ORIGINAL

Machined titanium disc decontamination using photodynamic therapy: an *in vitro* study

Descontaminação de discos de titânio com superfície maquinada por meio de terapia fotodinâmica: *estudo* in vitro

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ABSTRACT

Objective

This study investigated less invasive protocols that do not injure peri-implant tissues during implant surface decontamination and evaluated, *in vitro,* the efficacy of microbiological decontamination of machined surface titanium discs with photodynamic therapy.

Methods

Forty eight titanium disc contaminated with 10µL of a *Streptococcus sanguinis* suspension were randomly divided into groups: 1) titanium disc contaminated with Ss (titanium disc suspension) without treatment; 2) titanium disc suspension rinsed with saline solution; 3) titanium disc suspension rinsed with 0.2% chlorhexidine digluconate; 4) titanium disc suspension treated with Photosensitizer Methilene Blue; 5) titanium disc suspension treated with Photosensitizer Methilene Blue associated with laser diode; 6) titanium disc suspension treated with diode laser. After the treatments, the titanium disc were submersed in 3mL of sterile brain-heart

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infusion broth under aerobic conditions at 37ºC for 48 hours. Three petri plates were seeded per sample and maintained under aerobic conditions at 37ºC for 48 hours, after which the number of colony forming units per milliliter was counted.

Results

The Kruskal-Wallis test complemented by Dunn test showed that chlorhexidine digluconate eliminated titanium disc contamination ($p<0.05$). All the other groups (2, 4, 5, 6) had fewer colony-forming units than group 1 ($p<0.05$).

Conclusion

Within the limitations of this study, Photodynamic Therapy reduced titanium disc contamination but was not better than 0.2 % chlorhexidine digluconate rinsing.

Indexing terms: Chlorhexidine. Implant. Laser. Titanium.

RESUMO

Objetivo

Protocolos menos invasivos que não alterem superfície de implante durante a descontaminação têm sido pesquisados. Este estudo avaliou microbiologicamente a eficácia da descontaminação da superfície maquinada de discos de titânio após aplicação de terapia fotodinâmica.

Métodos

Quarenta e oito discos de titânio foram contaminados com 10µL da suspensão de células de Streptococcus sanguinis, e divididos em grupos da seguinte forma: 1) sem tratamento; 2) irrigados com soro fisiológico; 3) irrigados com digluconato de clorexidina a 0,2%; 4) tratados com fotossensibilizador azul de metileno; 5) tratados com terapia fotodinâmica; 6) tratados com laser díodo. Após os tratamentos, mantiveram-se os discos de titânio em 3mL de solução estéril Infusão de coração e cérebro em aerobiose a 37ºC por 48 horas. Semearam-se placas de Petri em triplicata para cada amostra, mantidas em aerobiose a 37ºC por 48 horas para contagem das unidades formadoras de colônia/mL.

Resultados

O teste de Kruskal-Wallis complementado pelo teste de Dunn demonstrou a eliminação da contaminação no grupo 3 (p<0,05), enquanto os grupos 2, 4, 5, 6 reduziram a contaminação quando comparados ao grupo 1 (p<0,05).

Conclusão

Nos limites deste estudo, a terapia fotodinâmica reduziu a contaminação dos discos de titânio, sem apresentar vantagens à irrigação com digluconato de clorexidina a 0,2%.

Termos de indexação: *Implante. Titânio. Laser. Clorexidina.*

INTRODUCTION

The significant increase in the number of dental implants led to many complications, such as peri-implantitis, which may be the main cause of implant failure. Around 14.14% of the dental implants done during a period of 5 years demonstrated peri-implant inflammations associated with bone loss[1].

Non-surgical treatments for peri-implatitis commonly relied on mechanical instrumentation, even though many studies had shown that this practice increases the roughness of the implant surface, which favors bacterial adhesion[2] and hinders decontamination of the implant[3], delaying or preventing the recovery of peri-implant tissues.

Many antimicrobial agents have been studied. Among them, 0.2% chlorhexidine digluconate has proven effective for decontaminating the implant surface, depending on the characteristics of bacterial growth[4,5]. However, resistent bacterial strains have led scientists to look for alternative descontamination methods[6].

Photodynamic Therapy (PDT) uses visible light and a photosensitizing agent[7] that, when photoactivated by a laser of specific wavelength[8], produces reactive oxygen species, singlet or triplet, and hydrogen peroxide, which then destroy the cellular components such as organelles, proteins and nucleic acids, killing the cell[9].

A clinical study using PDT in patients with periodontitis found that it reduces the number of pathogenic bacteria in periodontal pockets[10]. The bacterial biofilm associated with peri-implantitis is similar to that of periodontitis. Therefore, PDT could be an alternative solution for the decontamination of implant surfaces because it does not damage the surface within a certain wavelength range and has antibacterial effect[8].

This study tested *in vitro* a less invasive, harmless protocol for decontaminating implant surfaces and assessed the efficacy of PDT in reducing the contamination on machined Titanium Discs (TD).

METHODS

Titanium discs

Forty-eight machined pure-titanium discs with a width of 1 millimeter and diameter of 8 millimeters were fabricated (*Conexão, São Paulo, SP*, Brazil). The surface roughness of each disc was measured three times by a profilometer (Handsurf Modelo-E 35A, Seimitso Tokyo, Japan). The mean roughness of the machined titanium discs was 0.33µm.

The TD were then wrapped and autoclaved (Vitale 12L, Cristófoli Equipamentos de Biossegurança Ltda, *Campo Mourão*, PR, Brazil).

Bacterial culture and disc inoculation

The standard strain of *Streptococcus sanguinis* (IAL 1832) (Ss) purchased from Instituto Adolfo Lutz (*São Paulo*, SP, Brazil) was used for inoculating the TD. The bacterium was cultivated in petri dishes containing the culture medium Columbia Blood Agar (Laborclin, *Pinhais*, PR, Brazil) and incubated under aerobic conditions at 37ºC (culture incubator 502 - Oriom - Fanem, *São Paulo*, SP, Brazil) for 48 hours.

A 0.5 McFarland standard was prepared for disc inoculation (Tubidometer, Oxoid, Hampshire, United Kingdom). The final concentration was 1.5 X 10^8 cells per millimeter of sterile saline. Before inoculation, the cells in the solution were dispersed with a test tube shaker (AP56 Phoenix, *Araraquara*, SP, Brazil).

TD were contaminated by 10µL of saline containing 1.5 X 10^8 cells per millimeter which was placed at the center of the disc and spread by an automatic micropipette (Research, Eppendorf, *São Paulo*, SP, Brazil). After contamination, the TD were kept under aerobic conditions at 37ºC for 1 hour[11] to encourage bacterial growth.

Treatments

After contamination, the TD were randomly divided into groups, totalling 8 TD per group. The groups received the following treatments:

- Group 1: (negative control): contamination of TD with Ss;

- Group 2: TD contaminated with Ss and rinsed with 10mL of sterile saline (*Laboratório Tayuyna Ltda, Nova Odessa*, SP, Brazil) using an automatic pipette (Easypet, Eppendorf, *São Paulo*, SP, Brazil). Saline rinsing was done to determine its mechanical effect on TD contamination, if any, which would then serve as baseline for the 0.2% chlorhexidine digluconate rinsing.

- Group 3: (positive control): TD contaminated with Ss and rinsed with 10mL of a 0.2% chlorhexidine

Table 1. Protocol used for the semiconductor laser diode treatment of groups 5 and 6.

Wavelength (nm)	Energy density (J/cm^2)	Energy (J)	Power (W)	Irradiance (W/cm^2)	Time (sec)
660	14.4	7.2	0.04	0.08	180

digluconate solution (*Bioativa Farmácia de Manipulação, Araras*, SP, Brazil) using an automatic pipette;

- Group 4: TD contaminated with Ss and treated with an aqueous solution of 0.005% methylene blue photosensitizer (Chimiolux, Aptivalux, *Belo Horizonte*, MG, Brazil). A total of 3mL of photosensitizer were used. Two milliliters in a disposable plastic syringe were used to rinse the TD (BD Plastipak, Becton Dickinson Ind. Cirur. Ltda, *Curitiba*, PR, Brazil), and the TD were then submersed in 1ml of photosensitizer in a test tube for 5 minutes;

- Group 5: TD contaminated with Ss and treated with an aqueous solution of 0.005% methylene blue photosensitizer followed by irradiation with an InGaAlP semiconductor laser diode (Twin Laser, MMOptics, *São Carlos*, SP, Brazil). A total of 3mL of photosensitizer were used. Two milliliters in a disposable plastic syringe were used to rinse the TD and the discs were then submersed in 1mL of photosensitizer in a test tube for 5 minutes followed by irradiation by the semiconductor laser diode;

- Group 6: TD contaminated with Ss and treated only by irradiation with an InGaAlP semiconductor laser diode.

Irradiation of groups 5 and 6 were done as follows: the tip of the device was placed at the center of each disc, 5 millimeters away from its surface, with a circular motion along all its extension, scanning for 3 minutes. The light beam was perpendicular to the disc. The irradiation protocol is described in Table 1.

After the treatments, the TD were kept in 3mL of sterile brain-heart broth in a test tube, under aerobic conditions, at 37 ºC for 48 hours. Petri dishes containing the Columbia Blood Agar medium were seeded in triplicate for each disc and kept under aerobic conditions at 37ºC for 48 hours. The number of Colony Forming Units per milliliter (CFU/mL) was then counted. Scores were given to each group according to their number of CFU/mL, as shown in Table 2.

The entire experiment was done in asseptic conditions in a laminar flow cabinet.

Statistical analysis

The data were treated by descriptive analysis and the non-parametric Kruskal-Wallis and Dunn tests using the statistics software GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The significance level was set to 5%.

RESULTS

The scores obtained by each group regarding the number of colony forming units *per* milliliter were compared and tabulated (Table 3).

All TD groups were less contaminated than Group 1. There was no bacterial growth in Group 3, characterizing effective decontamination when compared with the other groups ($p<0.05$). Group 2 was significantly less contaminated than Group 6 ($p<0.05$).

Table 2. Scores used for bacterial count (CFU/mL).

Score	Number of CFU/ml
0	Absence of colonies
1	1 to 10
2	11 to 50
3	51 to 100
4	101 to 300
5	>300

CFU/mL: Colony Forming Units *per* milliliter.

Table 3. CFU/mL scores, descriptive analysis and Dunn's statistical test.

Groups	Scores of CFU/mL			Mean values	Standard deviations	Dunn*
	Median	Minimum	Maximum			
1	5	4	5	4.81	0.40	a
2	2	0	4	2.06	1.06	b
3	0	0	0	0	0	c
4	2	0	5	1.78	1.87	bd
5	3.5	0	5	2.68	2.05	bd
6	4	0	5	3.31	1.88	d

*Different low case letters show statistical significance between treated and untreated groups ($p<0.05$).

CFU/mL: Colony Forming Units *per* milliliter.

DISCUSSION

Contamination of the implant surface has the greatest impact on implant outcome; therefore, implantology studies have searched for less invasive decontamination protocols. This *in vitro* study was conducted to compare the effectiveness of machined TD decontamination by PDT and rinsing agents since there is a scarcity of studies that cover and compare these treatment protocols.

In this experiment, the planktonic form of the bacterial species Ss was chosen for TD contamination because this was one of the first microorganisms to colonize enamel and titanium surfaces in the oral cavity and provide conditions for the coaggregation of pathogenic bacteria, such as *Porphyromonas gingivalis*[12]. However, the present study used a different contamination time than other similar studies. Silva *et al.*[3] used a contamination time of 7 days, while Burgers *et al.*[12] used a contamination time of 2 hours. As Kreisler *et al.*[11], the present study also used a TD contamination time of 1 hour. According to the results of group 1 (not treated), this time was enough to ensure bacterial contamination.

The contamination time is also related with the bacterial, planktonic or biofilm growth, as well as biofilm age, whose characteristics affect microbial adhesion to the surface and their metabolism, which determines treatment impact[13]. According to Dobson & Wilson[14], the biofilm takes 3 to 4 days of incubation to form. In this study, Ss can be characterized by one planktonic growth phase on the surface of the TD after an incubation time of 1 hour.

Previous studies showed that PDT successfully eliminated the planktonic form of this microorganism[7,15] by producing singlet and triplet oxygen species or other molecules that destroy cellular components and cause cell death[9,16]. These reactions use the oxygen present in the medium or a photosensitizer compound in aqueous solution[17], so these procedures can be done *in vitro*. In the present study, an aqueous photosensitizer solution was used to treat groups 4 and 5.

Many microorganisms cannot absorb visible light, so a photosensitizer is necessary for the laser to penetrate the bacterial cell[14]. In this study, Group 6, which received only laser treatment, obtained one of the worst microbial decontamination score. This could be due to the light not penetrating the microorganism because of the absence of a photosensitizer and consequently not promoting the production of reactive oxygen species[17].

Methylene blue is a photosensitizer that modestly helps to destroy bacterial cell DNA and membrane. Exposure time to the photosensitizer can affect the location of the substance inside the cell and thereby affect photolytic effectiveness[15,18]. The contact time used in this study of 5 minutes and methylene blue characteristics may have contributed to the reduced bacterial contamination of group 4, coinciding with the results of other studies that used only a photosensitizer[14,15].

The degree of photolysis depends on the photosensitizer, concentration, bacterial species, and fluency and intensity of the laser[18]. The PDT used on group 5 reduced, but did not eliminate, bacterial contamination, differently from Bevilacqua *et al.*[15] who managed to eliminate bacterial contamination using PDT. *In vitro* PDT efficacy depends on the dosage used and bactericide action increases with increasing energy dosages[16].

Another hypothesis for the reduction, but not elimination, of the bacterial contamination present in groups 5 and 6 is the interaction between the laser and the metal surface. This interaction is determined by energy flow, degree of absorption, thermal conductivity and material composition. The reflective characteristics and absorption coefficient of each metal are similar, and depend on laser wavelength. The ability of titanium of reflecting light close to the infrared region varies between 50% and 60%, but increases to 96% when the wavelength reaches 10^3nm[11].

The 660nm laser used on the TD of groups 5 and 6 may have compromised its effectiveness because of reflection from the metal surface, which reduced but did not eliminate bacterial contamination.

According to Romanos *et al.*[8], some advantages of semiconductor laser diode are PDT, bactericidal action, and inability to affect the temperature of the implant and surrounding tissues. However, Street *et al.*[16] reported that a dosage of 9.4J/cm^2 increased the implant temperature by 3°C. In this study, the energy dosage was 14.4J/cm^2, so temperature changes should be investigated by future studies that use the same methodology.

According to the literature, a low to moderate-potency semiconductor laser diode with a wavelength <810nm will not modify the titanium surface, which is an advantage since increased surface roughness would facilitate further accumulation of plaque[8].

In vitro and *in vivo* bacterial adhesion on texturized titanium surfaces were primarily influenced by surface roughness, and less so by free surface energy[12]. Machined-titanium roughnesses of 0.15μm[12] and 0.17μm[3] had less Ss accumulation. According to Wennerberg *et al.*[19], machined titanium surfaces with a mean roughness ≤0.96mm would be satisfactory.

In this study, the machined TD roughness of 0.33μm and the short time given for planktonic bacterial growth could have hindered bacterial adhesion and facilitated mechanical TD decontamination by rinsing with 10mL of saline, the treatment given to Group 2, which is in agreement with Cousido *et al.*[20].

Of the antimicrobial rinsing compounds, chlorhexidine digluconate is preferred because of its efficacy, which has been demonstrated *in vitro* and *in vivo*[5,20]. In this study, rinsing with 10mL of 0.2% chlorhexidine digluconate completely eliminated the contamination of the TD of Group 3. This was the most effective treatment, confirming the results of Kreisler *et al.*[11] who compared laser diode irradiation and chlorhexidine digluconate for the decontamination of titanium surfaces.

The efficacy of a 0.2% chlorhexidine digluconate solution can be explained by its substantivity. Cousido *et al.*[20] found that rinsing a surface with 10mL of this solution for 30 seconds *in vivo* inhibited bacterial growth for 7 hours.

Other *in vitro* studies are needed to assess the effectiveness of these treatments, investigating other TD surface roughnesses, bacterial species and growth times for TD contamination.

Considering the methodology used in this *in vitro* study and its limitations, all treatments were capable of reducing the bacterial contamination of machined TD. PDT did not prove superior to a 0.2% chlorhexidine digluconate rinse. Chlorhexidine digluconate rinse was the most effective treatment because it completely eliminated the contamination placed on the machined titanium discs.

ACKNOWLEDGMENTS

The authors thank Prof. Dr. Ivana Barbosa Suffredini for her help in the laboratory.

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Recebido em: 1/8/2011
Aprovado em: 30/8/2011