Sabine Sennhenn-Kirchner
Sören Klaue
Nadine Wolff
Hamparsum Mergeryan
Margarete Borg von Zepelin
Hans Georg Jacobs

Decontamination of rough titanium surfaces with diode lasers: microbiological findings on *in vivo* grown biofilms

Authors' affiliations:
Sabine Sennhenn-Kirchner, Sören Klaue, Nadine Wolff, Hans Georg Jacobs, Department of Oral Surgery and Radiology, Georg-August University, Göttingen, Germany
Hamparsum Mergeryan, Department of Hospital Hygiene and Infection Control, Georg-August University, Göttingen, Germany
Margarete Borg von Zepelin, Institute of Medical Microbiology, Georg-August University, Göttingen, Germany

Correspondence to:
Sabine Sennhenn-Kirchner, DD
Abteilung zahnärztliche Chirurgie
Georg-August Universität
Robert-Koch-Strasse 40
37075 Göttingen
Germany
Tel.: +49 551 392 868
Fax: +49 551 399 217
e-mail: se.ki@med.uni-goettingen.de

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Key words: decontamination, diode laser, oral biofilm, titanium surface

Abstract

Objectives: The bactericidal efficacy of diode lasers has already been demonstrated *in vitro*. We investigated the reduction of aerobe bacteria – colonizing rough titanium samples in biofilms intraorally grown – by diode lasers of different wave lengths.

Material and methods: Twenty-two volunteers participated in the trial. They were fitted for 10 days with custom-made intraoral plastic splints carrying titanium sleeves. A part of the sleeves was then irradiated with diode lasers in different modes. The other part remained non-irradiated and served as control. Directly after irradiation, the sleeves were swabbed and the gained bacteria were first examined microscopically and then were cultured under aerobic conditions.

Results: The bacteria in the controls and in the treated samples were quantified. A comparison with the controls revealed a marked overall reduction of bacterial colonization in all irradiated sleeves. Continuous irradiation for 20 s reduced bacteria counts by 99.67% at 810 nm and 99.58% at 980 nm. Repeating the 20 s exposure five times reduced counts by 99.98% at 810 nm and by 99.39% at 980 nm. A 98.86% reduction was seen after irradiation in pulsed mode. A further analysis in respect of different isolated bacteria revealed that the streptococci group was reduced by 99.29–99.99%, while the staphylococci group was reduced to a lesser extent in the range 94.67–99.99%.

Conclusion: The results are of clinical relevance. In comparison with the mean bacterial counts of the untreated samples, all irradiation programs studied in this investigation reduced mean bacterial colonization in a biofilm on intraoral rough titanium surfaces by more than 98%. The actual extent of reduction was dependent on the bacteria species as well as on the irradiation mode.

Decontamination of the implant surface is one facet in the therapy of peri-implantitis, one of its goals is to reduce the number of colonizing pathogens as much as possible. The causal relationship between a persisting biofilm on the implant surface and the occurrence of peri-implant inflammation has been established clinically (Mombelli & Lang 1998; Hultin 2002; Shibli et al. 2003). Different microorganisms have been detected at peri-implant sites (Lee et al. 1997; Hultin 2002; Leonhard et al. 2003; Shibli et al. 2003). This bacterial contamination is connected to peri-implant infections and sometimes causes even implant failure (Rams & Link 1983; Becker et al. 1990; George et al. 1994; Piattelli et al. 1998; Leonhardt et al. 1999, 2003). The environmental conditions of the biofilm lead to increased resistance to antimicrobial treatment (Anwar et al. 1992; Larsen & Fiehn 1996;

Souli & Giamarellou 1998; Sbordone & Bartolaia 2003).

The antimicrobial activity of laser light, which depends on its photothermic effects, has been described by a number of authors *in vitro* (Deppe et al. 2001; Sennhenn-Kirchner et al. 2002; Kreisler et al. 2003; Soukos et al. 2003; Romanos et al. 2004) and *in vivo* (Moritz et al. 1997; Romanos & Nentwig 1999; Bach et al. 2000; Haas et al. 2000; Deppe et al. 2002). The antimicrobial efficacy of the diode lasers has been previously demonstrated *in vitro* (Sennhenn-Kirchner et al. 2002; Kreisler et al. 2003). The evaluation of the efficacy of diode laser light on biofilms induced *in vivo* is missing up to now. The present study investigated the decontaminating effect of five different irradiation programs of two different diode lasers (one emitting light at a wavelength of 810 nm and one at 980 nm) on intraorally grown biofilms on rough titanium surfaces. This study using a model of 'old' biofilm (Anwar et al. 1992) grown under *in vivo* conditions adds new data on proofs of laser efficacy published until now with *in vitro* models. The results prove the efficacy of both wavelengths with regard to the reduction of biofilm producing aerobe bacteria.

Material and methods

Laser and laser programs

The antimicrobial effect of five different diode laser irradiation programs with two different diode lasers: (1) 810 nm wavelength Ora Laser 01 I.S.T., Oralia, Konstanz, and (2) 980 nm wavelength Schütz WDL 2.5, Schütz Dental Group were studied.

(1) 810 nm wavelength, continuous wave (cw) mode with 1 W, 600 µm wave guide fiber for 20 s.
(2) 810 nm wavelength, cw mode with 1 W, 600 µm fiber for 20 s repeated five times with a 30 s pause after 20 s irradiation time.
(3) 980 nm wavelength, cw mode with 1 W, 500 µm fiber for 20 s.
(4) 980 nm wavelength, cw mode with 1 W, 500 µm fiber for 20 s repeated five times with a 30 s pause after each 20 s irradiation time.
(5) 980 nm wavelength, pulsed mode (1.5 W, 20 Hz, 3 ms), 500 µm fiber for 20 s repeated five times with a 30 s pause after each 20 s irradiation time.

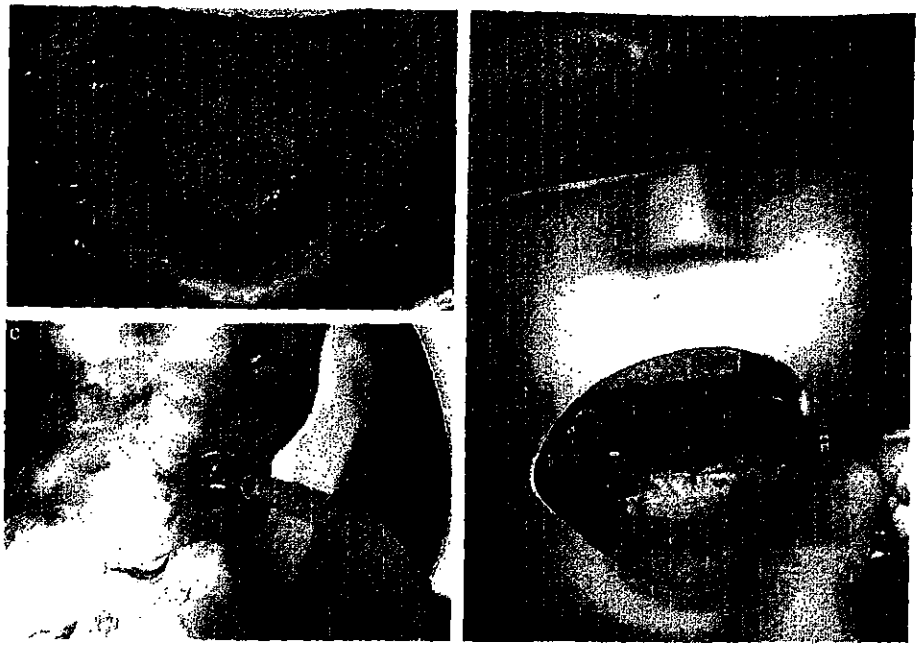

Fig. 1. (a) Titanium sleeves attached to custom-made plastic splints by light-curing resin. (b) Splint mounted on custom-made plaster model in a phantom head. (c) Application of the laser beam to the titanium surface.

Study objects and study design

Twenty-two volunteers participated in the trial. The study objects were titanium sleeves (Steco, Hamburg, Germany), normally used as drill guide for dental implantology (outside diameter of 3 mm, inside diameter of 2.35, 5 mm long) which were sand-blasted before use (Alcastral 150 µm, OmniDent, Rodgau Niederöden, Germany). In order to ensure a secure intraoral position, the sleeves were attached in vertical position to the buccal sides of custom-made mandibular plastic splints (Erkodur resin foil, 120 mm, Erkodent, Pfalzgrafenweiler, Germany) with light-curing resin (Triad Gel Clear Colorless, Dentsply, Konstanz, Germany). Figure 1a shows the top view on the splint *in situ*.

The splints were fitted to the patients mandible and they remained in place for 10 consecutive days and nights. They were removed from the oral cavity only for tooth brushing, interdental flossing, and for the intake of food and liquids, and then too for a longest time of 90 min at a time. During this time, they were stored in sterile plastic bags. The use of any kind of mouth rinse was prohibited during the entire period. After the 10-day period, the splints were removed and mounted on custom-made plaster models in a phantom head (Fig. 1b). Each voluntary participant carried at least three sleeves in his mouth.

One titanium sleeve from each splint was left untreated and served as control. Two sleeves from the same person were treated with two of the diode laser programs described above. The allocation of the different programs were random. Every program (1–5) was applied in eight test persons, so that eight different test parameters were gained for every program. All treatments were performed under identical conditions by the same investigator. The laser beam was applied to the inner titanium surface of the sleeves with an up-and-down motion in slight contact mode (Fig. 1c).

Samples and microbiology

Immediately after irradiation, swabs were obtained from each titanium sleeve with sterile tweezers and by scrubbing with sterilized interdental brushes (Cupaprox CPS 12 regular, Curaden, Kriens, Switzerland), exactly fitting in the sleeves in diameter, and with exactly 10 strokes per sleeve (Fig. 2). The swabs were placed in sterile Eppendorf tubes containing 1000 µl physiological saline according to Kite et al. (1997). The dissolved material was mixed

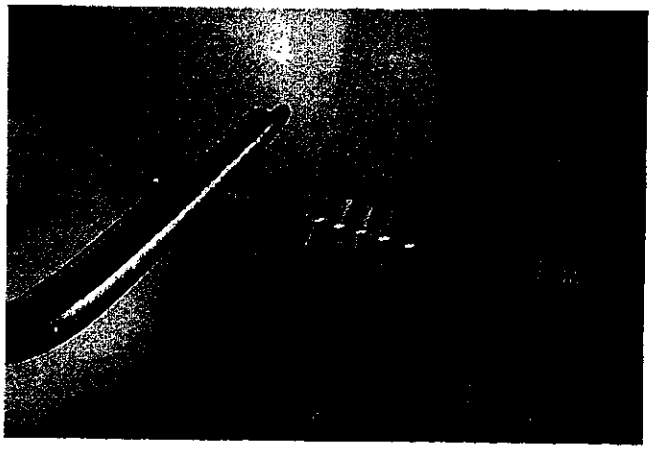

Fig. 2. Swabs were obtained from each titanium sleeve.

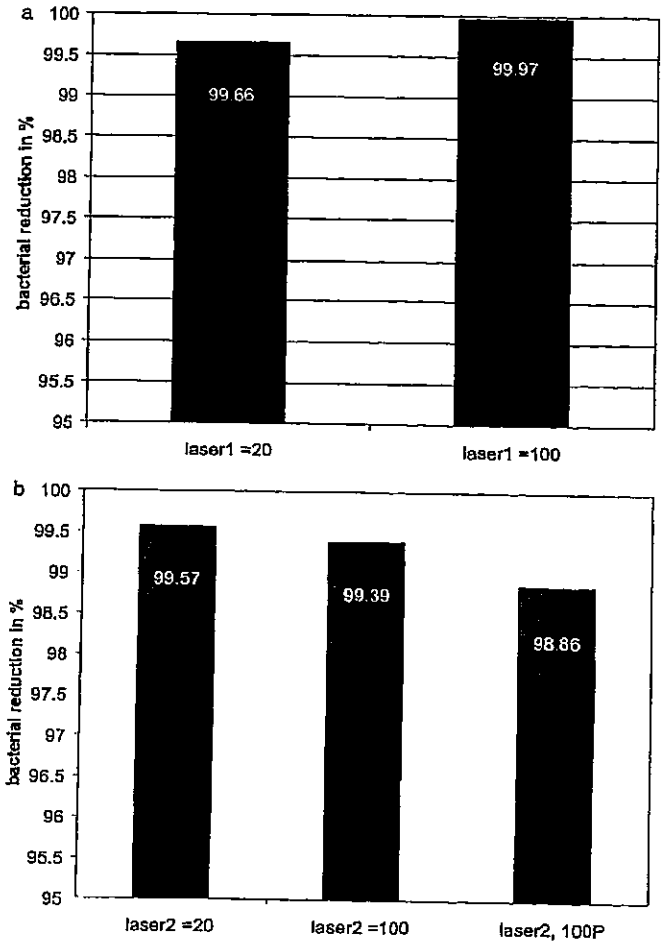

Fig. 3. (a) Effect of irradiation regimen by diode laser at 810 nm wavelength. Comparison of two application modes: continuous wave for 20 s (=20) and 100 s (=100). The figure shows the percentage of the mean bacterial reduction of each regimen compared with untreated controls that were set to 0%. To demonstrate the small differences between these two regimens, the *y*-axis starts at 95%. (b) Effect of the irradiation regimen performed with the diode laser at 980 nm wavelength. Comparison of three different application modes: 20 s (=20) and 100 s (=100) and pulsed mode (=100p). As in Fig. 3a the percentage of the mean bacterial reduction of each regimen is demonstrated and the *y*-axis starts at 95% to reveal the small differences between these regimens.

on a vortexer (VF 2, Anke and Klunke, Staufen i. Br., Germany) for 1 min. For the determination of the bacterial concentration of the different samples, the dissolved material was serial diluted in physiological saline (10^{-1}– 10^{-5}) following the method of Süßmuth et al. (1999). One hundred microliter aliquots of each dilution were then plated on blood agar plates (Columbia agar, Bio Mericux, No. 43049, Marcy L'Etoile, France) and incubated under aerobe conditions at 35 ± 1°C for 24 h (Reach-in incubator, Forma Scientific, Marietta, OH, USA). Colony-forming units (CFU) were counted by a colony counter (Bio, Kobe, Japan).

The CFU were analyzed for morphological differences on the agar plates and were first classified by Gram staining (Süßmuth et al. 1999). The bacteria were further differentiated by their metabolic properties with a commercially available identification system (BD BBL Crystal GP, No. 245140, Becton Dickinson, Heidelberg, Germany) after incubation for 24 h at 35 ± 1°C under aerobic conditions.

Statistics

The mean decontamination rates were calculated for each program separately and statistical analysis was performed as follows: In order to compare the paired observations of the modes of laser 1 (control, 20 and 100 s) Friedman's test and for comparisons in pairs the sign test were applied using the closure principle to adjust for multiple testing. For the independent observations of laser 2, a non-parametric ANOVA with two fixed factors (laser mode and group) was used. Again comparisons in pairs were adjusted for multiple testing using the closure principle.

Results

All laser irradiation regimens used in this investigation had marked antimicrobial effects on the detected bacteria gained from an intraorally grown biofilm when compared to the controls (Fig. 3a, b). The reduction rates were statistically significant.

- control/laser 1: $P<0.0001$,
- control/laser 2 cw: $P<0.0001$,
- control/laser 2 – 100p: $P<0.0001$.

Laser 1 (810 nm wavelength) induced an average CFU reduction of 99.66% with an

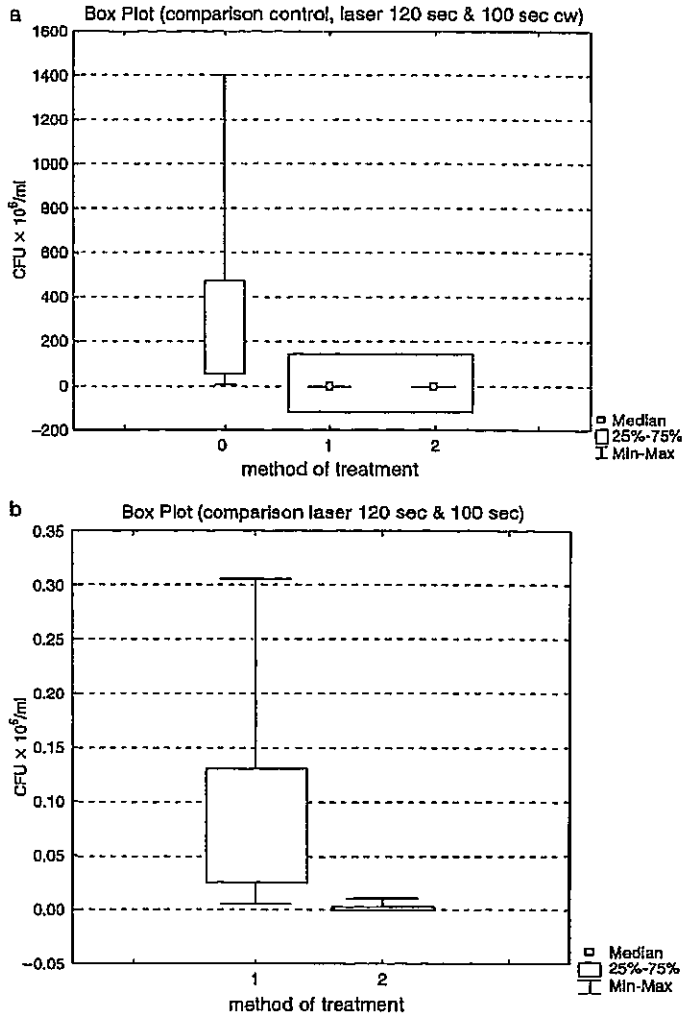

Fig. 4. Summary of the results gained by diode laser at 810 nm wavelength in detail. (a) Box plot of the results gained from at least eight different samples in each group. The bacterial counts of untreated controls (0) (median: 31.67 × 10^6 CFU/ml; interquartile range: 37.16; standard deviation: 22.77) is demonstrated, as well as the results obtained by the different programs 1 and 2 of this laser (compare (b)). (b) Box plot of the exact results gained with laser program 1 and program 2. To show the differences between both programs a different scale of the *y*-axis has been chosen. With program 1 a median of 0.034 × 10^6 CFU/ml (interquartile range: 0.29; standard deviation: 0.113) was obtained, while program 2 revealed a median of 0.00035 × 10^6 CFU/ml (interquartile range: 0.01; standard deviation: 0.0039). However, the differences between the programs, and between each program and the respective control were significant (Friedman test; sign test).

average reduction rate of 2.96 $\log_{10}$ steps when applied for 20 s at 1 W in cw mode.

Repeating the treatment five times increased the average CFU reduction rate to 99.98% (3.34 $\log_{10}$ steps). The increase in CFU reduction by the repetition of irradiation was statistically significant, $P = 0.0156$/Friedman's test, followed by sign test (Fig. 4a, b).

- laser 1 −20/−100: $P = 0.0156$.

Laser 2 (980 nm wavelength applied for 20 s at 1 W) induced an average CFU reduction of 99.57% with a rate of 2.89 $\log_{10}$ steps. Increasing the application time to five times 20 s showed an average CFU reduction of 99.39%, while the pulsed mode at 1.5 W and five times 20 s irradiation induced a reduction of 98.86%. The differences between these programs were not statistically significant:

- laser 2 −20/−100 cw: $P = 0.60$,
- laser 2 −100 cw/−100p: $P = 0.61$.

Various species of staphylococci and streptococci were detected in the biofilms. In all participants, streptococci occurred, which could not be identified by the used test kit. In combination with these streptococci, microorganisms could be detected. Sometimes they occurred alone and sometimes together with other bacteria as follows:

- *Staphylococcus aureus* (five cases) in combination with *S. lentus*, *Streptococcus pneumoniae* and *Str. vestibularis*,
- *S. crista* (three cases), once combined with *Str. pneumonia*,
- *Str. pneumoniae* (five cases) in combination with *Lactococcus lactis*, *S. aureus*, *S. crista* and *Str. vestibularis*,
- *Str. sanguis* (in two cases) and with *S. auricularis* and *S. hämolyticus*,
- *Aerococcus urinae* (in four cases) with *Micrococcus luteus*, *L. lactis* and *Str. Pneumonia* and
- *L. lactis* (in five cases).

Mean bacterial reduction rates were clinically relevant and could be measured between 94.67% and 100%. It depended on applied laser irradiation regimen and on bacterial species or even subtype; staphylococci showed minor decrease rates than other species. Comparison of medians and confidence intervals showed no relevant differences (Table 1).

Discussion

Following the demonstration of the antimicrobial efficacy of diode laser light *in vitro* by Sennhenn-Kirchner et al. (2002) and Kreisler et al. (2003), it is an open question whether it might be effective against bacteria protected by *in vivo* grown biofilms. Biofilms have been characterized by *in vitro* (Xu et al. 2000; Donlan & Costerton 2002; Pratten et al. 2003) and *in vivo* research (Marsh 1995; Bradshaw et al. 1997; Socransky et al. 1998, 2004; Soukos et al. 2003). Referring to these examinations and following the arguments of Costerton & Lewandowsky (1995) and Costerton et al. (1999), it can be assumed that pathogens associated with peri-implant infections are protected by biofilms (Bradshaw et al. 1997; Hultin 2002). Biofilm-producing bacteria are able to colonize all intraoral surfaces, particularly rough structures, such as the surface of implants (Krekeler et al. 1990; Marsh 1995; Bollen et al. 1996; Lee et al. 1997; Mombelli & Lang 1998; Groessner-Schreiber et al. 2004; Kuula et al. 2004). Biofilms protect

Table 1. **Mean reduction (MR) of different species of bacteria by different laser irradiation regimens in percent related to the mean basic bacterial counts of the untreated samples, medians and confidence intervals (CI)**

	Laser 1 = 20	Laser 1 = 100	Laser 2 = 20	Laser 2 = 100	Laser 2; 100 P
Staphylococci (11 P) MR	99.44	99.99	99.99	97.62	94.67
Median	99.44	100	99.99	99.99	99.13
CI	92.4–106.5	99.99–100	99.96–100	93.6–101.6	84.1–105.25
Streptococci (22 P) MR	99.29	99.99	99.8	99.94	99.94
Median	99.94	100	99.87	100	100
CI	98.2–100.37	99.98–100	99.65–99.96	99.82–100	99.87–100
Aerococcus urinae (4 P) MR	100	/	99.92	100	99.99
Median	100	/	99.94	100	99.99
CI			99.72–100.13		99.98–100.02
Lactococcus lactis (5 P) MR	99.75	99.95	100	100	99.95
Median	99.79	99.93	100	100	99.95
CI	99.25–100.25	99.83–100.06			99.57–100.33
All bacteria (22 P) MR	99.67	99.98	99.58	99.39	98.78
Median	99.93	100	99.95	100	99.98
CI	98.78	99.97–99.99	99.6–99.98	98.1–100.35	96.67–100.72

P, participants.

the colonizing microorganisms against a wide variety of exogenous influences (Anwar et al. 1992; Souli & Giamarellou 1998; Cochran et al. 2000; Sbordone & Bartolaia 2003; Soukos et al. 2003). Donlan & Costerton (2002) reviewed literature on survival mechanisms of clinically relevant microorganisms in biofilms. The microorganisms that grew in biofilms express a distinct phenotype that made them resistant to antibacterial agents and host response. Therefore the therapeutic success of infections caused by bacterial biofilm colonization of surfaces is more difficult to achieve. It has been shown that for the eradication of bacteria in biofilms, antimicrobial agents have to be overdosed up to 10 times (Wilson 1996; Socransky et al. 2002). In these cases the use/risk factor for the patient may easily shift to damage. So the efficacy of laser irradiation under different therapeutic aspects has to be investigated.

Following the arguments of Hejdenrijk et al. (2002) basing on studies of Quirinen & Listgarten (1990), Leonhardt et al. (1999) and Rosenberg et al. (1991), the simple presence of pathogens at peri-implant sites will not cause peri-implant infections consecutively as long as the number of these periodontal pathogens is kept at a low level and other potential (co)-factors are within normal limits. This emphasizes the necessity of reducing bacteria at peri-implant sites.

The present study investigated the decontamination efficacy of various diode laser irradiation programs on aerobic bacteria. The composition of subgingival biofilms has been described frequently (Socransky et al. 1998; Rutar et al. 2001; Leonhardt et al. 2003). A primary colonization has been demonstrated with cocci (Shibli et al. 2003; Li et al. 2004). Cocci seem to pave the way for colonization with anaerobic organisms (Rams et al. 1990; Wu-Yuan et al. 1995) and they are used for biofilm related studies. Anaerobes are very sensitive to oxygen. Therefore it has to be assumed that the yield of anaerobes gained by the microbrush technique might be too low leading to a false-positive effect of the laser therapy. Many studies on this topic focus on anaerobes considered to be involved in the etiology of peri-implant infections (Bollen et al. 1996; Lee et al. 1997; Rutar et al. 2001; Hultin 2002; Socransky et al. 2004), and rely on molecular biological analysis. However, some studies have demonstrated differing floras associated with periodontitis and peri-implantitis (Rams et al. 1990; Rutar et al. 2001; Leonhardt et al. 2003). Leonhardt et al. (1999, 2003) found approximately equal numbers of anaerobic microorganisms on the one hand, and aerobic cocci and yeasts on the other in infected peri-implant sites by cultivation and plating.

In this study we focused on cocci to evaluate the decontamination effects of laser light. These cocci had grown in biofilms on rough titanium surfaces which had been positioned intraoral in various voluntary persons. Therefore, the obtained biofilms showed differences in their composition of bacteria.

The efficacy of laser light of various wavelengths to decontaminate surfaces has been demonstrated repeatedly *in vitro* (Coffelt et al. 1997; Haas et al. 1997; Kreisler et al. 2002a, 2002b, 2003; Sennhenn-Kirchner et al. 2002).

Its clinical use in the treatment of peri-implantitis has been described (Bach et al. 2000; Haas et al. 2000; Shibli et al. 2003), but there are hardly any studies on the direct effects of laser light on biofilms as the literature reviews show (Roos-Jansacker et al. 2003; Esposito et al. 2004). Rovaldi et al. (2000), for example, found a 6 log bacterial decrease by photosensitation and following 662 nm laser irradiation *in vitro*. However, the same treatment mode applied on plaque bacterial biofilm samples of periodontal affected persons leads just to 75–92% reduction which means a ≤ 2 log decrease (Soukos et al. 2003).

As was shown previously *in vitro* (Haas et al. 1997; Goharkhay et al. 1999; Sennhenn-Kircher et al. 2002; Kreisler et al. 2003), applying diode laser light, either 810 or 960 nm wavelength in a continuous mode was highly effective. The light of the diode laser with 1 W of power has only little thermal penetration, which obviates possible injury to oral tissue or damage to the titanium (Romanos et al. 2000; Kreisler et al. 2002b). One would, therefore, expect no risks from its clinical application (Goharkhay et al. 1999; Kreisler et al. 2002b; Romanos et al. 2004).

The study design imitated the conditions encountered clinically in the treatment of peri-implantitis. However, there are differences between the surface structure of the study objects and the implants requiring treatment in clinical practice. In general,

cocci predominate in biofilm formation, especially at the beginning as Leonhardt and others were able to demonstrate.

A threaded implant has a far larger surface area than that of the roughened titanium sleeves, and not all areas are accessible in the same intensity by laser irradiation due to the threads. On the other hand, irradiation of the study objects was impaired not only by poor visibility but also by the small inside diameter, and that it was not possible to apply the light to the surface at the optimal angle of 90°.

The results of this study prove diode laser light highly effective, as had already been demonstrated *in vitro*. However, the successful eradication of biofilms is much more difficult (Anwar et al. 1992; Costerton & Lewandowsky 1995), but following the results of this study, pathogens grown intraorally in biofilms are highly injured by the application of laser light.

Conclusions

The results of this study prove the investigated treatment modes effective for the reduction of aerobic bacteria on rough titanium surfaces although protected by accumulation in intraorally grown biofilms. Compared with the mean bacterial counts of untreated controls (0% reduction), laser irradiation treatment reduced the mean bacterial counts in the range 98.86%–99.98%. Diode laser irradiation has been proven an instrument for significant bacterial reduction even when microorganisms are consolidated in a ten days old biofilm. It remains to be determined whether this treatment is just as effective in the clinical treatment of peri-implant defects in inaccessible areas and in decontaminating the implant threads.

要旨

目的：ダイオード・レーザーの殺菌効果はインビトロの研究において証明されている。我々は、異なる波長のダイオード・レーザーを用いて、口腔内で増殖したバイオフィルム中の粗いチタン試料上でコロニー化した好気性細菌の減少を調べた。

材料と方法：ボランティア２２名が本研究に参加した。被験者は１０日間チタン・スリーブを取り付けた特製のプラスチック・スプリントを口腔内に装着した。スリーブの一部に異なる様式でダイオード・レーザーを照射した。他の部分は照射を行わず対照とした。照射直後にスリーブを綿棒でこすって細菌を採取し、まず顕微鏡で検査し、次に有酸素下で培養した。

結果：対照試料と処置を行った試料の細菌数を数えた。対照と比較して、照射したスリーブでは全て細菌のコロニー化が顕著に減少していた。２０秒の連続照射では、細菌数が８１０nm で９９．６７%、９８０nm で９９．５８%減少した。２０秒照射を５回反復すると、細菌数は８１０nm で９９．９８%、980nm で９９．３９%減少した。パルス波照射後に９８．８６%の減少が認められた。異なる分離菌をさらに分析することによって、連鎖球菌群は９９．２９～９９．９９%減少したが、ブドウ球菌群は９４．６７～９９．９９%と減少の度合いが少ないことが分かった。

結論：同結果の臨床的な意義として、本研究の全ての照射プログラムは、未処置試料の平均細菌数に比べて、口腔内の粗いチタン表面上のバイオフィルム中の平均細菌コロニーを９８%以上減少させた。実際の減少の度合いは細菌種及び照射の様式に依存していた。

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