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ORIGINAL ARTICLE

Effects of Er:YAG laser on bacteria associated with titanium surfaces and cellular response in vitro

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Abstract This in vitro study examined (a) the anti-bacterial efficacy of a pulsed erbium-doped yttrium aluminum garnet (Er:YAG) laser applied to *Streptococcus sanguinis* or *Porphyromonas gingivalis* adhered to either polished or microstructured titanium implant surfaces, (b) the response of osteoblast-like cells and (c) adhesion of oral bacteria to titanium surfaces after laser irradiation. Thereto, (a) bacteria adhered to titanium disks were irradiated with a pulsed Er:YAG laser (λ=2,940 nm) at two different power settings: a lower mode (12.74 J/cm^2 calculated energy density) and a higher mode (63.69 J/cm^2). (b) After laser irradiation with both settings of sterile titanium, disks were seeded with 10^4 MG-63 cells/cm^2. Adhesion and proliferation were determined after 1, 4, and 24 h by fluorescence microscopy and scanning electron microscopy. (c) Bacterial adhesion was also studied on irradiated (test) and non-irradiated (control) surfaces. Adhered *P. gingivalis* were effectively killed, even at the lower laser setting, independent of the material's surface. *S. sanguinis* cells adhered were effectively killed only at the higher setting of 63.69 J/cm^2. Laser irradiation of titanium surfaces had no significant effects on (b) adhesion or proliferation of osteoblast-like MG-63 cells or (c) adhesion of both oral bacterial species in comparison to untreated surfaces. An effective decontamination of polished and rough titanium implant surfaces with a Er:YAG laser could only be achieved with a fluence of 63.69 J/cm^2. Even though this setting may lead to certain surface alterations, no significant adverse effect on subsequent colonization and proliferation of MG-63 cells or increased bacterial adhesion was found in comparison to untreated control surfaces.

Keywords Titanium surfaces · Er:YAG laser · Decontamination · Oral bacteria · Osteoblast-like cells

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Introduction

Rehabilitation and reconstruction of missing or displaced teeth by means of dental implants or orthodontic treatment has become an essential part of state-of-the-art dentistry to improve life quality of patients [1, 2]. However, a serious complication and risk factor for the long-term success of any intraoral biomedical device is that of bacterial infection. The high affinity and adhesion of microorganisms to implant surfaces impede normal physiologic or mechanical cleaning by means of saliva or oral hygiene [3]. Subsequent bacterial colonization and biofilm formation can lead to a tremendous loss of dental hard and soft tissue structures [4, 5]. Following this, the onset of severe inflammatory reactions like gingivitis and periodontitis around teeth or mucositis and peri-implantitis around dental implants is often inevitable [6].

Periodontitis as well as peri-implantitis are diseases characterized by inflammation, swelling, and bleeding of a soft tissue lesion; the severity and extent of these diseases are influenced by the microbial composition of the individual's indigenous oral flora and periodontal pathogens [7]. In the pathogenesis of peri-implant disease bacterial adhesion and colonization are considered to play a key role. These processes are initiated by early colonizers, like *Streptococcus sanguinis*, whenever an implant surface is exposed to the

oral environment. Bacteria adhering to the implant usually grow in a biofilm which make them difficult to treat [8].

Although various different therapeutic options have been advocated for the treatment and prevention especially for peri-implantitis, until now, no generally approved treatment concept could be established [8]. The initial infection rate of dental implants is still reported to be 5–8 % [9]. Moreover, a recent consensus meeting has concluded that peri-implantitis occurs at 12–40 % of sites of implants which makes peri-implantitis a true threat for the long-term success of dental implants [10]. The use of antimicrobial agents like chlorhexidine or antibiotics does not provide an effective countermeasure to peri-implantitis particularly over the long term. A crucial factor is that bacteria in biofilms are less sensitive against many antimicrobials, which make these infections difficult to treat. Besides classical mechanical removal strategies of the biofilm or novel approaches like application of gaseous ozone, different laser irradiation regimes have been commonly proposed as a promising treatment option [11]. Several clinical and experimental studies could demonstrate the advantages summarized in [10, 12] of laser irradiation on decontamination of implant surfaces. Beneath comfortable clinical handling, especially pulsed Er:YAG lasers have gained a growing popularity due to scientific evidence provided by animal studies in a canine model [13–15]. However, recent own experiments with a pulsed Er:YAG laser revealed certain surface alterations of polished and microstructured titanium implant surfaces by using clinically accepted laser settings [16].

Respecting the 3R principle (reduce, replace, refine) of animal welfare it is consequently desirable to evaluate such basic laser applications in an in vitro system to draw any possible conclusions. To obtain persistent success, the laser irradiation should (1) eliminate bacteria effectively, (2) enhance re-osseointegration, and (3) impede bacterial re-colonization of the surface.

Therefore, it was the aim of the present in vitro study to analyze the biological and microbiological potential of a pulsed Er:YAG laser, on *S. sanguinis* and *Porphyromonas gingivalis* adhered to either a polished or a sandblasted, large-grit, acid-etched (SLA) titanium surface in an approved disk model. The stated H_0 hypothesis was that irradiated implant surfaces do neither impede the proliferation of osteoblast-like cells nor advocate adhesion of *S. sanguinis* and *P. gingivalis* in comparison to non-irradiated surfaces.

Materials and methods

Test specimens and laser system

Disks (5.0 mm in diameter) of titanium (commercial pure, ASTM grade II) either sandblasted, large grit, acid etched (SLA), or polished (POL) (Straumann AG Basel, Switzerland) were sterilized by gamma irradiation. For the bacterial experiments, a total of 108 disks was coated by exposure to a sterile saliva–serum mixture (10:1) during 15 min at 35 °C [17].

Treatment of bacteria, adhered to the materials' surface, was carried out with an OpusDuo laser system (OpusDuo ECTM, Lumenis GmbH, Dreieich, Germany) with a fiber-optic delivery system. The Er:YAG laser, with a wavelength of 2.94 μm, allows variations of pulse energy up to 1,000 mJ, a pulse frequency of 7 to 20 Hz, and pulse duration of 250 to 400 μs. A 1,000-μm fiber tip (HPX Conical Sapphire Contact Tips) was used for application. The pulse frequency of 10 Hz and pulse durations between 250 to 400 μs were chosen as recommended by the manufacturer.

All titanium specimens were laser irradiated by hand-guiding the application tip at a constant distance of 0.5 to 1 mm. According to the findings of a previous study [16], the following two laser settings were chosen:

1. 100 mJ, 10 Hz, 10 s, calculated energy density 12.74 J/cm^2, which causes no surface alterations and
2. 500 mJ, 10 Hz, 10 s, calculated energy density 63.69 J/cm^2, which may cause certain surface alterations.

Microbiological experiments

The two bacteria examined were *S. sanguinis* (DSM 20068), an early colonizer, and *P. gingivalis* (ATCC 33277), frequently associated with peri-implantitis. Microbiological procedures were as previously described [17–19]. Bacteria used were suspended in the 10:1 saliva–serum mixture at a concentration of 10^8–10^9 colony-forming unit CFU/mL, to simulate in vivo conditions.

To irradiate adhered bacteria, the coated disks were placed on the bottom of 24-well plates (Becton Dickinson, Basel, Switzerland) and exposed to the bacterial suspensions for 2 h at 35 ° C. The disks were then placed for 1 min on a pad saturated with 70 % ethanol to inactivate bacteria adhered to the bottom side of the disks. Bacteria on the other side were then irradiated as described. Afterwards, the disks were suspended in 3 mL 0.9 % NaCl, vortexed for 60 s and sonicated for 15 s (30 W, 20 kHz; VibracellTM, Ultrasonic Processor, Sonics, Newtown, USA). Viable bacteria were determined by culture on blood agar plates and anaerobic incubation. For one bacterial suspension, three disks were used, one for the untreated control, and two for the laser applications. Each material with either *S. sanguinis* or *P. gingivalis* was tested in five independent experiments.

Sterilized uncoated disks were treated with the Er:YAG laser as above, then coated with the saliva–serum mixture and exposed to the bacterial suspensions for 2 h. Each material with either *S. sanguinis* or *P. gingivalis* was tested in four independent experiments. Viable bacteria adhered were enumerated by culture. Minimal CFU detectable was ≥30 CFU per disk.

Cellular experiments

In vitro experiments were performed by incubation of human osteosarcoma cells (MG-63, ATCC) on each material surface. Cells were maintained as subconfluent monolayers in Minimal Essential Medium supplemented with 10 % fetal calf serum and 1 % penicillin–streptomycin (all Sigma-Aldrich, Buchs, Switzerland) as described by Hauser-Gerspach et al. [18]. Cells were used for experiments no later than passage 4 and seeded on disks placed in 96-well culture plates (Techno Plastic Products AG, Trasadingen, Switzerland) at a density of 10,000 cells/cm^2. Determination of cell morphology, attachment, spreading, and proliferation was performed after incubation for 1, 4, and 24 h. All experiments were repeated in duplicates with $n=5$ for an independent experiment.

For the evaluation whether the substrate type affected cell morphology, the specimens ($n=2$) were examined by scanning electron microscopy (Philips XL-30, Eindhoven, Netherland). Images were recorded at ×500 magnification.

Cell attachment and spreading was examined by immunocytochemical staining of fixed cells ($n=3$). Nuclei staining was performed using 4′,6 – diamidin-2phenylindol (DAPI), and actin cytoskeleton was stained with Alexa Fluor® 488 phalloidin (both Sigma-Aldrich). Each five images were recorded of all material samples at ×10 magnification using a fluorescent microscope (Nikon, 90i, Egg, Switzerland), whereby areas were chosen randomly at five regions in the center of the disks, to the right, left, bottom, and top of it. Calculation of the area of attached cells as well as counts of nuclei was performed using the Visiopharm software Version 7 (Hoersholm, Denmark).

One-way ANOVA was used to determine whether there was a statistically significant difference between titanium SLA and polished surface. P values of <0.05 were considered to be statistically significant.

Results

Decontamination efficiency of Er:YAG laser

Overall, the Er:YAG laser had a superior decontaminating effect on sandblasted, large-grit, acid-etched (Ti-SLA) surfaces to polished (Ti-POL) titanium surfaces which had been seeded with *S. sanguinis* (Table 1). At the higher power setting of 63.69 J/cm^2, laser irradiation killed *S. sanguinis* adhered to Ti-SLA disks to below detection limit (>4 logs reduction) while bacteria adhered to polished surfaces (Ti-POL) were incompletely eliminated. Similarly, at the lower setting of 12.74 J/cm^2, laser irradiation caused more efficient killing of *S. sanguinis* on Ti-SLA (1 % survivors) than on Ti-POL surfaces (about 10 % survivors).

Table 1 Bactericidal effects of Er:YAG laser on bacteria adhered to titanium surfaces. Log reduction of adhered viable bacterial cells in relation to surface alterations observed after laser irradiation

Er:YAG laser parameters	Ti-SLA		Ti-POL	
	S. sanguinis	*P. gingivalis*	*S. sanguinis*	*P. gingivalis*
12.74 J/cm^2	2.0 logs[a]	≥3.5 logs[a]	0.9 logs[a]	≥3.6 logs[a]
63.59 J/cm^2	≥4.3 logs[b]	≥3.5 logs[b]	2.6 logs[b]	≥3.6 logs[b]

Mean and S.D. are given ($n=5$)

Surface alterations as reported by Stübinger et al. [16] are indicated by superscripted letters

Ti-SLA titanium sandblasted, large grit, acid-etched; *Ti-POL* titanium polished

[a] No visible alteration

[b] Surface alterations detected

On both titanium surfaces, Er:YAG laser irradiation of energy density 63.69 J/cm^2 was more strongly bactericidal to *S. sanguinis* than at the setting of 12.74 J/cm^2. Adhered *P. gingivalis* cells were very sensitive to laser applications: The bacteria were inactivated to below detection limit on either titanium surface by either laser setting (Table 1).

Cell spreading and proliferation on surfaces irradiated before

The MG-63 cells seeded on the Ti-SLA and Ti-POL surfaces showed a spherical morphology after 1 h, became gradually flattened after 4 h and were uniformly flattened after 24 h (Figs. 1 and 2). Cell spreading was quantified as percentage covered (Fig. 3a, b). The increase over 24 h was similar on both titanium surfaces without a significant effect of laser irradiations.

Proliferation of MG-63 cells as measured by average nuclei count was slightly higher on Ti-SLA surface and was not affected by the laser treatment in a significant way (Fig. 3c, d).

Attachment of bacteria to surfaces irradiated before

The other question addressed was whether Er:YAG laser irradiation would affect subsequent adherence of bacteria. Interestingly, adhesion of both bacterial species *S. sanguinis* and *P. gingivalis* to both titanium Ti-SLA and Ti-POL was statistically not significantly changed after irradiation of the surface in comparison to the untreated control (Table 2).

Discussion

Treatment of peri-implantitis aims at eliminating or substantially reducing the bacterial load on the implant in order to

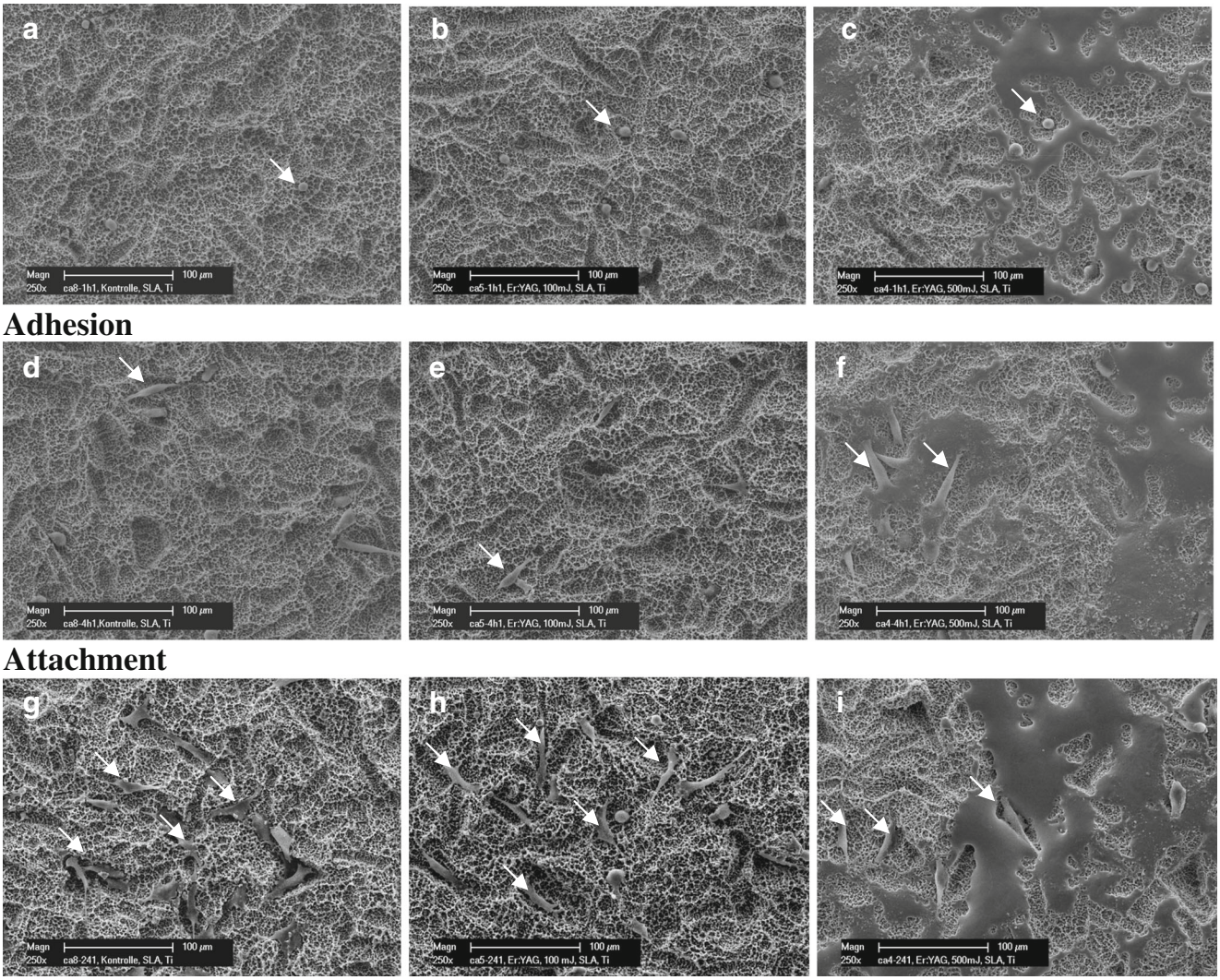

Fig. 1 Representative SEM imagines of osteoblastic cells (*arrows*) seeded on Ti-SLA (**a**, **d**, **g**, untreated; **b**, **e**, **h**, after laser treatment Er:YAG-1, 12.74 J/cm^2; **c**, **f**, **i**, Er:YAG-2, 63.69 J/cm^2) and incubated for 1 h (**a**–**c**), demonstrating the initial cell adhesion; 4 h (d–f), demonstrating cell attachment; 24 h (**g**–**i**), demonstrating cell growth

arrest disease progression. Therefore the first aim of this in vitro study was to investigate the bactericidal potential of Er:YAG laser on bacteria adhered to titanium surfaces. Indeed, differences in the anti-bacterial activity were detected between the two laser settings using *S. sanguinis*. Thus, for maximal decontamination the higher laser dose was required. However, this dose may lead to alterations of the surface [16].

The results also showed that not all bacterial species are eliminated with equal efficiency: Attached *P. gingivalis* was more sensitive to the Er:YAG laser tested than *S. sanguinis*, as there were marked differences at the lower laser settings.

The decontaminating potential of lasers has been explored in vitro using a range of different titanium implant or other surfaces, bacterial or fungal species, and laser systems [19–28]. Investigations using Er:YAG laser irradiation regimes are listed in Table 3. The data illustrate the enormous heterogeneity of the studies. The energy densities applied varied in a wide range of 0.04 to 63.69 J/cm^2 in the different studies. Therefore, any direct comparisons of results are hardly possible. Some of the surfaces materials irradiated with the higher doses underwent structural changes. The bacteria used represented different segments of the oral microbiota (Gram positive or negative, aerobic, microaerophilic, or anaerobic) and even bacteria of the same species could be of different strains (type strains from collections vs recent clinical isolates). Among the support/implant materials tested, titanium dominated, including different surface qualities, but glass and hydroxyapatite were also used. Some surfaces were conditioned with serum or saliva before microbial adhesion which was allowed to proceed for one to several hours. Alternatively,

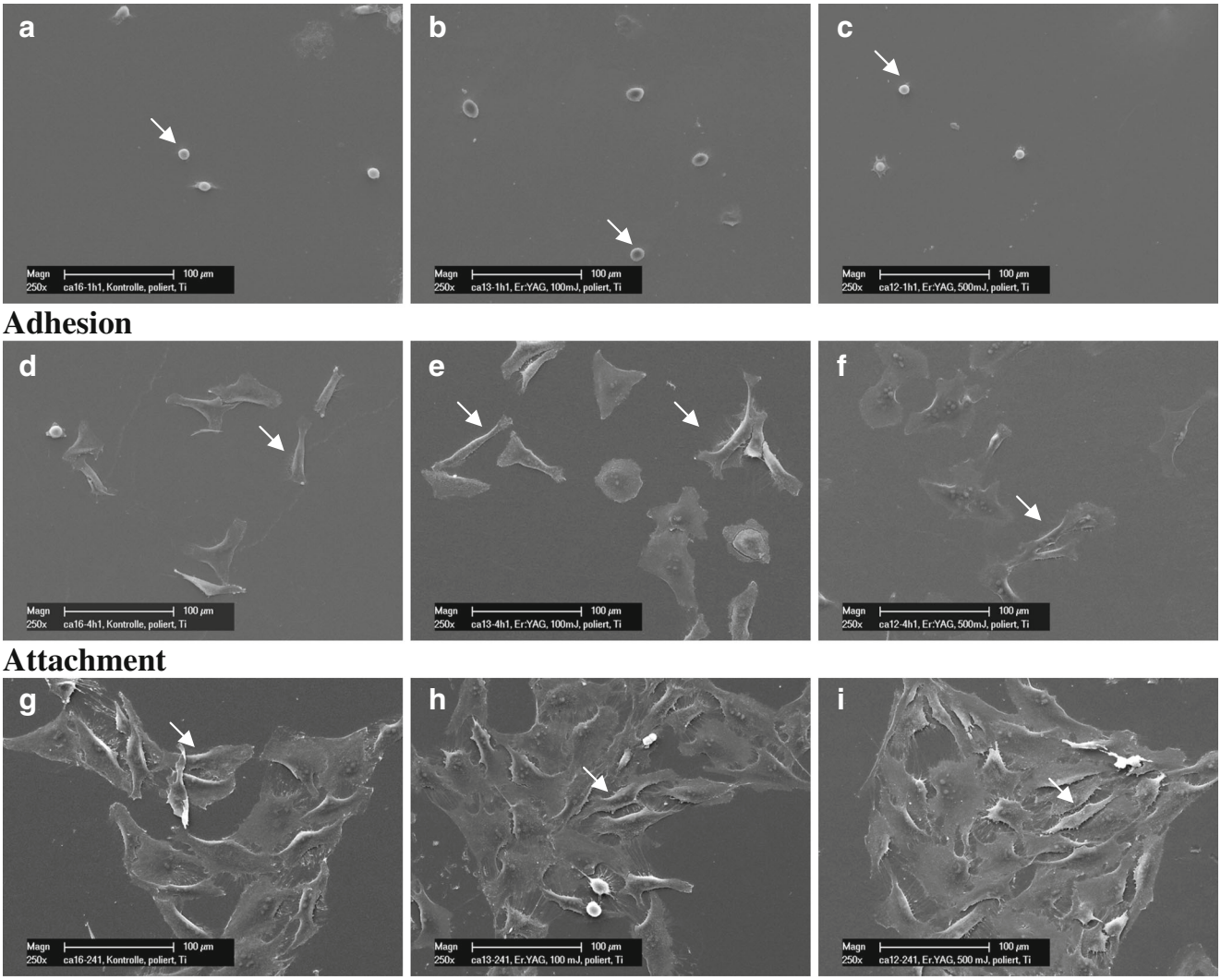

Fig. 2 Representative SEM imagines of osteoblastic cells (*arrows*) cultured on Ti-POL (**a**, **d**, **g**, untreated; **b**, **e**, **h**, after laser treatment Er:YAG-1, 12.74 J/cm^2; **c**,**f**,**i**, Er:YAG-2, 63.69 J/cm^2) and incubated for 1 h (**a**–**c**), demonstrating the initial cell adhesion; 4 h (**d**–**f**), demonstrating cell attachment; 24 h (**g**–**i**), demonstrating cell growth

incubation continued to yield a biofilm. Analyses of the laser effects involved light or electron microscopy (measuring dead and live cells), cultural techniques (measuring CFU) or enzyme assays (measuring total cell metabolic state). The results are difficult to compare because of the very diverse parameters, even though *P. gingivalis* and *S. sanguinis* are often among the bacteria tested. From these studies, it is difficult to draw general conclusions with respect to predictable clinical Er:YAG laser applications.

Another important point is the evaluation of the measurements documenting the antimicrobial efficacy of the laser system. Reduction of bacterial counts by laser irradiation in the order of 90 to 99 % (1 to 2 logs) are most likely statistically significant (Table 3). However, the relevant biological question remains unanswered: is a short-term drastic bacterial reduction (of e.g. 2 logs *P. gingivalis*) in the peri-implant region achieved by low laser energy sufficient for stable clinical improvements. A threshold level of *P. gingivalis* or any other peri-implant bacteria to obtain predictable clinical success is not known [29]. In periodontitis therapy, a statistically significant reduction of periodontopathogenic bacteria does not guarantee clinical success because reinfection from other oral sites may occur or bacteria may survive intracellularly at the treated site [30].

From previous studies, it is known that laser irradiation of titanium surfaces may lead to detectable alterations [16, 31–33]. Even after application of these higher laser dose conditions, neither initial cell adhesion nor spreading and proliferation of osteoblast-like MG-63 cells was altered on rough (Ti-SLA) and smooth (Ti-POL) surfaces. A slight

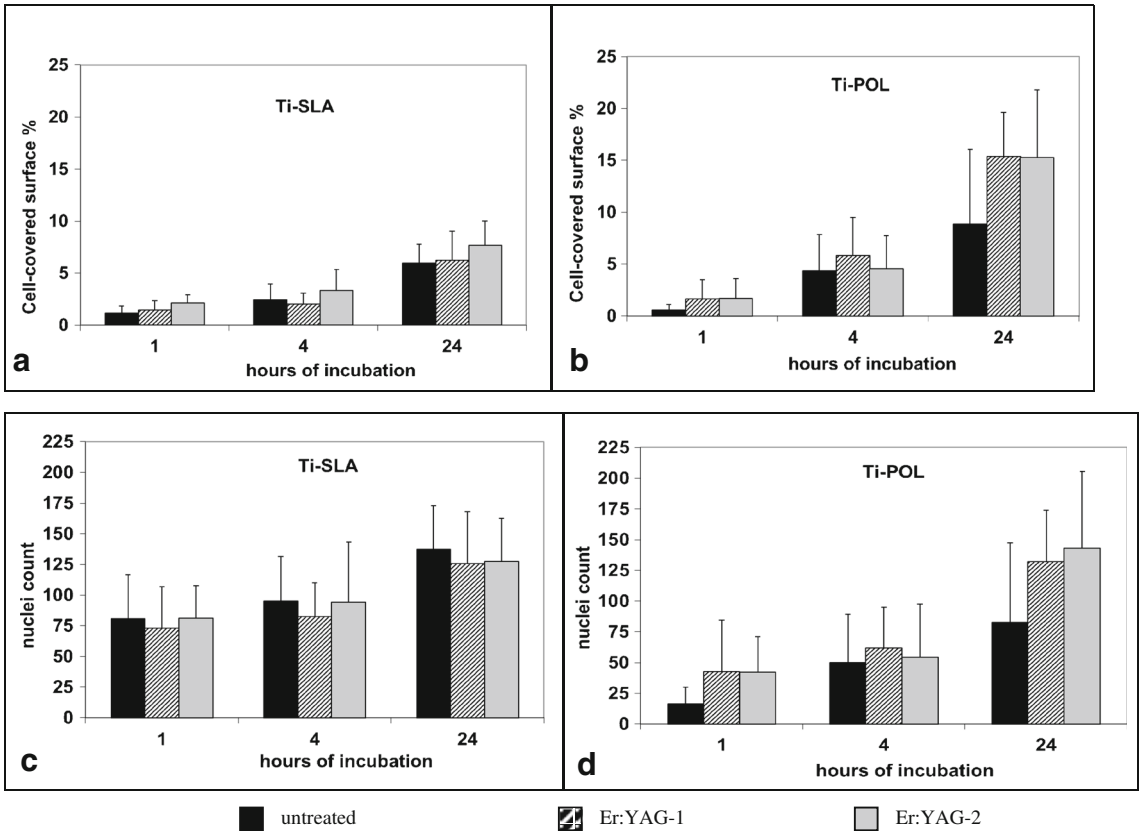

Fig. 3 Quantification of cell spreading per surface by actin staining. MG-63 cells cultured for 1, 4, and 24 h on different surfaces. **a** Ti-SLA and **b** Ti-POL without and after laser treatment. Quantification of nuclei per image area. MG-63 cultured for 1, 4, and 24 h on different surfaces. **c** Ti-SLA and **d** Ti-POL without and after laser treatment. Er:YAG-1, 12.74 J/cm^2; Er:YAG-2, 63.69 J/cm^2

decrease of initial cell adhesion could be assumed due to the surface energy alteration of untreated versus laser treated surfaces. However, a significant effect on MG-63 cells has not been determined. Similar results have also been reported in human osteoblastic Saos-2 cells on machined and sandblasted acid-etched surfaces following Er:YAG laser treatment observing no differences in cell proliferation after 24 h. This study also did not demonstrate any effect on the cell differentiation [34].

Similarly, the altered surfaces did not interfere with subsequent bacterial adhesion. No inhibition effect which would be desirable and no positive stimulation were observed. To date, there are only few studies evaluating the effects on bacterial adhesion on surfaces after Er:YAG laser treatment. Also Duarte et al. [31] did not find higher levels of *S. sanguinis* adhesion on titanium surfaces in vitro after Er:YAG laser treatment at 8.4 J/cm^2.

In the present in vitro study, effective decontamination of polished and rough titanium implant surfaces with a pulsed Er:YAG laser could be only achieved with an energy density of 63.69 J/cm^2. Even though these settings may lead to certain surface alterations, no significant adverse effect on subsequent colonization and proliferation of MG-63 cells or increased bacterial adhesion was found in comparison to

Table 2 Adhesion of *S. sanguinis* or *P. gingivalis* on titanium surfaces after laser irradiation of the surfaces (log)

	Control	Er:YAG laser parameters	
		12.74 J/cm^2	63.69 J/cm^2
Ti-SLA			
S. sanguinis log CFU per disk	5.9±0.07	5.8±0.14	5.8±0.12
P. gingivalis log CFU per disk	5.6±0.19	5.4±0.15	5.5±0.21
Ti-POL			
S. sanguinis log CFU per disk	5.1±0.20	5.1±0.22	5.2±0.24
P. gingivalis log CFU per disk	4.9±0.07	4.9±0.08	4.9±0.07

Mean and SD are given (n=4)

Ti-SLA titanium sandblasted, large grit, acid-etched; *Ti-POL* titanium polished

Table 3 Antimicrobial activities of Er:YAG laser application in vitro

Authors	Er:YAG laser	Bacteria/fungi			Results	Comment
	Calculated energy density	Species	Support surface	Measurements	Reduction in viability	
Ando et al. [24]	Single pulse at 0.04–10.6 J/cm^2	*P. gingivalis*, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*	Nutrient agar plates	Growth inhibition zones; survival in laser-irradiated colonies	Growth inhibition zones at ≥0.3 Jcm^{-2} for *P. gingivalis* + *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* 17 % survivors *P. gingivalis* at 10.6 J/cm^2	
Kreisler et al. [21]	26 or 52.2 J/cm^2 pulse	*S. sanguinis*	Bacteria in PBS adhered 1 h, 37° to titanium disks (3 surfaces)	CFU by conventional culture technique (after sonication)	1.8–2.4 logs at 26 J/cm^2 2.8–3.2 logs at 52.2-J/cm^2 values dependent on surface	Incomplete bacterial killing
Noiri et al. [25]	0.38 or 0.71 or 0.98 J/cm^2 pulse	4 aerobic G^+ and 3 anaerobic G^- species[a]	7-day aerobic, 14-day anaerobic monospecies biofilm on hydroxyapatite disks	CFU by conventional culture technique (after scraping off+sonication)	G^+: 0–3 logs at 0.71 J/cm^2; 0–4 logs at 0.98 J/cm^2 G^-: >3 logs at 0.71 and 0.98 J/cm^2	Viability species-dependent *Lactobacillus casei* most resistant
Quaranta et al. [27]	10.7 J/cm^2 pulse	*P. gingivalis*	48-h anaerobic incubation on different titanium implants	Counting bacterial cells by SEM	76.2–98.3 % decontamination values dependent on surface	Live or dead bacteria?
Sennhenn-Kirchner et al. [26]	12.0 or 15.2 J/cm^2 pulse	*Candida albicans* (clinical isolates)	5-day biofilm on glass and titanium disks in BHI	Colorimetric assay to determine mitochondrial dehydrogenase activity (metabolic state)	Significantly reduced enzyme activity	
Schwarz et al. [28]	12.7 J/cm^2		24-h ex vivo biofilm on titanium disks	Residual plaque area measured on microscopic images	97 % clean implant surface	
Hauser-Gerspach et al. this study	12.74 J/cm^2 or 63.69 J/cm^2 pulse	*S. sanguinis* *P. gingivalis*	Bacteria in human saliva/serum adhered 2 h, 37° to titanium disks (2 surfaces)	CFU by conventional culture technique (after sonication)	*S. sanguinis*: 0.9–2.0 logs at 12.74 J/cm^2 *P. gingivalis*: ≥ 3.5 logs at 12.74 J/cm^2 *S. sanguinis*: 2.6–≥4.3 logs at 63.69 J/cm^2 J/cm^2 *P. gingivalis*: ≥3.5 logs at 63.69 J/cm^2 Values dependent on surface	

[a] Gram positive: *Actinomyces naeslundii*, *Enterococcus faecalis*, *L. casei*, *Proprionibacterium acnes*; Gram negative: *Fusobacterium nucleatum*, *P. gingivalis*, *Prevotella nigrescens*

untreated control surfaces. Further detailed analyses of biomolecular and microbiological interactions and processes on laser-irradiated implant surfaces are necessary to draw any further conclusions on re-osseointegration and biofilm formation of dental titanium implants in vivo.

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