated the efficacy of PDT for removing bacterial biofilms from dental implants, most have focused on titanium surfaces. Therefore, this study aimed to assess the ability of *S. aureus* to form biofilms on zirconia surfaces and to evaluate the efficacy of PDT in removing *S. aureus* biofilms from zirconia surfaces.

Materials and Methods

Zirconia disk preparation

Zirconia disks (HASS, Gangneung, Korea) with a diameter of 12 mm and a thickness of 2.5 mm were prepared and surrounded with putty-type vinyl polysiloxane impression material (GC Corporation, Tokyo, Japan) to allow *S. aureus* biofilm formation on only one side of the disks. The disks were disinfected by immersion in 70% ethanol and then sterilized using hydrogen peroxide plasma.

Bacterial strains and culture conditions

S. aureus (ATCC 25923) was grown on tryptic soy agar (TSA) at 37°C for 24 h and then cultured in 10 mL tryptic soy broth for another 24 h to obtain a bacterial suspension. The suspension was adjusted to 1×10^8 colony forming units (CFU) per mL using a spectrophotometer. To assess biofilm formation, zirconia disks were placed in 12-well plates, and 100 μL bacterial suspension and 2 mL tryptic soy broth were added to each well. The plates were incubated at 37°C. The optimal culture time was determined based on the growth curve.

Determination of the optimal culture time

After incubation at 37°C for 6, 12, 24, 48, and 72 h, the putty surrounding the zirconia disks was removed, and the disks were rinsed twice with phosphate-buffered saline (PBS). The rinsed disks were placed in 12-well plates containing 2 mL PBS and sonicated twice for 20 s each to detach the biofilms. Each sample was diluted with PBS and cultured on TSA for 24 h at 37°C, after which the number of colonies was counted. The number of colonies was the highest on disks incubated for 48 h (Fig. 1). Therefore, zirconia disks seeded with the *S. aureus* suspension were cultured for 48 hours based on the growth curve.

Treatment protocol

Based on the growth curve, zirconia disks were incubated with the *S. aureus* suspension for 48 h at 37°C to allow biofilm formation. After incubation, the surrounding putty was removed, and the disks were rinsed twice with PBS. Thereafter, the disks were transferred to new 12-well plates and divided into six treatment groups, with each group consisting of seven disks. The experiment was repeated twice for consistency.

1. Control Group: Disks were treated with 100 μL PBS for 30 s.
2. Chlorhexidine Gluconate (CHX) Group: Disks were treated with 0.1% CHX (Bukwang Pharm. Co., Seoul, Korea) for 30 s.
3. Tetracycline (TC) Group: Disks were treated with a 50 mg/mL TC solution (Tetracyclin®, Chong Kun Dang, Seoul, Korea) for 30 s.
4. TBO Group: Disks were treated with 100 μg/mL TBO (Sigma-Aldrich Corp., St. Louis, USA) for 5 min in a dark room.
5. Cold Diode Laser (Laser) Group: Disks were treated with PBS for 5 min, followed by irradiation with a cold diode laser (Periowave system; Ondine Biomedical, Vancouver, Canada) with a wavelength of 670 nm at 160 mW in continuous mode for 60 s.
6. TBO + Cold Diode Laser (PDT) Group: Disks were treated with 100 μg/mL TBO for 5 min, followed by irradiation with a cold diode laser under the same parameters as in the Laser group.

After treatment, all disks were rinsed twice with PBS. Bacterial cell viability was assessed through cell counting and confocal laser scanning microscopy (CLSM). One disk sample from each group was selected for CLSM imaging to determine the biofilm viability.

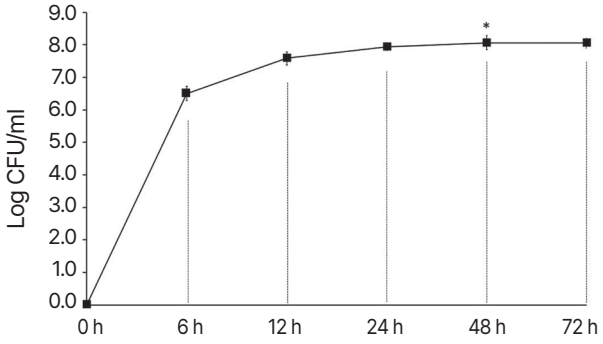

Fig. 1. Growth curve of the *S. aureus* biofilm on a zirconia disk.
* The number of bacteria peaks at 48 h.
CFU, colony-forming unit.

Bacterial cell viability test

Treated disks were placed in 12-well plates containing 2 mL PBS and sonicated twice for 20 s each to detach the biofilms. The samples were diluted with PBS (1 : 100) and cultured on TSA for 24 h at 37°C. The colony-forming units (CFU) were counted using an automated colony counter (IUL, Barcelona, Spain).

Confocal laser-scanning microscopy

CLSM was performed to assess the viability of the bacteria after treatment. Biofilms were stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes Inc., Eugene, USA) according to the manufacturer's instructions. The stained biofilm samples were examined using a confocal laser-scanning microscope (TCS SP8 STED, Leica Microsystems,

Mannheim, Germany), and the images were analyzed using an image processing program (LAS X, Leica Microsystems).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to assess differences between groups, and the Tukey honestly significant difference test was applied for post hoc analysis. Statistical significance was set at 5%.

Results

Fig. 1 illustrates the growth curve of the *S. aureus* biofilm over time. Bacterial numbers increased rapidly until 6 h, after which the rate of increase slowed. The bacterial count peaked at 48 h and remained constant thereafter.

The results are summarized in Table 1. Compared to the control group, the bacterial reduction was 95.3%, 95.0%, 55.3%, 10.1%, and 93.8% in the CHX, TC, TBO, Laser, and PDT groups, respectively. The CHX, TC, and PDT groups showed extremely significant reductions in bacterial counts ($P < 0.001$), whereas the TBO group showed a significant reduction ($P < 0.05$). No significant difference between the Laser group and the control group was observed, and no significant differences were observed among the CHX, TC, and PDT groups.

CLSM images showing the viability of bacteria are presented in Fig. 2. A greater number of dead bacteria were observed in the CHX, TC and PDT groups compared to the other groups.

Discussion

PDT has been proposed as an alternative treatment for peri-implantitis, and several studies have reported its efficacy in elimination of bacteria.[19-21] This study demonstrated that PDT is effective for reducing *S. aureus* biofilms cultured on zirconia disks. In the PDT groups, a significant reduction in CFU concentrations of *S. aureus* compared to those in the control group was observed, and dead bacteria were observed in CLSM images.

CHX and TC are effective in reducing bacterial counts and are commonly used to treat peri-implantitis.[22,23] In this study, CHX, TC, and PDT reduced bacterial counts by 95.3%, 95.0%, and 93.8%, respectively, and the differences among the three treatments were not statistically significant. Therefore, PDT can be considered as effective as CHX and TC and may serve as an alternative treatment.

In this study, the growth curve of *S. aureus* on the zirconia surface increased exponentially until 6 h, followed by a gradual increase from 6 to 48 h, and the number of bacteria remained constant thereafter. According to Harri et al.,[24] under ideal conditions, the growth curve of *S. aureus* comprises three phases: lag, exponential, and stationary. During the exponential phase, bacterial metabolism is rapid, leading to continuous growth. Eventually, as *S. aureus* cells age and stop proliferating, the bacterial count stabilizes

Table 1. Reduction in bacteria on zirconia surfaces after treatment

Group	Log CFU/mL (mean ± SD)	Bacterial reduction (%)	*P* value
Control	7.54 ± 0.31	-	-
CHX	6.21 ± 0.14	95.3**	< 0.001
TC	6.24 ± 0.07	95.0**	< 0.001
TBO	7.19 ± 0.50	55.3*	< 0.05
Laser	7.49 ± 0.12	10.1	0.987
PDT	6.33 ± 0.07	93.8**	< 0.001

CHX, chlorhexidine; TC, tetracycline; TBO, toluidine blue O.; Laser, cold diode laser; PDT, photodynamic therapy.
* Significant differences compared to control group (PBS group) ($P < 0.05$) using ANOVA.
** Significant differences compared to control group (PBS group) ($P < 0.001$) using ANOVA.

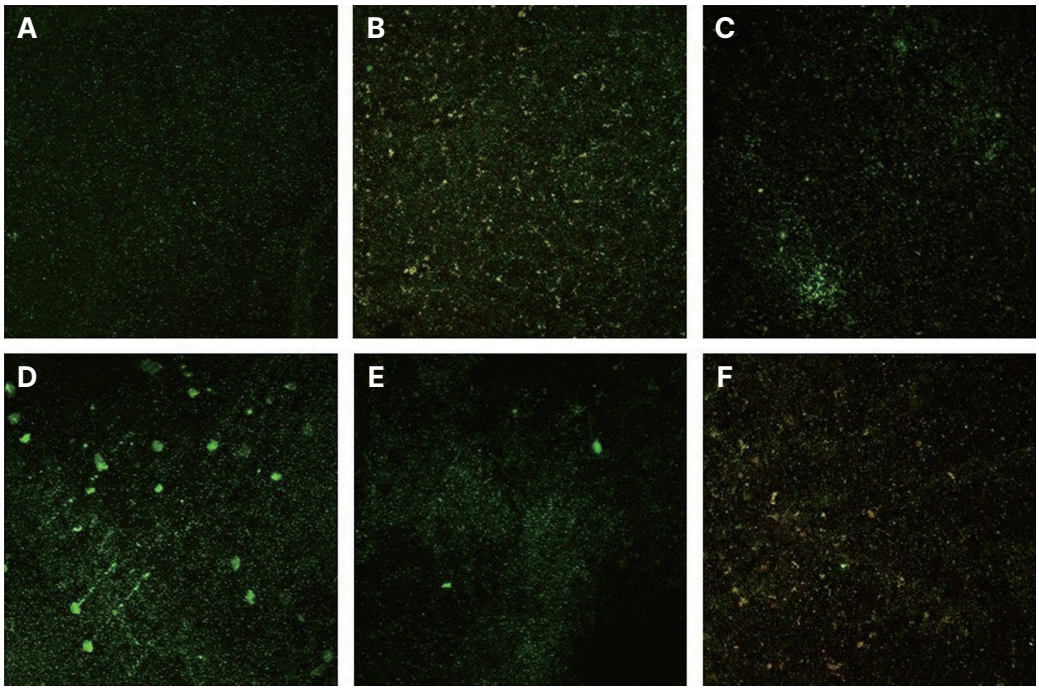

Fig. 2. Confocal laser scanning microscopy images of *S. aureus* attached to the zirconia disks (original magnification: 10×). Live bacteria appear green, dead bacteria appear red, and the overlap areas appear yellow. CLSM revealed a greater number of dead bacteria in the CHX, TC and PDT groups compared to that in other groups. (A) Control group, (B) CHX group, (C) TC group, (D) TBO group, (E) Laser group, (F) PDT group.
CHX, chlorhexidine; TC, tetracycline; TBO, toluidine blue O; Laser, cold diode laser; PDT, photodynamic therapy.

The continuous growth from 0 to 48 h followed by stabilization observed in this study is consistent with the findings of Harri et al.[24] Additionally, comparison with a study that cultured *S. aureus* on titanium surfaces revealed that the growth curve for *S. aureus* on zirconia was similar to that on titanium, with peak growth at 48 h.[25] This suggests that the biofilm was mature at this point, justifying the 48-h incubation period for bacteria on zirconia disks.

Azizi et al.[26] reported that using a laser and photosensitizer together significantly reduced the *Prevotella intermedia, Actinomyces actinomycetemcomitans*, and *Porphyromonas gingivalis* counts and the total bacterial load on contaminated zirconia implants. Anil et al.[27] reported that PDT exhibited a higher antibacterial effect against *P. gingivalis* and *T. forsythia* on zirconia blocks than CHX and hydrogen peroxide without altering the zirconia surface topography. These findings suggest that PDT effectively reduces potential pathogens associated with peri-implant disease around implants with zirconia abutments or fixtures.

Compared to a study by Park et al.[25], which evaluated the effect of PDT on *S. aureus* on titanium disks in a similar experimental setup, in this study, the bacterial count per unit area was lower, and the number of bacteria on zirconia was smaller in CLSM images. Roehling et al.[28] compared the biofilm formation of three species (*S. sanguinis, F. nucleatum, P. gingivalis*) with that of human plaque samples on zirconia and titanium surfaces. They found that the biofilm was significantly thinner on zirconia than on titanium, and the mass of biofilms from human plaque samples was also reduced. They suggested that these differences could be due to variations in surface topography, material composition, and the hydrophilicity of metals versus oxide ceramics. Pérez-Tanoira et al.[29] compared the adhesion of *S. aureus* on titanium surfaces to that on zirconia-coated titanium surfaces. The mean proportion of zirconia-coated titanium surfaces covered with strains of *S. aureus* was lower than that of uncoated titanium surfaces. Thus, zirconia implants or abutments might be more effective

in preventing peri-implant diseases because biofilm formation is lesser than that on titanium.

This study was conducted using a single bacterial strain in an in vitro environment. However, peri-implant diseases result from complex interactions among various pathogens, including numerous oral bacteria, viruses, and fungi. Thus, this study lacks biological complexity. Additionally, real oral conditions, such as constant saliva flow and fluctuating temperature and pH, cannot be fully replicated in vitro. Therefore, further research considering these complex interactions is necessary. Nevertheless, this study is valuable as it addresses the limited research on the efficacy PDT against bacteria on zirconia surfaces.

Conclusion

Within its limitations, this study demonstrated that *S. aureus* can form biofilms on zirconia surfaces. PDT effectively reduces *S. aureus* biofilms on zirconia disks and can serve as an alternative to conventional treatments such as CHX and TC.

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지르코니아 표면에 형성한 *Staphylococcus aureus* 바이오필름에 대한 광역학 치료의 항미생물 효과

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목적: *Staphylococcus aureus*는 생체재료 표면에 침착된 세포외 기질 성분과 혈장 단백질에 부착하여 바이오필름을 형성할 수 있다. 따라서 *S. aureus*의 관리는 임플란트 주위 질환의 예방과 치료에 중요하다, 이번 연구는 *S. aureus*가 지르코니아 표면에서 바이오필름을 형성하는 능력을 평가하고, 광역학요법(phtodynamic therapy; PDT)이 이러한 바이오필름을 감소시키는 데 효과적인지 평가하였다.

연구 재료 및 방법: 시간에 따른 바이오필름 성장 분석 결과 48시간에 최적의 바이오필름이 형성됨을 확인하였다. 따라서 멸균된 지르코니아 디스크에 *S. aureus* ATCC 25923를 접종하여 48시간 동안 배양하여 바이오필름을 형성하였다. 형성된 바이오필름은 각각 7개의 디스크로 구성된 군으로 나누어, 대조군, 클로르헥시딘군(CHX), 테트라사이클린군(TC), 톨루이딘 블루군(TBO), 다이오드 레이저군(Laser), 광역학요법군(PDT; TBO + Laser)로 처리하였다. 군에 따른 처리 후 세균을 배양하여 세균수(colony forming unit; CFU)를 계산하고, 바이오필름을 공초점 현미경을 통해 분석하였다. 통계 분석은 CFU값을 로그 값으로 환산한 후 분산분석방법을 시행하였다.

결과: 실험군 중 CHX (95.3%), TC (95.0%), PDT (93.8%) 군에서 대조군에 비해 세균 수가 유의하게 감소하였다($P < 0.001$). 공초점 현미경 분석 결과 CHX, TC와 PDT 군에서 다른 군에 비해 더 많은 사균이 관찰되었다.

결론: 이번 연구를 통해 *S. aureus*가 지르코니아 표면에서 바이오필름을 형성할 수 있음을 확인하였고, 광역학요법은 CHX 및 TC와 같은 기존 항균 치료와 유사한 수준으로 *S. aureus* 바이오필름을 감소시키는 효과를 확인하였다.

(구강회복응용과학지 2024;40(4):241-8)

주요어: 바이오필름; 광역학요법; *Staphylococcus aureus*; 지르코니아

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