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Supervisors:
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Associate Professor Ana Budimir MD, PhD

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Sveučilište u Zagrebu
STOMATOLOŠKI FAKULTET

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PROCJENA ANTIMIKROBNE UČINKOVITOSTI RAZLIČITIH VRSTA FOTODINAMSKE TERAPIJE NA UZROČNIKE PERIIMPLANTITISA *IN VITRO*

DOKTORSKI RAD

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I dedicate this thesis to my parents.

SUMMARY

The aim of this study was to evaluate the efficacy of antimicrobial photodynamic therapy (aPDT) against a bacterial suspension prepared from three different bacterial species on titanium and zirconia dental implants, and to analyze the possible alterations of the implant surfaces as a result of aPDT.

The study was conducted on 72 titanium dental implants and 72 zirconia dental implants contaminated with a bacterial suspension prepared from three bacterial species. The contaminated implants were randomly divided into four experimental groups and two control groups (n=12 each), according to the following treatment protocols: Group 1 (PDT1): PDT (660 nm, 100 mW, 60 seconds) with toluidine blue; Group 2 (PDT2): PDT (660 nm, 100 mW, 60 seconds) with phenothiazine chloride dye; Group 3 (PDT3): light emitting diode (LED) with toluidine blue; Group 4 (TB): treatment with only toluidine blue for 60 seconds. In the positive control (PC) group, the implants were treated with a 0.2% chlorhexidine-based solution for 60 seconds, and in the negative control (NC) group no treatment was used.

In the titanium implants, the highest bacterial reduction was recorded in the PDT1 (98.3%) and PDT2 (97.8%) groups. Results of this study showed that there was a statistically significant reduction of bacteria in the PDT1 and PDT2 groups compared to the NC group (<0.05), individually for each bacterial species, as well as for all three species together. PDT3 was less effective than PDT1 and PDT2, and did not show a statistically significant difference compared with NC or any other treatment group. TB was the least effective treatment in terms of both the total bacterial count and the individual count for each bacterial species.

In the zirconia implants all the study groups had significantly lower bacterial counts (>99.9%) when compared with NC for the total bacterial count and each bacterial species separately. Among them the highest bacterial reduction was recorded in PDT1, PDT2 and PDT3, and all of them were also significantly different from TB.

PDT1 and PDT2 protocols showed high efficacy against a three-day old bacterial biofilm on titanium dental implants, while PDT1, PDT2 and PDT3 showed high efficacy on zirconia implants.

Keywords: Photodynamic therapy, titanium implants, zirconia implants, antimicrobial efficacy, implant surface, laser, LED

PROŠIRENI SAŽETAK

Svrha rada

Cilj ove studije bio je procijeniti učinkovitost antimikrobne fotodinamske terapije (aPDT) u trodnevnoj bakterijskoj suspenziji pripremljenoj od tri različite bakterijske vrste primijenjene na površinu titanskih i cirkonskih dentalnih implantata, te analizirati moguće promjene površina implantata kao rezultat djelovanja aPDT-a.

Materijali i postupci

Istraživanje je provedeno na 72 titanska dentalna implantata i 72 cirkonska dentalna implantata kontaminirana bakterijskom suspenzijom pripremljenom od tri bakterijske vrste: *Prevotella intermedia, Aggregatibacter actinomycetemcomitans* i *Porphyromonas gingivalis.* Kontaminirani implantati su inkubirani u anaerobnim uvjetima kroz 72 sata, a potom slučajnim odabirom podijeljeni u četiri eksperimentalne skupine i dvije kontrolne skupine (n = 12 po skupini) prema sljedećim protokolima tretiranja: Grupa 1 (PDT1): PDT (660 nm, 100 mW, 60 sekundi) s toluidinskim modrilom; Grupa 2 (PDT2): PDT (660 nm, 100 mW, 60 sekundi) s fenotiazinskim kloridnim bojilom; Grupa 3 (PDT3): svjetleća dioda (LED) s toluidinskim modrilom; Skupina 4 (TB): tretiranje samo toluidinskim modrilom kroz 60 sekundi. U pozitivnoj kontrolnoj skupini (PC) implantati su tretirani s 0,2%-tnom klorheksidin-baziranom otopinom tijekom 60 sekundi, a u negativnoj kontrolnoj skupini (NC) nikakav tretman nije korišten.

Rezultati

U skupini titanskih implantata, najviše redukcije bakterija zabilježeno je u skupinama PDT1 (98,3%) i PDT2 (97,8%). Rezultati ovog istraživanja pokazali su statistički značajno smanjenje bakterija u skupini PDT1 i PDT2 u usporedbi s NC grupom (<0,05), pojedinačno za svaku vrstu bakterija, kao i za sve tri vrste zajedno. PDT3 je bio manje učinkovit nego PDT1 i PDT2 i nije pokazao statistički značajnu razliku u usporedbi s NC ili bilo kojom drugom skupinom tretiranja. TB je bio najmanje učinkovit tretman, s obzirom na ukupni broj bakterija i pojedinačni broj za svaku pojedinu vrstu bakterija.

U skupini cirkonskih implantata sve ispitivane skupine imale su znatno niži broj bakterija (> 99,9%) u usporedbi s NC za ukupni broj bakterija i svaku bakterijsku vrstu odvojeno. Među

njima je najveće smanjenje bakterija zabilježeno u slučaju korištenja PDT1, PDT2 i PDT3, od kojih su svi bili statistički značajno različiti od TB.

Zaključak

PDT1 i PDT2 protokoli pokazali su visoku djelotvornost protiv trodnevnog bakterijskog biofilma na titanskim dentalnim implantatima, dok su PDT1, PDT2 i PDT3 pokazali veliku učinkovitost na površini cirkonskih implantata.

Ključne riječi

fotodinamska terapija, titanski implantati, cirkonski implantati, antimikrobni učinak, površina implantata, laser, LED

The list of abbreviations

Abbreviation	Term
$^{3}O_{2}$	Ground state Oxygen
Al2O3	Aluminium oxide
ALA	5-aminolevulinic acid
aPDT	Antimicrobial photodynamic therapy
ATZ	Alumina-toughened zirconia
BOP	Bleeding on probing
BPD	Benzoporphyrin derivative
BRONJ	Bisphosphonate-related osteonecrosis of the jaw
CAL	Clinical attachment level
CFU	Colony forming units
CO2	Carbon dioxide
CpTi	Commercially pure titanium
Er:YAG	Eribium: yttrium-aluminium-garnet
GaAlAs	Gallium-aluminium-arsenide
HA	Hydroxyapatite coating
HGF	Human gingival fibroblasts
HiVac	High vacuum
HO:YAG	Holmium: yttrium-aluminium-garnet
HV	High voltage
KTP	Potassium titanyl phosphate
LED	Light-emitting diode
LLLT	Low level laser therapy
LS11	Talaporfin sodium
LTD	Low temperature degradation
MRONJ	Medication-related osteonecrosis of jaws
mTHPC	Temoporfin
MVD	Microvessel density
NaOCl	Sodium hypochlorite

ND:YAG	Neodymium:yttrium-aluminium-garnet
NOS	Nitrous oxide synthase
PDT	Photodynamic therapy
ROS	Reactive Oxygen Species
SCC	Squamous cell carcinoma
SEM	Scanning electron microscopy
SnET2	Tinethyletiopurpurin
Ti	Titanium
TPS	Titanium plasma-sprayed
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
Y-TZP	Yttria tetragona zirconia polycristalline
ZTA	Zirconia-toughened alumina

Contents

1. Introduction

Dental implants are becoming a routine procedure in modern dentistry. They can potentially last a patient's entire life. The increase in the number of dental implants placed every year has also been accompanied with an increase in the number of peri-implant diseases. Peri-implantitis is a serious complication that can lead to the disintegration and early loss of the implants. Currently there are no standardized treatment protocols for treating peri-implant diseases. This gives rise to many new challenges in finding a successful treatment method and establishing an effective treatment protocol. Photodynamic therapy has recently emerged as a potential effective treatment choice for peri-implantitis due to its non-invasiveness and good antimicrobial effects.

1.1 Photodynamic Therapy

1.1.1 Historic overview

The first use of light for therapeutic purposes dates from around 1400 BC when the sunlight was used as a source for treating skin diseases (1).

In 1801 ultraviolet (UV) rays were discovered and scientists began to understand the therapeutic effect of the sunlight. Later during the 19th century the interest for heliotherapy increased in the scientific community and different scientists started using the sunlight to treat different diseases such as rachitis, peritoneal tuberculosis, lupus vulgaris, etc. (1).

Towards the end of the 19th century, Lahmann constructed and used the first artificial light sources in Germany. His construction was made from a carbon arc lamp in combination with a parabolic mirror. He successfully treated a patient with lupus vulgaris of the nose and recorded an improvement in another patient that had the same condition (2).

In the beginning of the 20th century, Niels Finsen received the Nobel Prize for his therapeutic results in treating lupus vulgaris with concentrated doses of UV radiation from a carbon arc lamp. This was regarded as the beginning of modern phototherapy (1).

In the mid of the 20th century scientists and doctors started using artificial light sources for treating neonatal jaundice, psoriasis, and many different skin conditions (3).

Nowadays this technology is known as phototherapy. Phototherapy can be used with or without the use of a photosensitizer. When used together with a photosensitizer, phototherapy is known as photochemotherapy (2).

Photodynamic therapy (PDT) is a type of photochemotherapy which involves three components: light, a photosensitizer and oxygen. The therapeutic possibilities of photodynamic therapy were first introduced in the 19th century, but it was not until the 1990s when the first photosensitizers were approved for clinical use (2,4,5).

Currently photodynamic therapy is mostly used in the treatment of cancers (6–8), however, there are numerous recent studies that have shown that photodynamic therapy also has antimicrobial effect (9–12).

1.1.2 Photochemistry of Photodynamic Therapy

Photodynamic therapy (PDT) is a treatment in which a photosensitive dye (photosensitizer) is activated by light, which leads to selective toxicity for the desired treatment (13).

As a result of the light activation, the sensitizer is transformed from ground state to the first excited state. In this state the photosensitizer has to have enough stability so that it can cross to the triplet excited state (T1), which is an even more stable state. Subsequently two different reaction processes, both involving molecular oxygen, can take place. In the first type (Type I), the Reactive Oxygen Species (ROS) are formed as a result of the interaction of ground state oxygen with the resultant radical which is created from the hydrogen abstraction or electron transfer between an excited sensitizer and an adjacent sensitizer molecule. In the second type (Type II), singlet oxygen species are formed as a result of the energy transfer directly from T1 to the ground state oxygen ($^3O_2$). This can happen only if the sensitizer and the ground state oxygen are in the same triplet state multiplicity (2,13). Both types of processes are shown schematically in Figure 1.

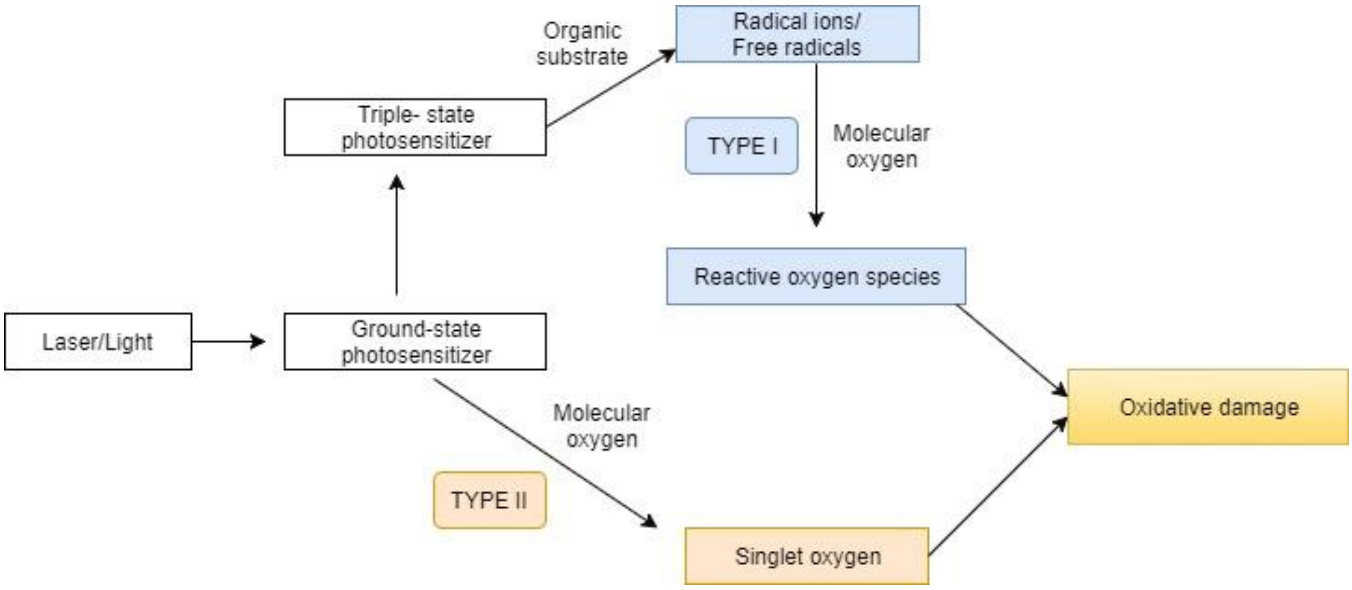

Figure 1. Photochemical mechanisms in photodynamic therapy (Type I and Type II).

The Type I reaction mainly happens in anoxic or hypoxic environments. In anoxic environments the excited photosensitizer reacts directly with organic substrates, producing an oxidized substrate and a reduced photosensitizer. In hypoxic environments the reduced photosensitizer reacts with oxygen and superoxide anions are produced which, as a result, can form highly reactive hydroxyl radicals (4).

Type II reactions are dependent on oxygen concentration. They are commonly associated with the formation of singlet oxygen, however, there are also other compounds which have triplet-ground state similar to oxygen which can be involved in this type of reaction (nitric oxide and vitamin A) (4, 14). Type II reactions are dominant during PDT treatments, however Type I reactions might become dominant under hypoxic conditions or in the presence of highly concentrated photosensitizer (2, 4).

1.1.3 Oxygen in Photodynamic Therapy

Oxygen is one of the three components of photodynamic therapy. In its ground state, oxygen has two unpaired electrons that are positioned on the outermost orbitals. Depending on the presence

or absence of magnetic field, these electrons can have three different configurations: both spins aligned up, both spins aligned down or in opposite directions. Because of these three possible configurations, the ground state of oxygen is also called a triplet state (4).

The predominant agent produced from photodynamic therapy is the singlet oxygen (2). This is a highly reactive form that happens as a result of pairing electrons into antibonding orbital which makes the molecule unstable. The lifetime of singlet oxygen is very short due to its reactiveness, and as a result of this short lifetime the energy created and the oxidative damage induced by PDT is highly localized (2,4) (Figure 2).

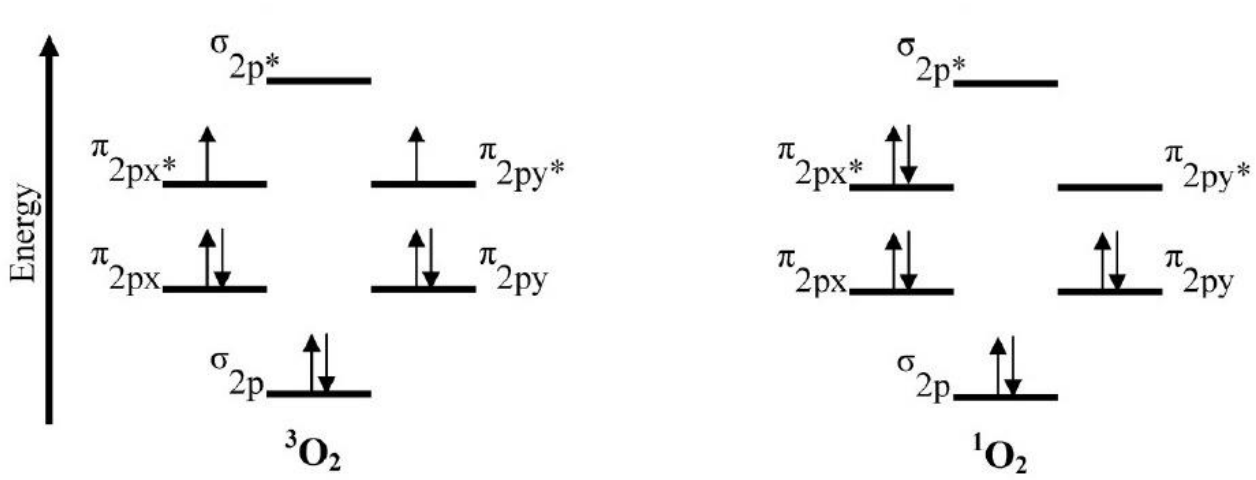

Figure 2. States of oxygen. Triplet ($^3O_2$) and singlet ($^1O_2$) oxygen (2).

1.1.4 Photosensitizers in Photodynamic Therapy

The first photosensitizers used for photodynamic therapy were porphyrins, chlorins and bacteriochlorins. These dyes have the strongest light absorption in the red portion of the electromagnetic spectrum. Among them there are differences in the absorption spectra ranging from around 400 nm (called the Soret band) to around 800 nm, however, the most useful absorption range for PDT is between 600 nm and 800 nm. These photosensitizers are highly efficient singlet oxygen generators. The production efficiency of singlet oxygen is called singlet-oxygen quantum yield (Φ) (2,4,5).

An optimal photosensitizer should have the following properties (15):

1. availability in pure form

2. it should be synthesizable and easily reproduced

3. high singlet-oxygen quantum yield

4. strong absorption in the spectrum (680-800 nm)

5. effective accumulation in tumor tissue

6. stability and solubility in the organism

7. excretion from the body after completing the treatment.

The first commercial photosensitizer was Photofrin®. It belongs to porphyrin group of photosensitizers. Its longest wavelength absorption maximum is relatively weak. At 630 nm it can be activated up to about 5 mm in the tissues. At the beginning it was approved only for treating bladder cancer, but later it was also approved for treating many other cancers (esophageal, lung, head, neck, abdominal cancer etc.) (4,5,15).

In an attempt to create a better photosensitizer, many new compounds have been synthesized. The second generation of photosensitizers includes 5-aminolevulinic acid (ALA), benzoporphyrin derivative (BPD), lutetium texaphyrin, temoporfin (mTHPC), tinethyletiopurpurin (SnET2), talaporfin sodium (LS11), etc. These compounds are more potent than the first generation and due to their potency they can cause pain and lead to severe skin photosensitivity. The third generation of photosensitizers includes modified drugs that are currently available, but more studies are needed to verify the potential of these photosensitizers (2,4,5,15).

Even though most of the photosensitizers belong to the porphynoid groups, there are also several non-porphyrin photosensitizers. These compounds include: antharquinones, phenothiazines, xanthenes, cyanines etc. (2).

In dental medicine most commonly used photosensitizers belong to the phenothiazines. Methylene blue and toluidine blue are the most commonly used to treat chronic periodontitis. Methylene blue is used in addition against melanoma, basal cell carcinoma and Kaposi's sarcoma (13,16). The structures of methylene blue and toluidine blue are presented in Figure 3.

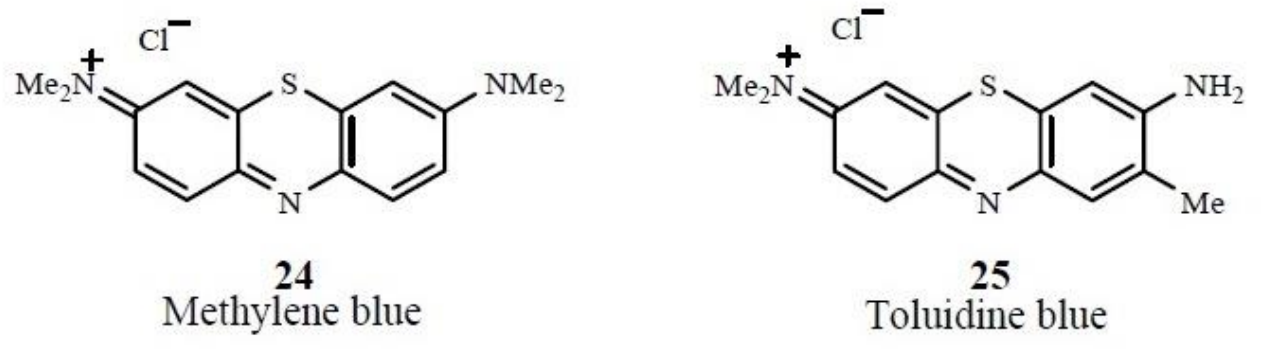

Figure 3. Structure of Methylene blue and Toluidine blue (2).

Methylene blue and toluidine blue belong to the phenothiazinium family with similar chemical and physicochemical characteristics. Methylene blue is a redox indicator which is blue in an oxidizing environment and upon reduction becomes colourless (16). At first it was used as a medicine against malaria (17). The best absorption of methylene blue occurs at the wavelength of 666 nm (2). Its mechanism of action is due to its positive charge and it includes the interleaving of methylene blue cations in the structure of nucleic acids. A disadvantage of methylene blue is the fact that this chromophore is easily reduced in biological systems, which as a results reduces the antimicrobial activity (18). However, many studies report methylene blue to be effective in killing *Helicobacter pylori, Candida albicans,* the influenza virus and periodontal bacteria (19–22).

Toluidine blue has a blue-violet colour. It is capable of inactivating gram-positive and gram-negative bacteria. It interacts with lipopolysaccharides that are present in the cell membrane of Gram-negative bacteria even without light application (16,23,24). When exposed to a wavelength of 630 nm, it shows maximum absorption and is effective in killing various types of microorganisms (13,16). Its mechanism of action is due to its chemical and physical properties that allow it to pass freely across the bacterial membrane and act directly on the mitochondria (18). It has been shown in different studies that toluidine blue is effective against periodontal bacteria both in planktonic cultures (25,26) and biofilms (22).

1.1.5 Light Sources

The first light sources used for PDT were argon-pumped dye lasers, potassium titanyl phosphate (KTP)- or neodymium:yttrium aluminium garnet (Nd:YAG)-pumped dye lasers, and gold vapor- or copper vapor-pumped dye lasers. All these devices are expensive and complex, which is why nowadays diode laser systems are predominantly used. Diode lasers are easy to handle, portable and less expensive compared to previously used devices (5,27).

For an effective PDT treatment, the light source should be capable of activating the photosensitizer at a specific wavelength. Red light is the most efficient one regarding the use in human tissues. As a result, most of the sensitizers used are between 630 and 700 nm. This corresponds to a light penetration depth of up to 1.5 cm (27,28). This limited depth of penetration prohibits the uniform illumination of larger and solid tumors (7,29).

Recently, non-laser light sources, such as light-emitting diodes (LED), have also been applied in PDT procedures. These light sources are much less expensive and are small, lightweight, and highly flexible (9,30–32).

The PDT, depending on the pathology treated, can be applied superficially, interstitially, intra-operatively, and intra-cavitary.

Depending on the location and morphology of the lesion, sources used for light delivery can be fiber-optic catheters or lenses for flat-field applications (7,29).

The light should be precise and uniform allowing for an effective treatment. The fiber tip can have different shapes in order to allow light diffusion in all directions. Important issues related to the light source for PDT are the accurate calibration, sensitizer and light dosimetry. Such devices would greatly advance PDT as a routine clinical treatment (33).

1.1.6 Limitations of Photodynamic Therapy

In order for PDT to be effective it requires the light to be directed to the appropriate site and tissue depth. Optimal light delivery with lasers and the coordination between different clinicians

is complex and sometimes the availability of the light sources is a major issue. Currently there are portable light sources which have simplified the process. PDT is an ablative procedure and the treatments do not provide material for histopathological diagnosis. That is why prior to the application of PDT, a treatment diagnosis should be made by other methods. Another limitation of PDT is the inability of the light to penetrate deep in tumors which makes it less effective for treating large tumors. Since it is a local treatment, it is also impossible to be used for treating metastasized cancers (34–36).

Photosensitivity is another issue that can last for some time after the application of certain photosensitizers. It is dependent on the method of application of the photosensitizer. When administered systematically, skin photosensitivity may last for several days or weeks. Patients are instructed to avoid exposure to sunlight, protect the skin and the eyes until the drug is completely eliminated (5).

1.1.7 Antimicrobial Photodynamic Therapy (aPDT)

The antimicrobial potential of PDT has been known since the beginning of the last century. However, it was not until the emergence of antibiotic-resistant strains of bacteria that scientists were stimulated to look for alternative treatments, especially for localized infections of the skin and oral cavity (5,37,38).

The products of photodynamic therapy cause damage to various components of the microbial cells or they can alter the metabolic activity irreversibly. This results in microbial elimination. This mechanism of action is based on the energy absorbed through intracellular photosensitization which is transferred to the oxygen molecule in order to damage the oxidative reaction pathways in the plasma membrane and the genetic material of the microbial cells (39,40). This effect is limited only on the microbial cells without any toxic effects for the host cells (41).

The efficiency and reliability of aPDT is due to the relatively simple basic principles behind it. If all the components of aPDT (light, oxygen and photosensitizer) are present in sufficient amounts

during the application of this therapy, then this technique can be highly effective and cause damage to the target cells (42).

The antimicrobial effect of photodynamic therapy has been shown to be effective in many studies and against a number of different microorganisms: *Agerggatibacter actinomycetemcomitans, Prevotella intermedia, Staphyloccocus aureus. Streptococcus pneumoniae, Enterococcus faecalis, Escherichia coli, Candida albicans, Pseudomonas aeruginosa, Porphyromonas gingivalis, Streptococcus sanguis* etc. (9,18,23,30,43). It is unlikely that these microorganisms can develop resistance to aPDT. This is due to the fact that singlet oxygen and the free radicals interact with several cell structures and different metabolic pathways in the cellular level (44).

1.2 Application of Photodynamic therapy in dental medicine

The application of PDT in dental medicine is growing rapidly and it is being used for the photodynamic diagnosis of malignant transformation of oral lesions, the treatment of head and neck cancer, as well as bacterial and fungal infections (5,45).

1.2.1 Application in Periodontology

Periodontitis is a chronic inflammatory disease affecting tissues surrounding the teeth. It is induced by bacterial infection and causes major destruction of the periodontium which can eventually lead to the loosening of the teeth and their subsequent loss (46,47).

The basic treatment of periodontitis consists of the mechanical debridement, often combined with the use of chemical decontamination or the use of systemic or local antimicrobial therapy.

The mechanical debridement alone cannot remove all the infections due to the difficulty in reaching deep pockets and as a result, a residual plaque can remain even after the treatment.

When mechanical debridement is repeatedly used, it can lead to cumulative scuffing of the root surface (48).

The systemic delivery of antimicrobial therapy has some side effects, including antibiotic resistance which should always be considered when treating periodontitis. The local application of the antimicrobial therapy also has some disadvantages, such as the necessity to repeat the treatments, the effect appears only in a limited part of the periodontium and decalcification and root surface softening can occur (49–51). Furthermore, mechanical debridement can open dentinal tubes and the remaining periodontal bacteria are able to penetrate into the dentinal tubes (13,52,53).

The limitations of the traditional periodontal therapy have shifted the focus towards aPDT as an alternative treatment for periodontal diseases (54–56). It has been confirmed in many studies that aPDT is an effective antimicrobial therapy (12,36).

The advantages of aPDT in comparison with other treatments are that the photosensitizer can be placed directly into the pocket and then activated with an optical fiber tip. Another advantage is that aPDT is effective only against microbial cells, avoiding damage to the host tissues. This makes it a safe procedure against periodontal microbiota (57,58).

Many studies have shown potential improvements after the use of aPDT in conjunction with mechanical debridement (59–61), however there are also some studies that report different results (55,62–64). In his meta-analysis Atieh (61) showed potential improvements after using aPDT together with scaling and root planning. A reduction in probing depth and greater clinical attachment gain was seen in association with those two treatments. Similarly, in their study Sgolastra et al. reported that the combination of aPDT and conventional treatment provides additional benefits by reducing the pocket depth and increasing the clinical attachment level (CAL) (59).

1.2.2 Application in Oral and Maxillofacial surgery

In the oral and maxillofacial region, photodynamic therapy is used for its anti-cancer therapeutic potential and for its antimicrobial potential. The main focuses of the anti-cancer photodynamic therapy in this region are head and neck cancers. Most of the head and neck cancers are squamous cell carcinomas (SCC). Oral SCC is the most frequent tumor in the oral cavity (65). Conventional methods used for treating head and neck cancer have not shown any significant improvements in the 5-year survival rate of these cancers. They also cause different side effects such as jaw pain, mouth sores, difficulty in chewing, swallowing, etc. (66).

Pre-malignant lesions of the oral mucosa are considered as one of the developing factors of oral SCC. Erythroplakias and dysplastic leukoplakias are the most common pre-cancerous lesions. Around 50% of oral SCC are associated with leukoplakias (67).

Photodynamic therapy has therapeutic potential not only for the head and neck cancer, but also against pre-malignant, primary, recurrent, and metastatic lesions (68,69).

Compared to conventional treatments, PDT has an advantage due to its minimal invasiveness and selective tumor destruction without affecting the healthy tissues. In addition, it can be applied in combination with conventional therapy to increase the overall treatment success (29). The mostly used and studied photosensitizer is Photofrin®, but it has limited application for treatment of large and solid tumors. The clinical results obtained from the use of Photofrin® for head and neck cancer are generally excellent (70). A major side-effect after Photofrin® use is the photosensitivity of the skin which can last for up to 6 weeks after treatment (29,70).

Focsan® is a second-generation photosensitizer, the aims of which include: local destruction of the tumor, preservation of organ function, relief of symptoms and avoiding disease-related complications. Similarly to other photosensitizers, it causes photosensitivity after treatment. This can last for approximately 15 days (70).

A newer generation of photosensitizer is ALA, which is a precursor of the photosensitizer protoporphyrin IX. It is mostly used for superficial lesions due to its limited depth and light penetration. The photosensitivity after treatment lasts less than 24 hours. This photosensitizer has been widely used in the treatment of pre-malignant lesions in the oral cavity (27,71–73).

Regarding the antimicrobial effect of PDT in oral and maxillofacial surgery, it is mostly used for the disinfection of soft tissue or bone during final surgical phases as an additional means of prevention. In their study Neugebauer et al. (74) showed that after the use of aPDT there is a significantly lower incidence of alveolar ostitis. Nagayoshi et al. (75) concluded that photodynamic therapy had nearly the same antimicrobial effect as 2.5% NaOCl without adverse effects on surrounding tissues. Their study was conducted on *in vitro* periapical lesion model.

Photodynamic therapy has recently also been used as an adjuvant therapy for the treatment of medication-related osteonecrosis of jaws (MRONJ), which is highly related to bisphosphonate-related osteonecrosis of the jaw (BRONJ). Minamisako et al. (76), according to their results, suggest that both LLLT (low level laser therapy) and PDT are beneficial in the treatment and clinical management of the MRONJ. Similarly, Rugani et al. (77) in their review conclude that photodynamic therapy can be used as an adjunct therapy in BRONJ treatment before or after surgery. It can also be used as a primary treatment option in cases of very early BRONJ or if surgery is not indicated.

1.2.3 Application in Endodontics

In endodontics aPDT is used for the disinfection of the root canal. Conventional root canal treatment consists of a combination of mechanical instrumentation, the use of disinfecting solutions for irrigation and the placement of medicaments in between appointments. However, due to the root canal anatomy, sometimes the complete disinfection of the root canal is very difficult while using mechanical and chemical methods (78,79).

In an *in vitro* study by Bago et al. (80), the antimicrobial efficacy of photodynamic therapy was shown to be more effective in reducing root canal infection compared to high-power diode laser and conventional irrigation with NaOCl (sodium hypochlorite).

The use of aPDT has also been shown effective as an adjunct therapy for the root canal disinfection in many studies. Raymond at al. (78) in their *in vitro* study tested the efficacy of conventional chemo-mechanical treatment together with photodynamic therapy. Their results showed that this combination is more effective than the use of only chemo-mechanical treatment.

Rios et al. (81) in their study used a light-emitting diode in combination with toluidine blue O. They concluded that photodynamic therapy has the potential to be used as an adjunctive antimicrobial procedure in endodontics. Similarly, Bago et al. (82) in their clinical study concluded that the of the effect of aPDT, in addition to the conventional chemo-mechanical root canal preparation, led to a significant reduction of the bacteria and in some samples the complete elimination of bacteria.

1.3 Dental Implants

Per-Ingvar Brånemark in 1978 presented two-stage threaded implants made from titanium in a root-form. With his research that started in 1965 he established the basis for modern implantology. The first four implants placed by Brånemark in 1965 integrated within six months and remained in place for over 40 years (83).

The concept of osseointegration of the implants was first brought during the 1950s and 1960s after observing in many studies that there was a bone growth in contact with titanium in many animal studies. Brånemark defined osseointegration as: *"A direct connection between living bone and a load-carrying endosseous implant at the light microscopic level."* (84).

Since then dental implants have become a long-term reliable treatment option for replacing missing teeth (85). An ideal implant should have the following properties: biocompatibility, adequate toughness, strength, corrosion resistance, facture and wear resistance (86,87). Materials for producing dental implants can be categorized according to the biological response or the chemical composition. Regarding their chemical composition, they can be produced from metals, ceramics or polymers (88).

Up to date, titanium implants and their biomedical alloys are the "gold standard" for producing dental implants. This is due to the excellent biocompatibility and long-term survival rate of titanium implants (89–91). However, there are some concerns regarding their dark gray colour, which can be visible through the peri-implant soft tissue, especially when a thin biotype of gingiva is present, or when a resorption of the buccal plate occurs (90,92). Furthermore, titanium

dental implants might cause galvanic side effects after contact with saliva, and even though they are rare, allergic reactions might be possible (93,94). Due to these reasons, many scientists have shifted their focus to producing ceramic implants (95).

1.3.1 Titanium Dental implants

There are six types of titanium available as implant material. They are classified by the American Society for Testing and Materials into four grades of commercially pure titanium (CpTi) and two titanium (Ti) alloys (85,96).

CpTi has mechanical and physical properties different from the two titanium alloys, mainly due to the oxygen residuals in the metal. They are classified from Grade I to Grade IV pure titanium. In CpTi there are usually some trace elements, such as carbon, oxygen, nitrogen and iron, which improve the mechanical properties of pure titanium. Their amount increases with the increasing grade from I to IV (85,96).

The titanium alloys used in dentistry have three structural forms depending on the elements mixed with titanium: alpha (α), beta (β) and alpha-beta. These structural forms can coexist depending on the composition and the heat treatment of titanium (86,97). The most commonly used structural form for the production of dental implants is the alpha-beta combination. This is known as Ti-6Al-4V and contains 6% aluminium and 4% vanadium. These alloys have low density and are strong and resistant to corrosion and fatigue (85). They have a module of elasticity similar to bone, which enables a more favorable stress distribution at the bone-implant interface (96).

In addition to enhancing the titanium properties, many modifications to the implant surface have been made with the purpose of decreasing the healing time for osseointegration. The only part that is in contact with the bioenvironment is the surface of the dental implant and it is this interaction that affects the implant/tissue interface (83,98).

Surface treatment of the dental implants increases the functional surface area of the implant-bone interface and stress is more effectively transferred. There are also surface treatments that promote

bone apposition. The most common treatments include mechanical treatments, chemical treatments, electrochemical treatments, thermal treatments and laser treatments (99,100).

Increasing the surface roughness of the implant has been shown to influence the production of cytokine and growth factors by osteoblasts. It also increases osteoblast cell propagation by transforming growth factors (101). This suggests that the implant structure directly influences the interaction between the metal and the surrounding living tissue (102,103).

The first implants that were produced after Brånemark introduced the concept of osseointegration were the ones with smooth machined surface, and they were also called machined implants. These implants required a longer osseointegration period due to their smooth surface, but in accordance with Brånemark's two stage concept. These implants show good long-term results when they are used in the areas with sufficient bone, allowing for a two-stage process (83,104,105).

Later ceramics were introduced in dental implantology for coating endosseous implants in order to improve osseointegration (106). Calcium phosphates, bioglasses, inert ceramics and zirconium oxide have been used for this purpose. The thickness of the coating varies from 1 to 100 μm, depending on the coating method. The coating can be done using plasma spraying, sputter-deposition, sol-gel coating, electrophoretic deposition or biomimetic precipitation (106,107). In many studies bioactive ceramics have been shown to enhance bone apposition as compared with inert ceramics and metallic surfaces. This is due to the release of calcium phosphate ions around the implants (106–109). The most popular calcium phosphate coating materials today are hydroxyapatite and fluorapatite.

Hydroxyapatite coating (HA) is a surface treatment of titanium dental implants in order to form a stronger bond between the bone and the implant (110). The HA is mostly applied to the titanium surface by plasma spraying which allows a uniform thickness of 40-50 micrometers (111).

A major concern regarding the plasma spraying is that hydroxyapatite may resorb eventually, which might ultimately cause loosening of the titanium particles. Another concern regarding this method is the implant failure due to microbial infection (112–115).

Another surface treatment is the etching of the implant surface with strong acids like hydrochloric or sulfuric acid. This procedure provides an equal roughness, active surface area and better adhesion (116). It also eliminates the oxide layer from the implant surface (117). Acid etching

has improved the osseointegration according to Cho et al. (118) and Wong et al. (119) in their respective studies.

In addition to these methods, some manufacturers use fluoride treatment, or laser ablation for the implant surface (83,120). The use of antibiotics has also been studied on the implant surface in order to prevent the infection of the surgical site. For this purpose, tetracycline has shown good results and it supports osseointegration of the implant (121,122).

Recently, titanium implant coatings with antimicrobial properties are being evaluated in many studies (123,124). Kulkarni Aranya et al. (124) investigated different modifications of calcium phosphate coatings on titanium discs. According to their results, the growth, colonization and adherence of *P.gingivalis* were inhibited.

1.3.2 Zirconia Dental Implants

As an alternative to titanium dental implants, recently novel implant technologies that produce ceramic implants have been introduced (95).

Initially ceramic implants were made from mono or polycrystalline aluminium oxide (Al2O3), however due to its mechanical weakness it resulted in poor clinical outcome (125).

Nowadays, ceramic dental implants are produced from yttria stabilized tetragonal zirconia polycrystalline (Y-TZP) (126). Compared to other ceramics, zirconia holds a unique place due to its excellent mechanical properties and it exhibits superior corrosion and wear resistance. It also has a high flexural strength compared to other dental ceramics (126,127). This makes them suitable substrates for the production of dental implants (125).

In preclinical studies the biological and physical properties of Y-TZP implants have been shown to be comparable to titanium implants (128–131).

Recently several clinical studies have addressed the outcome of implants produced from Y-TZP. Promising results have been found in a recent review regarding the survival rate and the marginal bone loss after one year in function (132). However, long-term clinical results are still missing.

The main concern regarding zirconia as implant material is low temperature degradation (LTD). This process is also known as ageing and it occurs by a slow surface transformation of the tetragonal crystals to stable monoclinic structure when water or water vapor is present. To a certain degree this transformation improves the mechanical properties of Y-TZP, however between the improvement and destruction there is a narrow range which seldom results in property deterioration of the material (133). In order to minimize LTD, many manufacturers have included the addition of small amounts of silica, reduced the grain size, increased the stabilizer content, used yttria-coated powder instead of co-precipitated powder, or even formed composites with aluminium oxide (134–136). The addition of alumina to zirconia stops the ageing or at least reduces its kinetics drastically. This is a result of the changes in the grain-boundary chemistry and the limitation to the tetragonal grain growth during sintering. The outcome of this is a more stable structure of the material (135).

In order that an optimal zirconia implant design can be developed, biomechanical failure modes of the zirconia should be understood (137). The physical mechanism of ceramic implant failure can be mechanical and/or chemical (138). Mechanical failure can occur during the placement of the implant or after loading the implant (137,139).

The manufacturing process of zirconia implants is more complicated and imperfections or flaws during the production can compromise their strength (107,137). There are many manufacturers of zirconia implants available today, however only three types are being used in dentistry: yttrium-stabilized tetragonal zirconia polycrystals (Y-TZP), alumina-toughened zirconia (ATZ) and zirconia-toughened alumina (ZTA) (107).

Altogether, zirconia implants are becoming an alternative to titanium implants. They have tooth-like colour, high strength and higher fracture toughness compared to other ceramics (90,126,140). According to some studies, zirconia implants induce smaller inflammatory response and bone resorption compared with the titanium particles, which suggests good biocompatibility comparable to titanium implants (92,141).

1.3.3 Peri-implant tissues around zirconia and titanium implants

The soft tissues around the implant form a crucial seal between the implant surface, the bone and the oral environment (142,143). In contrast to teeth, there is no supracrestal connective tissue attachment around the implants. This makes the seal fragile and when subjected to bacterial or mechanical challenge, the destruction of the soft connective tissue around the implant can be faster and more devastating process compared to the periodontium of the teeth (144,145).

The inflammatory response of the soft tissues around the implant and the plaques adhesion on the implant surface has been studied in many *in vitro* and *in vivo* studies. Scarano et al. (146) in their study evaluated the coverage of titanium and zirconia discs by bacteria and found that there is a significant difference in bacterial adhesion between these types of surfaces. In another study the expression of the vascular endothelial growth factor (VEGF), nitrous oxide synthase (NOS) and microvessel density (MVD) in titanium and zirconia healing caps was examined. They found out that all of these had higher values and greater extension of inflammatory infiltrate in the titanium specimens (147).

Kohal et al. (130) in their study in a monkey model, found no difference in soft tissue integration around rough zirconia and titanium implants. In another study the attachment, growth behavior and the effect of human gingival fibroblasts (HGF) cultured on zirconia and titanium surfaces was investigated. In this study the biological response to zirconia and titanium were comparable (148).

Consequently, enhancing the seal formed by the peri-implant soft tissues, especially that of the titanium/connective tissue interface may be an important factor in implant survival.

1.4 Peri-implantitis and aPDT

Peri-implant diseases can be presented in two forms: peri-implant mucositis and peri-implantitis. Peri-implant mucositis is the reversible inflammatory process of the soft tissue around the implant, which is followed by reddening, swelling and bleeding on probing (149).

Peri-implantitis is an inflammatory process affecting the soft and hard tissue around an osseointegrated implant, resulting in the loss of supporting bone (150).

The literature provides widespread evidence of the microbial etiology of peri-implantitis (151). The microorganisms found in peri-implantits are very similar to those found in advanced periodontitis (152–154). Most of them are spirochetes and non-motile anaerobic Gram-negative bacteria such as: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannarella forsythia, Treponema denticola* etc. (155,156).

The colonization of the implant surfaces with bacterial biofilms occurs rapidly in the oral cavity. In the early stages of peri-implantitis there are no significant symptoms, hence it is usually diagnosed during routine dental check-up. The early diagnosis of peri-implantitis is of great importance in order to stop the progression of the disease and to establish good osseointegration (157–159).

According to Teughels et al. (160), in addition to the chemical composition, the surface roughness of the implan has a significant impact on the quantity and quality of plaque formation and bacterial adhesion on implant surfaces.. Rough surfaces and those with greater surface free energy accumulate more plaque. Furthermore, the areas with high wettability and pits and grooves in the roughened surfaces also attract initial bacterial adhesion which is difficult to remove.

There are many treatment modalities proposed in literature for treating peri-implantitis. They can be summarized in two groups: resective and regenerative therapies (161).

Resective treatments attempt to eliminate the etiologic factors of peri-implantitis and maintain optimal conditions. These treatments are mainly done by cleaning and decontaminating implant

surfaces. Regenerative treatments consist in using bone grafts, membranes and growth factors in order to reconstruct the pre-existing hard and soft tissues around the implants (161,162).

Similarly to treating periodontitis, resective treatments are mainly done by mechanical removal of biofilm from the surface of the implant, which is of utmost importance when treating peri-implantitis. For this purpose plastic curettes, ultrasonic scalers, air-powder abrasive and ablative lasers are used (163). The objective is to create a clean surface which can stop the progression of the disease and promote re-osseointegration of the implant. However, the implant surface roughness facilitates bacterial adhesion and colonization which makes mechanical debridement very difficult (164).

Some authors suggest elimination of the implant threads and smoothing the implant surface (implantoplasty) in order to achieve better decontamination. This procedure allows better maintenance and facilitates the oral hygiene when threads are exposed (165,166).

The use of plastic curettes is recommended over the use of metallic curettes because metallic curettes can alter surface roughness favoring bacterial colonization, whereas plastic curettes produce very little damage or none at all. When metallic curettes are used, titanium curettes are preferred over stainless steel curettes (167). Abrasive sandblasting systems are also reported to be effective for mechanical cleaning of the implant without producing adverse effects (168).

Laser decontamination of the implant surfaces has been widely studied recently. In their study Kreisler et al. (169) evaluated the mechanical effects produced by Nd:YAG, Ho:YAG (holmium:yttrium-aluminium-garnet), Er:YAG (Eribium: yttrium-aluminium-garnet), CO2 (Carbon dioxide) and GaAlAs (Gallium-aluminium-arsenide) lasers on four types of implant surfaces. Their results showed that Nd:YAG and Ho:YAG lasers cause significant damage to the implant surfaces. On the other hand, CO2 and Er:YAG lasers can be used in specific power settings and GaAlAs laser did not cause any damage to the surface in any power settings.

In addition to mechanical methods of treating peri-implantitis, the use of chemical decontamination and antibiotic therapy is promoted as adjunct therapy to mechanical decontamination in order to improve the treatment outcome. The most commonly used antimicrobial solutions are chlorhexidine, tetracycline or minocycline, citric acid, hydrogen peroxide, and phosphoric acid (170).

Recently aPDT has emerged as a potential treatment option or adjuvant treatment to peri-implantitis. It has generated much interest for its potential to decontaminate implant surfaces without damaging the surface and the surrounding tissues around the implant. Furthermore, it is more effective than the application of laser alone (164,171).

In their study Hayek et al. (172) concluded that aPDT is effective and non-invasive method compared to conventional therapy during surgical treatment of peri-implantitis with elevated mucoperiosteal mucosa flaps.

The possibilities of aPDT for treating peri-implantitis are opening new challenges ahead in establishing optimal conditions for the clinical application of aPDT. It holds a promise as a novel and non-invasive method that can be effective when applied alone or as adjunct therapy to conventional methods for treating peri-implantitis (45).

2. The aim and the hypotheses

2.1 The aim of the study

The aim of this research is to test and compare the effectiveness of antimicrobial photodynamic therapy (aPDT) using three different devices on titanium dental implants and zirconia dental implants contaminated with anaerobic and facultative anaerobic bacteria.

In addition, our aim was to evaluate if aPDT causes damage and alteration to the implant surfaces which would interfere with the re-osseoinegration of the implants in clinical conditions.

2.2 The hypotheses

1. There is no difference in the treatment outcomes between the study groups and the control groups.

2. The effect of the photodynamic therapy is not dependent on the device and photosensitizing dye used.

3. The treatment outcome is not affected by the implant material/surface.

4. There are no surface alterations after the use of aPDT

3. Materials and Methods

3.1 Study sample

The study sample consisted of 144 sterile dental implants out of which 72 were titanium dental implants (BlueSky, Bredent®, Senden, Germany) and 72 zirconia dental implants (whiteSKY, Bredent®, Senden, Germany). The approval for the study was obtained from the Ethics Committee of the School of Dental Medicine, University of Zagreb (05-PA-26-4/2016).

3.1.1 Titanium Dental Implants

The titanium dental implants used were with a diameter of 4.0 mm and 12 mm of length (Figure 4). The implants used were with sandblasted and acid etched surface. Each of the implants was in an unopened sterile packaging.

Figure 4. Titanium dental implant, BlueSky, Bredent®, 4.0x12mm

3.1.2 Zirconia Dental Implants

The zirconia dental implants used were with a diameter of 4.0mm and 12 mm of length (Figure 5). The implants used were with sandblasted surface. Each of the implants was in an unopened sterile packaging.

Figure 5. Zirconia dental implant, WhiteSky, Bredent®, 4.0x12mm

3.2 Bacterial contamination of dental implants

All microbiological procedures were performed at the laboratory of the Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb.

A bacterial suspension was prepared from three bacteria: *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis.*

The strain of *Prevotella intermedia* was isolated from a clinical sample at the Clinical Hospital Centre in Zagreb. *Aggregatibacter actinomycetemcomitans* (ATCC® 33384) and *Porphyromonas*

gingivalis (ATCC® 33277) were purchased from The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Germany as dry frozen cultures.

The bacteria were grown separately in Columbia Agar for 72 hours and then, using thioglycolate broth, a bacterial suspension was prepared for each of the bacteria and mixed together in a joint suspension. A density of 600 nm equivalent of $1x10^8$ CFU/ml (Colony forming units per mililiter) was set by optical densitometer (Densimat, Biomerieux, Marcy l'Etoile, France) (Figure 6).

Figure 6. Optical densitometer, Densimat Biomeraux

Every single implant was put in sterile Eppendorf tubes (Eppendorf, Hamburg, Germany) containing 300 µl of the prepared bacterial suspension (Figure 7) and incubated under anaerobic conditions for 72 hours using GasPak® anaerobic system (Becton, Dickinson and Co, Maryland, USA) (Figure 8). The bacterial suspension covered the entire lengths of the implants in the Eppendorf tubes.

Figure 7. Implants placed in Eppendorf tubes containing bacterial suspension. Implants covered in their entire length by the bacterial suspension

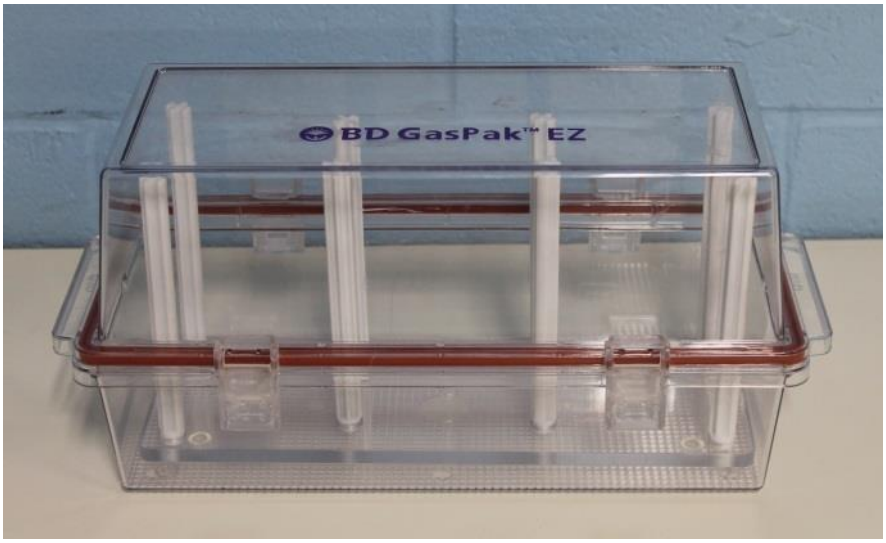

Figure 8. GasPak® anaerobic system

3.3 Antimicrobial protocols

After the incubation period, the implants were taken out of anaerobic chamber conditions and randomly divided into four study groups (n=12 implants/group) and two control groups (n=12).

3.3.1 Group 1. LaserHF treatment group (PDT1)

The implants were treated with a diode laser (660 nm, Laser HF®, Hager Werken, Duisburg, Germany) with 320 µm optical flat fiber tip and a toluidine blue-based dye (155 µg/ml, LaserHF® Paro-PDT solution) (Figure 9). The laser parameters are presented in Table 1.

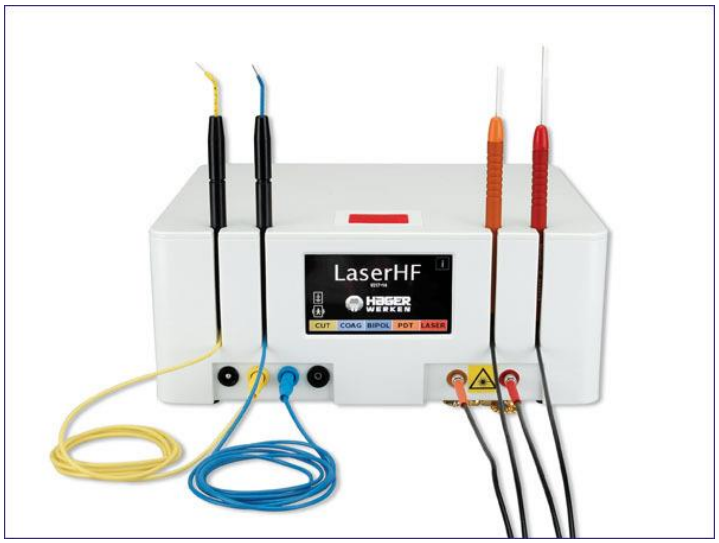

Figure 9. Diode laser, Laser HF® (Hager Werken, Duisburg, Germany)

Table 1. Treatment parameters of PDT1

Wavelength: 660nm
Fiber tip: 320 µm optical fiber tip
Power output: 100 mW
Power density: 124.3 W/cm^2
Irradiation Time: 60 seconds
Distance from the implant: 5mm

3.3.2 Group 2. Helbo treatment group (PDT2)

The implants were treated with a diode laser (660 nm, Helbo® Therapielaser, Helbo Photodynamic Systems GmbH & Co KG, Grieskirchen, Austria) and a 3D fiber optic tip with a spot size of 0.06 cm in diameter (HELBO 3D Pocket Probe, Helbo Photodynamic Systems GmbH & Co KG), with phenothiazine chloride dye (10 mg/ml, Helbo® Blue photosensitizer) (Figure 10). The laser parameters are presented in Table 2.

Figure 10. Diode laser Helbo® (Helbo, Grieskirchen, Austria).

Table 2. Treatment parameters of PDT2

Wavelength: 660nm
Fiber tip: 3D pocket probe
Power output: 100 mW
Power density: 35.37W/cm^2
Irradiation Time: 60 seconds
Distance from the implant: 5mm

3.3.3 Group 3. Light-emitting diode treatment group (PDT3)

The implants were treated with LED curing light (Optilight Ld®, Gnatus, Brazil). The curing light was modified with a red LED light, (660 nm, LZ1-00R205, Ledengin,Inc.®, San Jose, USA). A toluidine blue solution (Biognost®, Zagreb, Croatia) was used as a photosensitive dye. The diameter of the tip was 6 mm (Figure 11). The laser parameters are presented in Table 3.

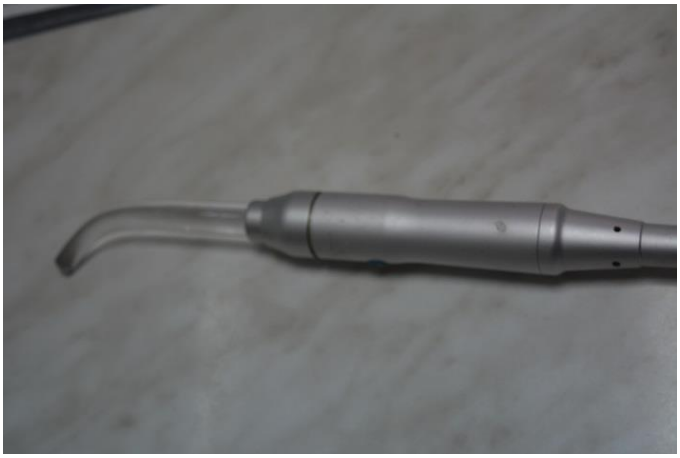

Figure 11. LED lamp (Optilight Ld®, Gnatus, Brazil)

Table 3. Treatment parameters of PDT3

Wavelength: 660nm
Fiber tip: 6mm LED composite curing tip
Power output: 200 mW
Power density: 0.71 W/cm^2
Irradiation Time: 60 seconds
Distance from the implant: 5mm

3.3.4 Decontamination procedures for PDT1, PDT2 and PDT3

The implants were first coated with the photosensitive dye and left for 60 seconds, and then rinsed with sterile saline solution in order to remove the excess of the photosensitive dye. In order to standardize the irradiation treatment protocols for all implants, the implants were placed in a rotational electric motor (Shenzhen Powerful Electronics, Shajing, China), with a power of 12 V, 120 mA with a rotating speed of 10 rounds per minute.

The titanium implants were fixed to the electric motor using an insertion drill (SKY TK Mounter long) which was glued to the motor and then the implants were placed on that drill.

For the zirconia implants, the implant holder from the packaging was used for the same purpose. It was glued to the rotational motor and then the implants were placed in the holder in the same manner as the titanium implants. The light source was placed approximately 5 mm away from the surface of the rotating implant in a static holder and the treatment time was 60 seconds. The treatment procedures for titanium and zirconia implants are shown in Figures 12 and 13.

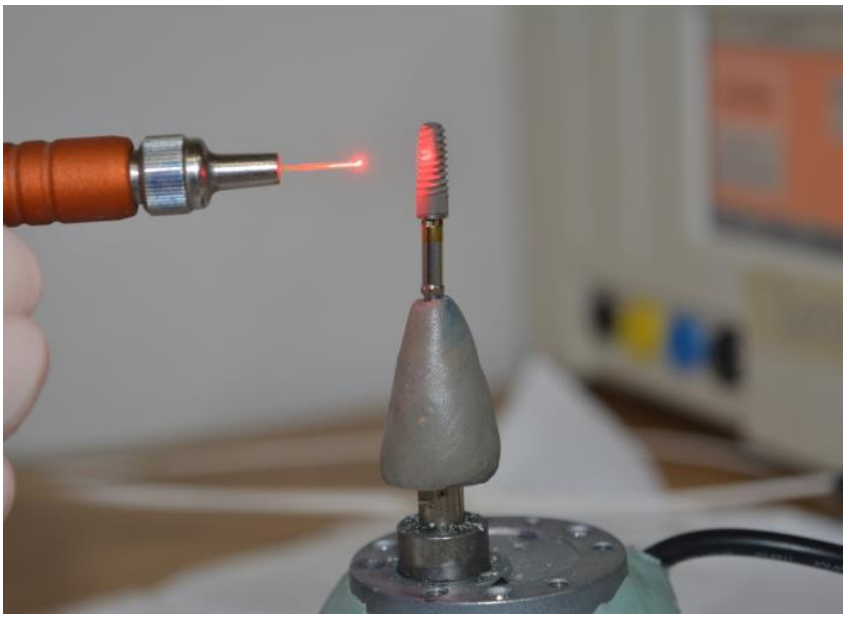

Figure 12. Titanium implant placed in a rotational motor and treated with PDT1.

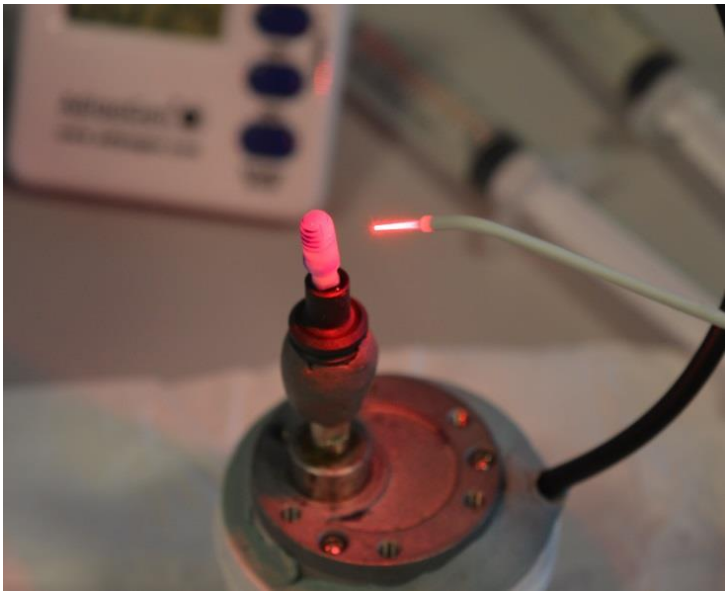

Figure 13. Zirconia implant placed in a rotational motor and treated with PDT2.

3.3.5 Group 4. Toluidine blue treatment (TB)

The implants were immersed in a photosensitive dye (toluidine blue; Biognost®, Zagreb, Croatia) solution (1 mg/ml) for 60 seconds and then they were rinsed with sterile saline solution to remove the excess dye.

3.3.6 Control Groups

In the negative control group (NC), the implants did not receive any treatment, and after their removal from the bacterial suspension, the implants were kept in room conditions for 60 seconds before microbiological analysis.

In the positive control group (PC), the implants were immersed in 0.2% chlorhexidine gluconate solution (Curasept ADS® Curaden International AG, Kriens, Switzerland) for 60 seconds (Figure

14). After their removal from the chlorhexidine solution, the implants were rinsed with sterile saline to remove the remaining solution.

Figure 14. Titanium dental implant immersed in 0.2% chlorhexidine (PC group).

3.4 Microbiological analysis

Immediately after the treatment procedures, every implant was placed in a 1.5 ml Eppendorf test tube containing 500 µl of phosphate buffered saline (PBS) and vortexed for 60 seconds (Vortex, Genius 3, IKA, Germany) to remove the remaining bacterial cells from their surfaces (Figure 15).

Figure 15. Vortex, Genius 3, IKA, Germany.

From each tube, 100 µl were transferred to 100 µl of Mueller Hinton broth, and a volume of 20 µl of PBS was also transferred to a microplate well containing 180 µl of broth creating a 10-fold dilution. Ten-fold serial dilution was performed by using 96-well microtiter plates; 30 µl of suspension from each well was then inoculated to Brucella agar plates (Figures 16 and 17).

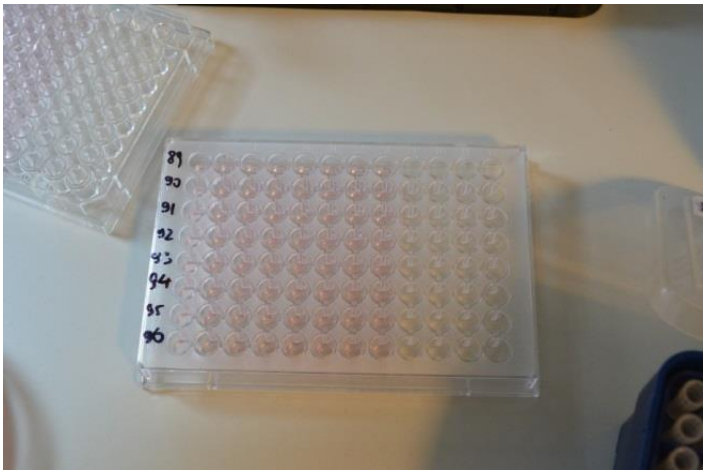

Figure 16. 96-well microtiter plate used for ten-fold serial dilution.

Figure 17. Brucella agar plate with marked dilution prior to inoculation of the suspension.

The plates were incubated in anaerobic conditions for 72 hours and the colony forming units per mililiter (CFU/ml) were counted on Brucella agar plates (Figure 18). Macroscopically distinctive colonies were confirmed with MALDI Biotyper (Bruker Daltonics, Germany).

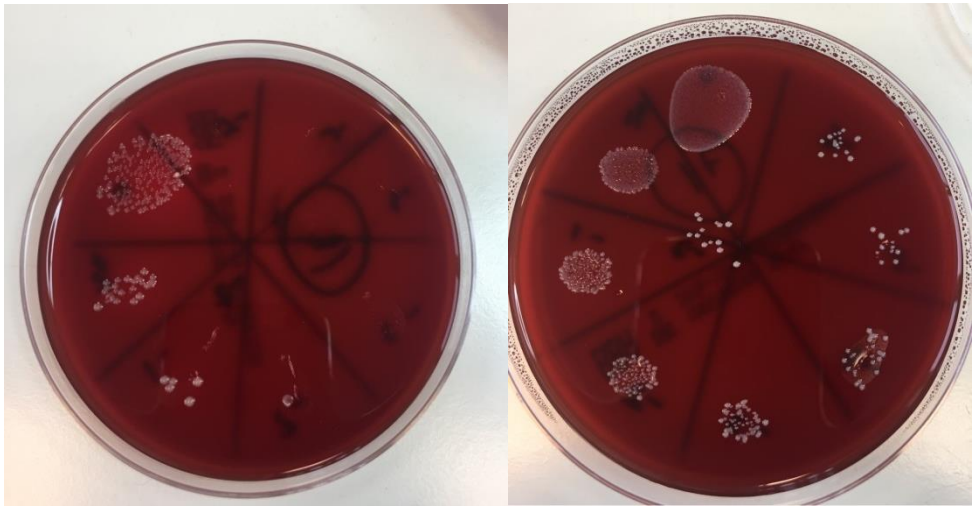

Figure 18. Brucella agar plates with visible colonies of bacteria.

3.5 Scanning electron microscopy analysis

After microbiological analysis, one random implant was chosen from each of the treatment groups and one sterile non-treated implant was chosen for scanning electron microscopy (SEM). The implants for SEM were stored in paraformaldehyde 2% for 2 hours. Then, the implants were dehydrated in increasing concentrations of ethanol (60%, 75% and 95%), for 30 minutes in each and were then left for drying all night. The surfaces of the implants were observed using SEM (Vega TS5136MM, Tescan, Brno, Czech Republic) (Figure 19). The SEM images were taken at 1:250 magnifications under high vacuum (HiVac) with a high voltage (HV) of 30kV. All the images were taken between the fourth and the fifth thread of the implants.

Figure 19. Scanning Electron Microscope (Vega, Brno, Czech Republic).

As for the zirconia implants, they are non conducting material and in order to make the samples conductive and avoid charging of the sample surface, the implants were coated with gold and palladium sputter (SC7620 Mini Sputter Coater, Quorum Technologies Ltd, UK) (Figure 20).

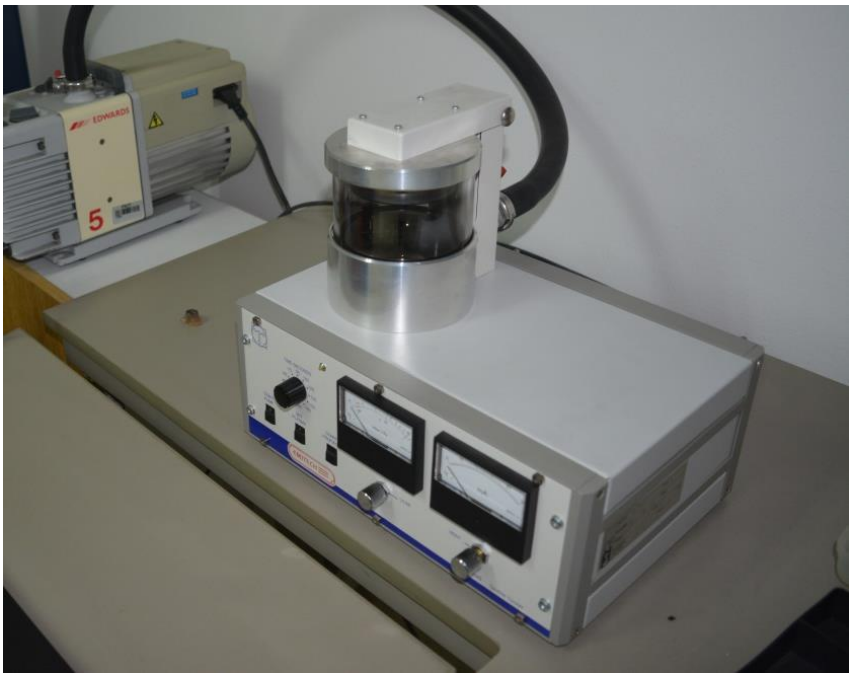

Figure 20. Gold and palladium sputter for the zirconia implants (SC7620 Mini Sputter Coater, Quorum Technologies Ltd, UK).

3.6 Statistical analysis

To determine the difference between the groups for each bacterial species separately and for the total count of bacteria, the obtained data were compared by analysis of variance test (ANOVA). Multiple comparisons between the applied methods were done by Tukey test. The level of significance was set at 5%. Due to the large differences in the standard deviations between the groups, the data were transformed according to the following equation:

$$L=\log_{10}(N+1).$$

To calculate the bacterial log reduction and the reduction in percentage compared to the NC group, the following formulae were used:

$$1-\frac{T}{C}=100\cdot\left(1-\frac{T}{C}\right)\%. \qquad \log_{10}\frac{C}{T}=\log_{10}C-\log_{10}T.$$

Here, T stands for the Treatment group and C stands for the negative control group (NC).

All calculations were performed using the statistical package SAS system for Windows (Release 8.02, SAS Institute Inc., Cary, NC, USA).

4. Results

4.1 Descriptive statistics

Descriptive statistics for each bacteria separately and the total bacterial count for both titanium and zirconia implants are presented in Tables 4-11. They are presented in CFU/ml (colony forming units per mililiter) and also transformed in logarithmic form.

Table 4. Titanium implants; Descriptive statistics for *A.Actynomycetemcomitans* (mean and standard deviation) in Cfu/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	208,457	(331,464)	3.3	(2.2)
PDT2	192,427	(570,940)	3.1	(2.0)
PDT3	60,520,200	(170,844,881)	5.4	(2.3)
TB	84,338,383	(117,993,238)	6.2	(2.3)
PC	44,172,684	(87,638,353)	4.7	(2.7)
NC	121,192,667	(289,930,108)	6.5	(1.7)

Table 5. Titanium implants; Descriptive statistics for *P. gingivalis* (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	6,643,624	(20,035,962)	3.7	(2.5)
PDT2	254,624	(588,104)	2.8	(2.4)
PDT3	28,306,092	(61,302,933)	5.2	(2.2)
TB	34,253,542	(43,280,627)	6.2	(2.0)
PC	16,670,794	(31,716,093)	4.7	(2.3)
NC	328,280,033	(678,053,479)	6.8	(1.9)

Table 6. Titanium implants; Descriptive statistics for *P. intermedia* (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	6,683,834	(12,299,136)	4.3	(2.4)
PDT2	16,801,042	(57,693,469)	3.6	(2.4)
PDT3	158,917,850	(267,478,190)	5.4	(3.1)
TB	179,250,925	(270,408,827)	6.7	(2.4)
PC	75,007,679	(121,537,940)	4.9	(2.7)
NC	342,008,583	(554,467,604)	7.0	(2.2)

Table 7. Titanium implants; Descriptive statistics for the total number of bacteria (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	13,534,248	(24,854,759)	4.7	(2.3)
PDT2	17,239,759	(58,193,987)	3.9	(2.3)
PDT3	247,744,142	(466,066,101)	6.1	(2.5)
TB	297,842,850	(352,941,629)	7.0	(2.2)
PC	135,851,158	(202,357,373)	5.4	(2.6)
NC	791,481,283	(1,496,114,298)	7.4	(1.8)

Table 8. Zirconia Implants; Descriptive statistics for *A.Actynomycetemcomitans* (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	19	(57)	0.4	(0.8)
PDT2	7	(12)	0.4	(0.6)
PDT3	61	(107)	0.8	(1.1)
TB	7,270	(15,323)	2.4	(1.3)
PC	128	(287)	1.2	(1.0)
NC	2,858,333	(5,731,009)	5.9	(0.7)

Table 9. Zirconia Implants; Descriptive statistics for *P. gingivalis* (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	19	(57)	0.4	(0.8)
PDT2	3	(5)	0.3	(0.5)
PDT3	21	(57)	0.6	(0.7)
TB	1,908	(5,726)	1.9	(1.1)
PC	127	(233)	0.9	(1.2)
NC	6,804,167	(19,961,421)	5.7	(1.0)

Table 10. Zirconia Implants; Descriptive statistics for *P. intermedia* (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	31	(57)	0.8	(0.9)
PDT2	10	(17)	0.5	(0.7)
PDT3	40	(75)	0.8	(0.9)
TB	2,809	(6,240)	2.3	(1.2)
PC	736	(1,199)	1.5	(1.5)
NC	13,321,833	(29,535,046)	5.9	(1.3)

Table 11. Zirconia Implants; Descriptive statistics for the total number of bacteria (mean and standard deviation) in CFU/ml and transformed data (mean and standard deviation) in logarithmic form.

Group	Mean	St. dev	Mean (log)	St. dev (log)
PDT1	69	(169)	0.9	(1.0)
PDT2	20	(32)	0.7	(0.8)
PDT3	122	(183)	1.1	(1.2)
TB	11,987	(24,717)	2.9	(1.2)
PC	991	(1,472)	1.8	(1.5)
NC	22,984,333	(37,486,543)	6.7	(0.9)

4.2 Results of Multivariate analysis of variance (MANOVA)

To determine the difference among the groups and between the two types of implants, MANOVA test was applied. Results of the test are given in Table 12.

Table 12. Results of MANOVA analysis.

	A.actynomycete-mcomitans			*P. gingivalis*			*P. intermedia*			**Total**			Wilks' lambda
Factor	DF	SS	p*	DF	SS	p*	DF	SS	p*	DF	SS	p*	p
Implants	1	328.8	**< 0.001**	1	378.7	**< 0.001**	1	403.2	**< 0.001**	1	419.5	**< 0.001**	**< 0.0001**
Group	5	336.2	**< 0.001**	5	337.2	**< 0.001**	5	304.8	**< 0.001**	5	359.6	**< 0.001**	**< 0.0001**
Implants*Group	5	55.3	**0.003**	5	50.3	**0.005**	5	44.8	**0.045**	5	60.5	**0.003**	**0.0001**

*-p-value for ANOVA test

Wilks's lambda statistic showed that there was a significant difference in the number of bacteria between types of implants and between different groups ($p<0.0001$ for both factors, MANOVA test). Interaction between groups and implants type was also significant ($p=0.0001$, MANOVA test). Since all factors and interactions were significant, ANOVA test was applied to the number of each bacterium separately and to the total number of bacteria. All ANOVA tests showed that two factors and their interaction were significant (Table 12).

The comparison between titanium and zirconia implants, regardless of the study groups, showed that for all three types of bacteria separately, as well as for the total number of bacteria, there was a significantly lower number of bacteria on zirconia implants (Table 13).

Tukey test was applied for the comparison among the study groups regardless of the type of implant. Regarding the total number of bacteria, the lowest number of bacteria was found in PDT1 and PDT2, followed by PDT3 and PC without significant difference among them. NC group had significantly the largest number of bacteria when compared to the other groups (Table 13).

Identical results were obtained for the number of *A. actynomycetemcomitans*, *P. gingivalis* and *P. intermedia* separately (Table 13).

Table 13. Number of bacteria by factors.

	A. actynomycetemcomitans					***P. gingivalis***				
Factor	N	mean	st.d.		p*	N	mean	st.d.		p*
Implants										
Zirconia	72	1.9	(2.1)		**<0.0001**	72	1.6	(2.1)		**<0.0001**
Titanium	72	4.9	(2.5)			72	4.9	(2.6)		
Group										
PDT1	24	1.9	(2.2)	b	**<0.0001**	24	2.0	(2.5)	b	**<0.0001**
PDT2	24	1.8	(2.0)	b		24	1.6	(2.1)	b	
PDT3	24	3.1	(2.9)	ab		24	2.9	(2.8)	ab	
TB	24	4.3	(2.7)	a		24	4.0	(2.7)	a	
PC	24	2.9	(2.7)	ab		24	2.8	(2.6)	ab	
NC	24	6.2	(1.3)			24	6.2	(1.6)		
	P. intermedia					**Total**				
Factor	N	mean	st.d.		p*	N	mean	st.d.		p*
Implants										
Zirconia	72	2.0	(2.1)		**<0.0001**	72	2.3	(2.3)		**<0.0001**
Titanium	72	5.3	(2.7)			72	5.8	(2.5)		
Group										
PDT1	24	2.6	(2.5)	b	**<0.0001**	24	2.8	(2.6)	b	**<0.0001**
PDT2	24	2.0	(2.3)	b		24	2.3	(2.4)	b	
PDT3	24	3.1	(3.2)	ab		24	3.6	(3.2)	ab	
TB	24	4.5	(2.9)	a		24	5.0	(2.7)	a	
PC	24	3.2	(2.7)	ab		24	3.6	(2.7)	ab	
NC	24	6.4	(1.8)			24	7.1	(1.5)		

The total number of bacteria for the different groups and two implants type are shown in Figure 21. The difference between zirconia implants instead of titanium implants is not the same for all groups. The smallest difference between them in the number of bacteria is for the control group. The impact is almost the same for PDT1, PDT2, PC and TB, while the largest difference between titanium and zirconia implants were in the PDT3 group. The results for each of the bacteria separately are shown in Figures 22-24.

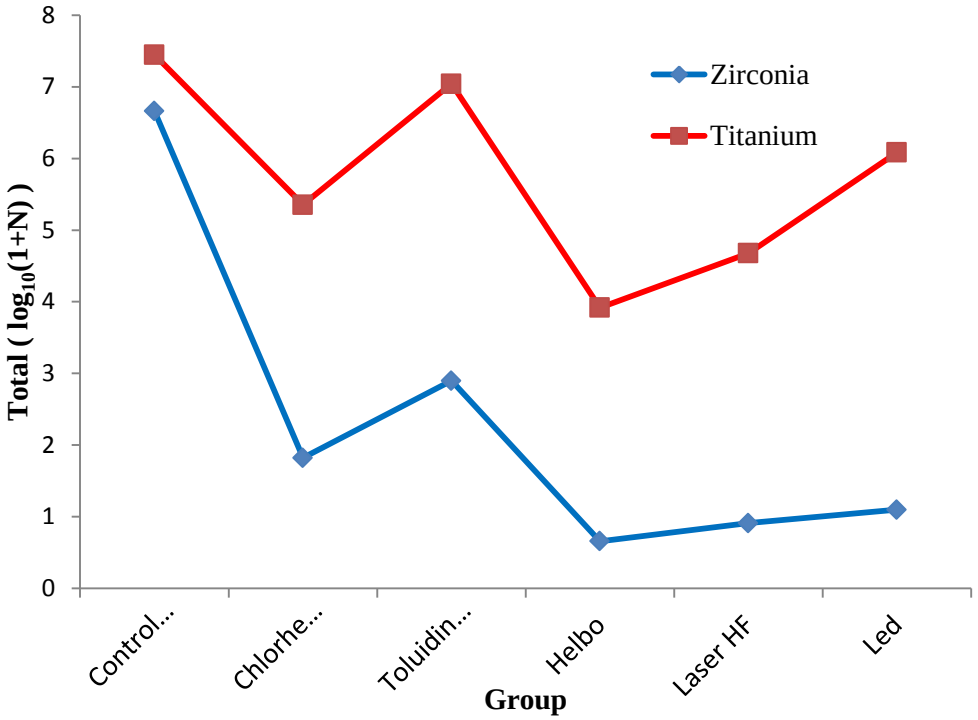

Figure 21. Comparison of total number of bacteria by type of implant and group.

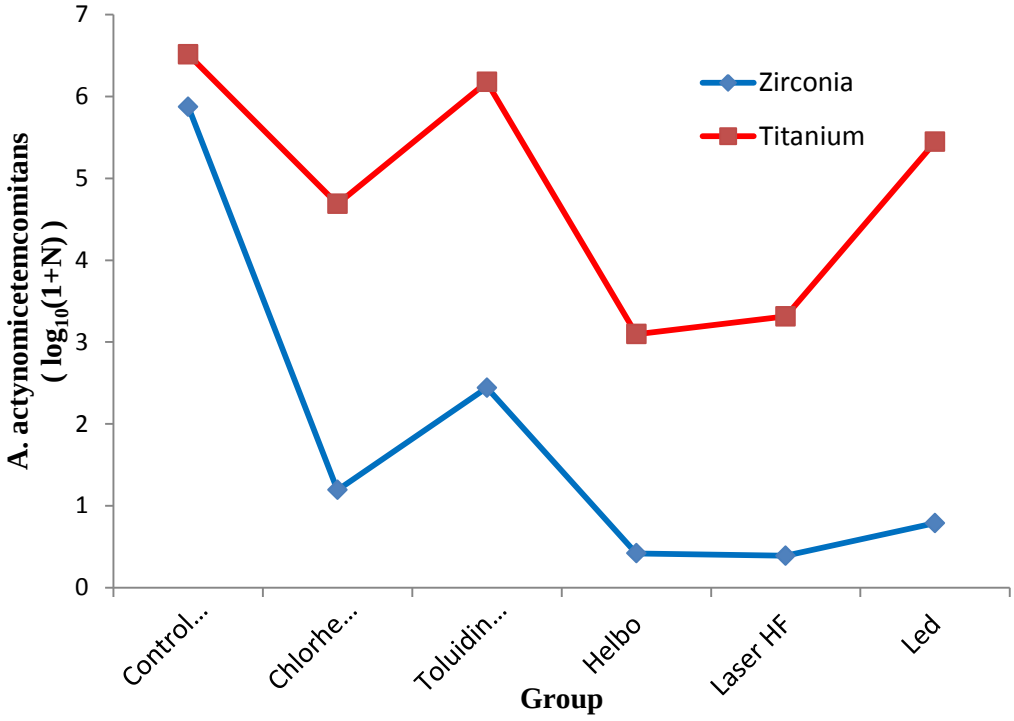

Figure 22. Comparison of the number of A. actinymycetemcomitans by type of implant and group.

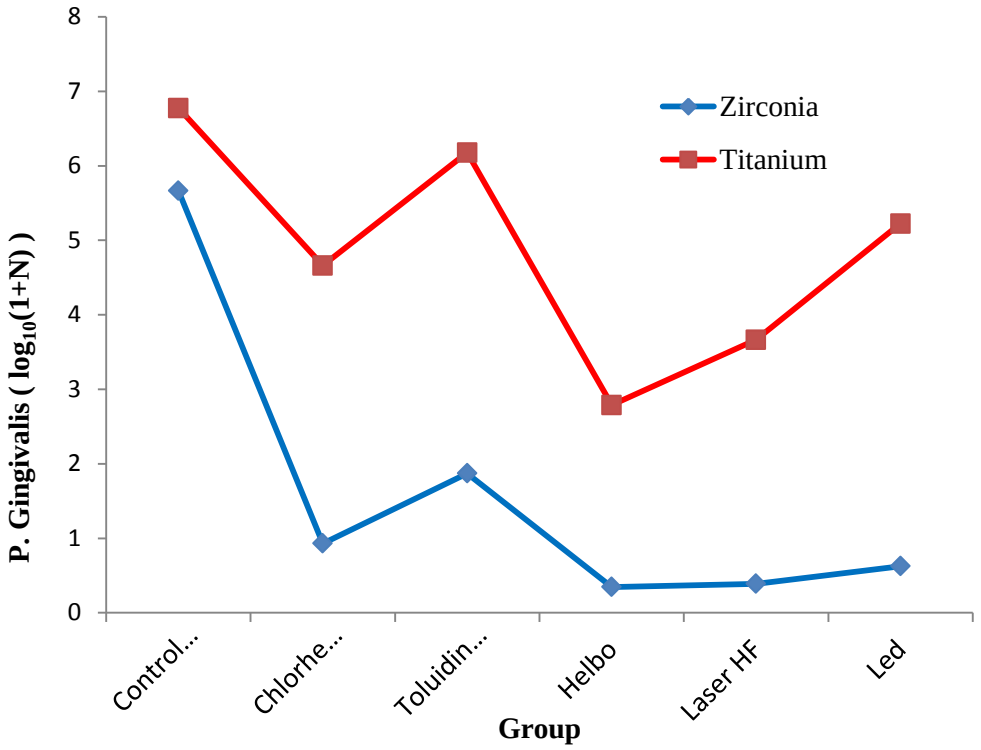

Figure 23. Comparison of the number of P. gingivalis by type of implant and group.

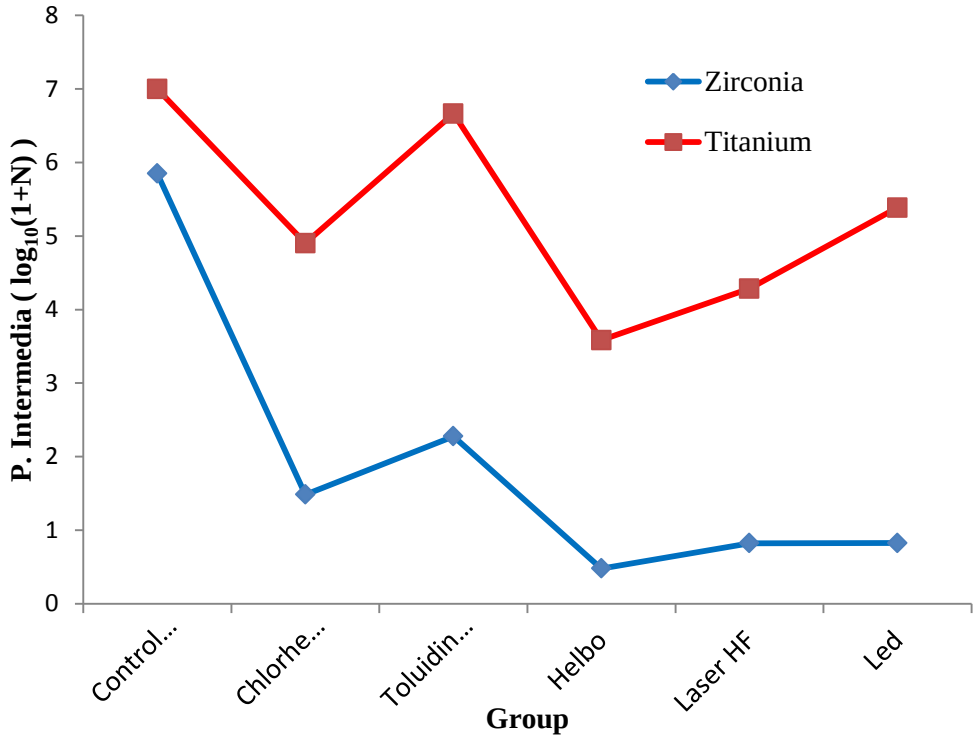

Figure 24. Comparison of the number of P. intermedia by type of implant and group.

4.2.1.1 Titanium implants

For the titanium implants, the results showed that there were statistically significant differences among the groups for each bacterial species separately and also for the total number of bacteria (p=0.0022). These data are presented in logarithmic form in Table 14. The bacterial reduction compared to the negative control group, expressed in percentage and log reduction, is shown in Table 15. The largest bacterial reduction in term of the total count of bacteria was recorded in the PDT1 (98.3%) and PDT2 (97.8%) groups. These two groups were significantly superior compared to NC ($p<0.05$). PDT3 group caused a 68.7% (Table 15) bacterial reduction and did not show significant differences when compared to NC (Table 14).

Table 14. Results of ANOVA and Tukey's post hoc test for the Titanium implants

	A. actynomycetemcomitans					***P. gingivalis***					Wilks' lambda
Group	N	mean	st.d.		p*	N	mean	st.d.		p*	p
PDT1	12	3.3	2.2	b	**0.0006**	12	3.7	2.5	bc	**0.0003**	**0.0026**
PDT2	12	3.1	2.0	b		12	2.8	2.4	c		
PDT3	12	5.4	2.3	ab		12	5.2	2.2	abc		
TB	12	6.2	2.3	a		12	6.2	2.0	ab		
PC	12	4.7	2.7	ab		12	4.7	2.3	abc		
NC	12	6.5	1.7	a		12	6.8	1.9	a		
	P. intermedia					**Total**					Wilks' lambda
Group	N	mean	st.d.		p*	N	mean	st.d.		p*	p
PDT1	12	4.3	2.4	ab	**0.0096**	12	4.7	2.3	bc	**0.0022**	**0.0026**
PDT2	12	3.6	2.4	b		12	3.9	2.3	c		
PDT3	12	5.4	3.1	ab		12	6.1	2.5	abc		
TB	12	6.7	2.4	a		12	7.0	2.2	ab		
PC	12	4.9	2.7	ab		12	5.4	2.6	abc		
NC	12	7.0	2.2	a		12	7.4	1.8	a		

* - p-value for ANOVA test

abc - result of post-hoc comparison (Tukey test). Means with the same letter are not significantly different.

The PDT1 and PDT2 groups also showed the largest bacterial reduction when compared to each of the bacteria separately. Compared to the NC, the PDT1 group was significantly more effective in the eradication of *A. actinomycetemcomitans* and *P. gingivalis* ($p<0.05$), however without significant difference in the eradication of *P. intermedia*. The PDT2 group was significantly more effective in the eradication of each of the bacteria when compared to the NC group ($p<0.05$).

The toluidine blue group (TB) was the least effective compared to other study groups with only 62.4% bacterial reduction; moreover, it did not differ significantly compared to the NC in terms of the total number of bacteria or for each of the bacteria separately.

Table 15. Bacterial reduction compared to the NC group in percentage and log reduction for the titanium implants.

	A.actynomycetemcomitans				***P. gingivalis***			
Group	Mean	(St.d.)	Reduction %	Log reduction	Mean	(St.d.)	Reduction %	Log reduction
PDT1	2.08E+05	3.31E+05	99.8	2.9	6.64E+06	2.00E+07	98	1.5
PDT2	1.92E+05	5.71E+05	99.8	2.7	2.55E+05	5.88E+05	99.9	3.1
PDT3	6.05E+07	1.71E+08	50.1	0.2	2.83E+07	6.13E+07	91.4	1
TB	8.43E+07	1.18E+08	30.4	0.4	3.43E+07	4.33E+07	89.6	1.2
PC	4.42E+07	8.76E+07	63.6	0.5	1.67E+07	3.17E+07	94.9	1.3
NC	1.21E+08	2.90E+08			3.28E+08	6.78E+08		
	P. intermedia				**Total**			
Group	Mean	(St.d.)	Reduction %	Log reduction	Mean	(St.d.)	Reduction %	Log reduction
PDT1	6.68E+06	1.23E+07	98	1.7	1.35E+07	2.49E+07	98.3	1.8
PDT2	1.68E+07	5.77E+07	95.1	1	1.72E+07	5.82E+07	97.8	1.4
PDT3	1.59E+08	2.67E+08	53.5	0.3	2.48E+08	4.66E+08	68.7	0.5
TB	1.79E+08	2.70E+08	47.6	0.3	2.98E+08	3.53E+08	62.4	0.6
PC	7.50E+07	1.22E+08	78.1	0.7	1.36E+08	2.02E+08	82.8	0.9
NC	3.42E+08	5.54E+08			7.91E+08	1.50E+09		

4.2.1.2 Zirconia implants

For the zirconia implants, the results showed statistically significant differences among the groups for each bacterial species separately and also for the total number of bacteria ($p<0.0001$). The bacterial reduction results showed a huge bacterial reduction for every group compared to the NC. The results are presented in Table 16 and the bacterial reduction in percentage is presented in Table 17.

Table 16. Results of ANOVA and Tukey's post hoc test for the Zirconia implants

	A. actynomycetemcomitans					***P. gingivalis***					Wilks' lambda
Group	N	mean	st.d.		p*	N	mean	st.d.		p*	p
PDT1	12	0.4	0.8	a	**0.0001**	12	0.4	0.8	b	**0.0001**	**0.0001**
PDT2	12	0.4	0.6	a		12	0.3	0.5	b		
PDT3	12	0.8	1.1	a		12	0.6	0.7	b		
TB	12	2.4	1.3			12	1.9	1.1	a		
PC	12	1.2	1.0	a		12	0.9	1.2	ab		
NC	12	5.9	0.7			12	5.7	1.0			
	P. intermedia					**Total**					Wilks' lambda
Group	N	mean	st.d.		p*	N	mean	st.d.		p*	p
PDT1	12	0.8	0.9	b	**0.0001**	12	0.9	1.0	b	**0.0001**	**0.0001**
PDT2	12	0.5	0.7	b		12	0.7	0.8	b		
PDT3	12	0.8	0.9	b		12	1.1	1.2	b		
TB	12	2.3	1.2	a		12	2.9	1.2	a		
PC	12	1.5	1.5	ab		12	1.8	1.5	ab		
NC	12	5.9	1.3			12	6.7	0.9			

* - p-value for ANOVA test

abc - result of post-hoc comparison (Tukey test). Means with the same letter are not significantly different.

In terms of the total count of bacteria every group had a statistically significant difference when compared to the NC ($p<0.05$). The bacterial reduction was more than 99% in each group. The PDT1, PDT2 and PDT3 had the largest bacterial reduction for each bacterium separately, as well as for the total count of bacteria. In addition to the difference to the NC group, these three groups also had statistically significant differences compared to the TB group ($p<0.05$). Between them, however, the differences in bacterial reduction were small and there was no statistically significant difference neither for each of the bacteria separately nor for the total count of bacteria ($p>0.05$).

Table 17. Bacterial reduction compared to the NC group in percentage and log reduction for the titanium implants.

	A. actynomycetemcomitans				***P. Gingivalis***			
Group	Mean	(St.d.)	Reduction %	Log reduction	Mean	(St.d.)	Reduction %	Log reduction
PDT1	1.92E+01	5.73E+01	99.9	5	1.92E+01	5.73E+01	99.9	5.5
PDT2	6.67E+00	1.23E+01	99.9	5.7	3.33E+00	4.92E+00	99.9	6.6
PDT3	6.08E+01	1.07E+02	99.9	4.7	2.08E+01	5.66E+01	99.9	5.5
TB	7.27E+03	1.53E+04	99.7	2.6	1.91E+03	5.73E+03	99.9	3.5
PC	1.28E+02	2.87E+02	99.9	4.3	1.27E+02	2.33E+02	99.9	4.9
NC	2.86E+06	5.73E+06			6.80E+06	2.00E+07		
	P. Intermedia				**Total**			
Group	Mean	(St.d.)	Reduction %	Log reduction	Mean	(St.d.)	Reduction %	Log reduction
PDT1	3.08E+01	5.66E+01	99.9	5.7	6.92E+01	1.69E+02	99.9	5.3
PDT2	1.00E+01	1.65E+01	99.9	6.3	2.00E+01	3.19E+01	99.9	6.1
PDT3	4.00E+01	7.53E+01	99.9	5.6	1.22E+02	1.83E+02	99.9	5.3
TB	2.81E+03	6.24E+03	99.9	3.7	1.20E+04	2.47E+04	99.8	3.2
PC	7.36E+02	1.20E+03	99.9	4.4	9.91E+02	1.47E+03	99.9	4.4
NC	1.33E+07	2.95E+07			2.30E+07	3.75E+07		

The TB group had the lowest bacterial reduction for every bacterium separately and also for the total bacterial count. Moreover, the TB group did not have a statistically significant difference for *A. actinomycetemcomitans* compared to the NC ($p>0.05$). The PC group had lower bacterial reduction compared to PDT1, PDT2 and PDT3, but without statistically significant differences among them. It also did not differ significantly compared to the TB in terms of the total bacterial count, *P. gingivalis* and P. *intermedia*. It had a significant difference compared to the TB only for *A. actinomycetemcomitans.*

4.3 Scanning Electron Microscope analysis

The SEM images obtained from the PDT1, PDT2, and PDT3 groups visually did not show any surface alterations, cracks, or damage when compared to the images obtained for the sterile implants, and their surface appeared to be very similar to the surface of the sterile implant. The same results were obtained for both titanium and zirconia implants. The obtained images are shown in 1:250 magnification in Figures 25-32.

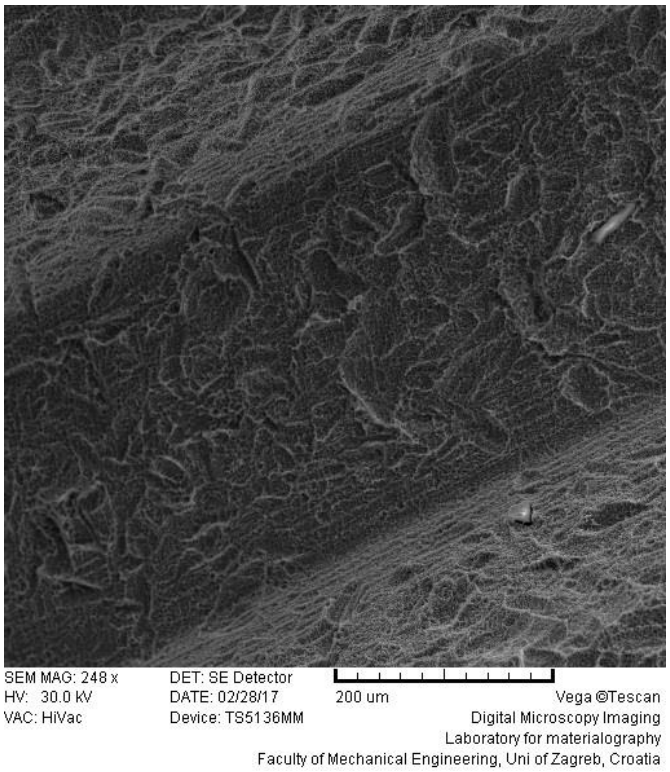

Figure 25. Sterile titanium implant; magnification 1:250.

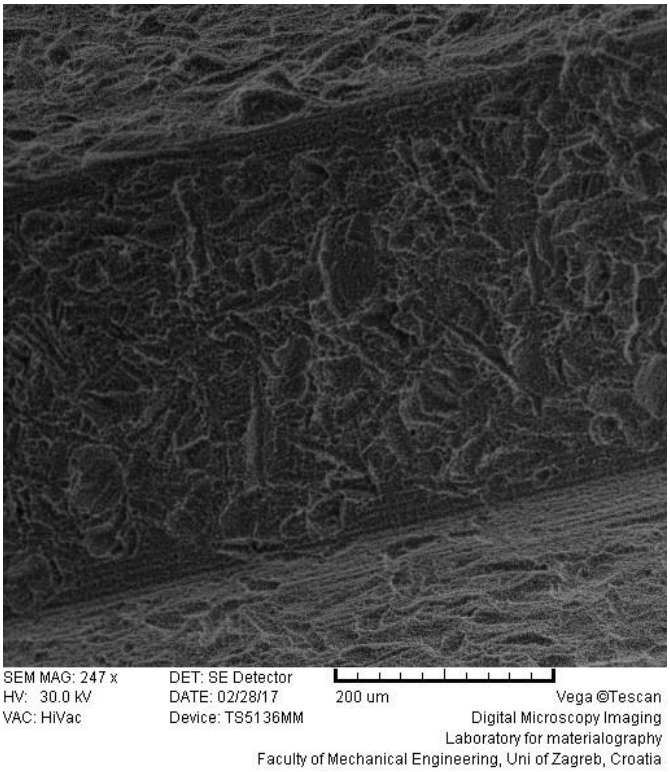

Figure 26. Titanium implant treated with PDT1; magnification 1:250.

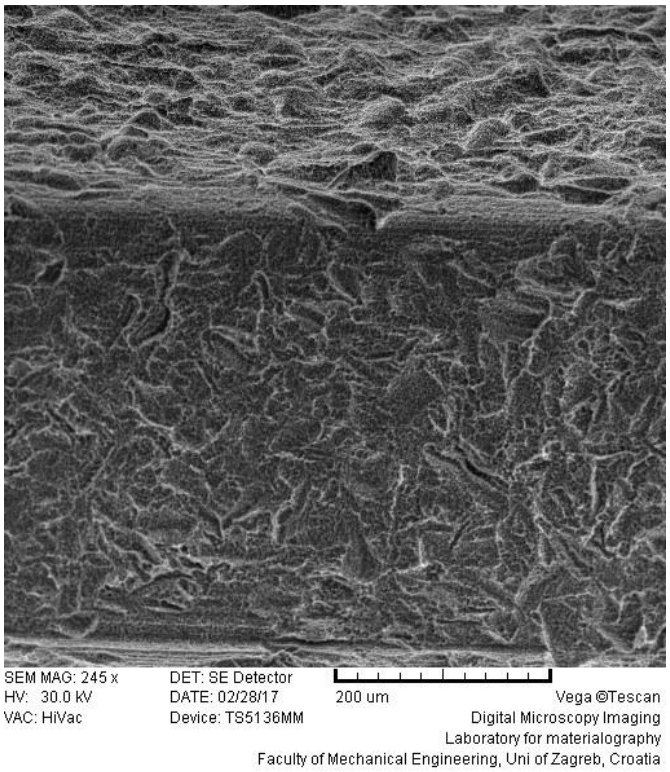

Figure 27. Titanium implant treated with PDT2; magnification 1:250.

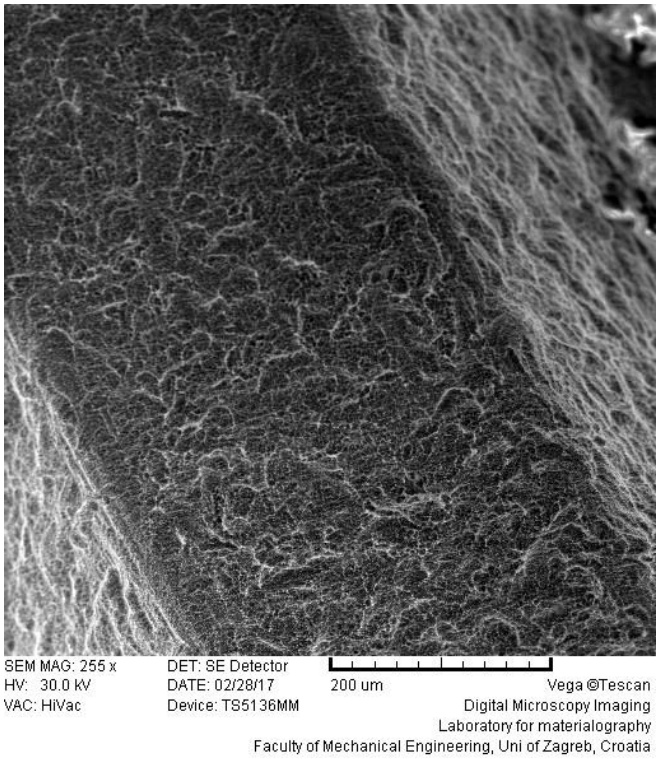

Figure 28. Titanium implant treated with PDT3; magnification 1:250.

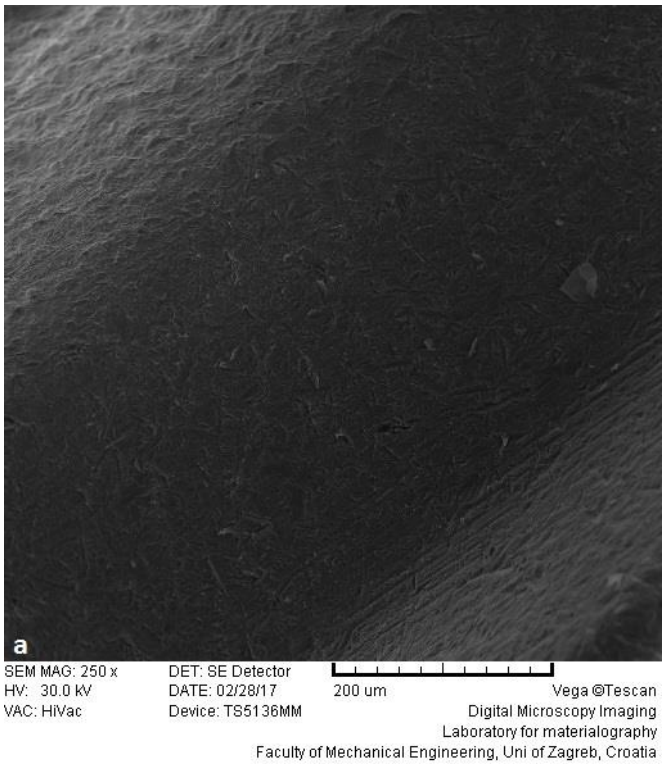

Figure 29. Sterile zirconia implant; magnification 1:250.

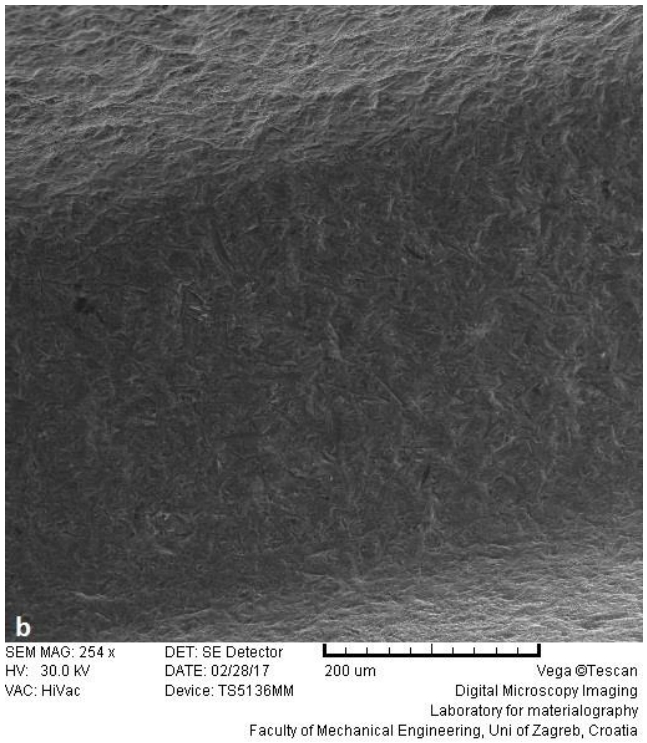

Figure 30. Zirconia implant treated with PDT1; magnification 1:250.

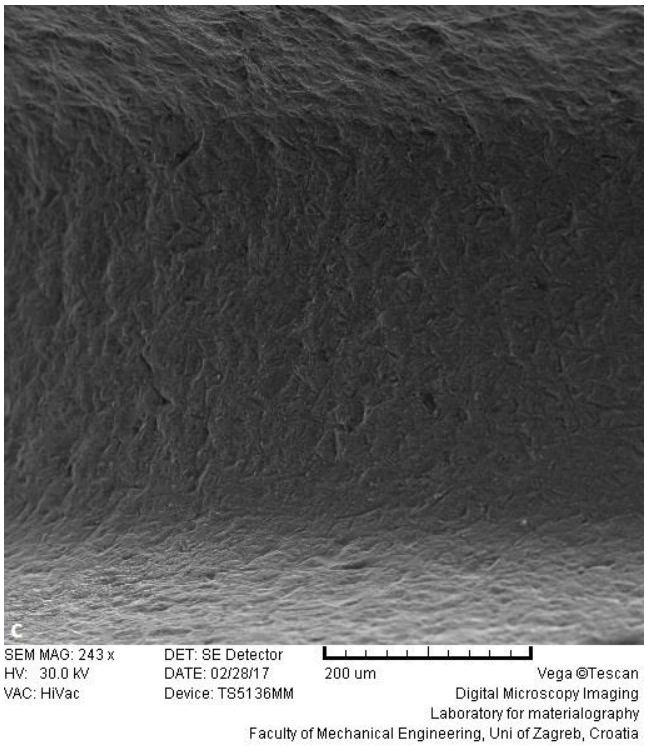

Figure 31. Zirconia implant treated with PDT2; magnification 1:250.

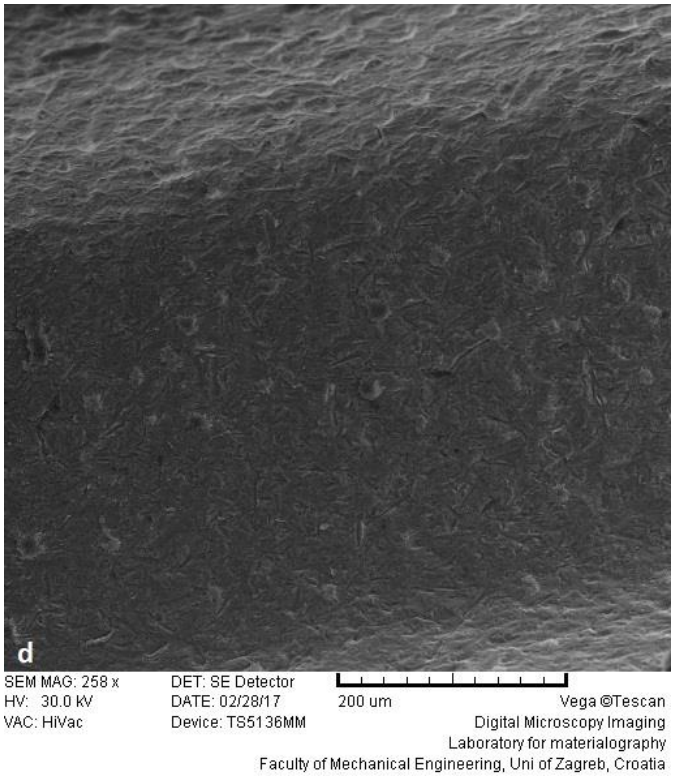

Figure 32. Zirconia implant treated with PDT3; magnification 1:250.

5. Discussion

Peri-implantitis is considered to be one of the main causes of implant failure. There are numerous studies that report various prevalence rates of peri-implant disease due to different reporting methods and study characteristics (173–176). Van Velsen et al. (173) reported a 7% rate of peri-implantitis in their 10-year prospective cohort study. Meijer et al. (174) in their study reported that 29.7% of patients after 10 years were affected by peri-implantitis. According to Atieh et al. (175), in their meta-analysis peri-implantitis affects 18.8% of patients and 9.6% of the implants. Fardal et al. (176) report an even higher number of patients affected with peri-implantitis. In their retrospective study they conclude that patients initially treated for periodontitis have a prevalence of peri-implantitis of 53.5% at the patient level and 31.1% at the implant level.

The lack of a clear protocol for treating peri-implantitis has increased the focus of the scientific community towards the use of photodynamic therapy as a treatment option or an adjuvant treatment for peri-implantitis in the recent years (11,177,178).

Photodynamic therapy is a promising alternative when treating periodontal diseases and peri-implant diseases. In the present study, the effect of photodynamic therapy was evaluated on artificially contaminated dental implants under *in-vitro* conditions. The contamination of the implants was performed in order to reproduce the adhesion stage of biofilm formation. A similar methodology has been used in many other studies with *in vitro* contamination and decontamination of titanium implants (11,177,179).

The main focus of the study was to determine if photodynamic therapy is efficient as compared to the negative control group (NC) and to the conventional disinfection with chlorhexidine solution (PC). Furthermore, the focus was to investigate if different types of devices and photosensitizers affect the results of aPDT and whether different bacteria react differently to aPDT depending on the photosensitizer and the light source.

When decontaminating the implant surfaces it is of utmost importance not to cause damage to the implant surface which might interfere with the re-osseointegration of the implant (9,180). For this purpose, in this study the implants were examined under scanning electron microscope in order to evaluate if aPDT causes any surface alterations.

5.1 The effect of aPDT on titanium implants

Recent advances in the production of titanium dental implants aim to achieve a faster osseointegration through roughening the implant surfaces. The challenges of decontaminating rough titanium surfaces lies in the fact that the surface roughness in addition to better and faster osseointegration, also enables the bacteria to adhere more to the surface, which makes it difficult for the conventional treatment methods to successfully eliminate the bacteria from the implant surface (160).

The obtained results from the present study showed that PDT1 and PDT2 groups caused great bacterial reduction when compared to the NC group (98.3% and 97.8%). The NC group, as expected, had the greatest bacterial count among all other groups. Both PDT1 and PDT2 groups were a combination of a diode laser as a light source and a photosensitizer.

The results of this study are in accordance with other *in vitro, in vivo* and clinical studies (9,11,177,181,182). In a similar *in vitro* study, Marotti et al. (11) showed that aPDT using a GaAlAs low-level diode laser in combination with methylene blue dye is effective against the bacteria present in peri-implantitis. The irradiation time did not influence the results, as both subgroups of aPDT with respective irradiation time of 3 minutes and 5 minutes had similar results without significant differences between them. The effect of aPDT was comparable to the positive control with 0.12% chlorhexidine solution. Similar results were obtained in our study when PDT1 and PDT2 were compared to the PC group, even though the concentration of chlorhexidine used in our study was 0.2%.

In another study Haas et al. (9) showed that short term exposure (60 seconds) to light and photosensitizer can effectively kill *A. actinomycetemcomitans, P.gingivalis* and *P.intermedia.* In their study they used a 905nm light source with a power output of 7.3mW and toluidine blue as a photosensitizer. Even though we obtained similar results in the present study, the light source used was between 600-700nm and a higher power output was used.

In an *in vivo* study, Shibli et al. (181) evaluated the effect of GaAIAs low-level diode laser with a wavelength of 685nm for a duration of 80 seconds in combination with toluidine blue on dogs. They concluded that this combination shows a significant reduction and in some cases the

elimination of the pathogenic bacteria associated with peri-implantitis. In another study by Shibli et al. (182), guided bone regeneration and aPDT was used to treat ligature-induced peri-implantitis in dogs. According to their results, there was up to 41.19 % better re-osseointegration in the test groups compared to the control group, which suggests a very good effect of aPDT.

In human studies, the combination of a diode laser with a wavelength of 690 nm with toluidine blue O for 60 seconds has been shown to be effective against *P.gingivalis, P. intermedia and A. actinomycetemcomitans* in decreasing their count by 92%. However, the complete elimination of the bacteria was not demonstrated (177).

In the present study the effect of aPDT was also evaluated on each bacterium separately. With regard to *A.actinomycetemcomitans and P. gingivalis,* the results were almost identical to the total bacterial count with PDT1 and PDT2, both being significantly different compared to the NC. However, regarding the effect on the bacterial count of *P.intermedia,* only PDT2 was significantly different when compared to the NC. PDT1 and PDT2 had very similar effect without significant difference between them.

PDT3 was the least effective treatment group among the PDT groups and did not differ significantly compared to the NC or to the PC groups, having even lower bacterial reduction than the PC group regarding the total number of bacteria. The same results were obtained for the number of each bacterium separately. However, it must be pointed out that in contrast to PDT1 and PDT2, the implants belonging to the PDT3 group were treated using a modified dental LED curing light and not with a laser light source. This was done in order to test the LED light as an alternative light source to lasers.

Many recent studies have tested the efficacy of LED lights as a photodynamic light source, yet only a few of them have been conducted on implant surfaces. The results of these studies vary and are dependent on the study design, power output, irradiation time, and the photosensitizer used. Cho et al. (30) in their study tested the efficacy of a green LED light with the power output of 150 mW/cm2 in combination with erythrosine against *A.actinomycetemcomitans*. According to their results, this combination is very effective in reducing the bacteria attached to different titanium surfaces, with the reduction reaching up to 92.4% for a 60 second irradiation of the implant surface. The irradiation was done on titanium discs on only one surface providing a uniform irradiation of the surface. On the other hand, in the present study the light was applied in

a rotating implant in order to copy as much as possible the clinical conditions of applying the light source around the dental implant. This might be the reason why there is a difference in the efficacy of aPDT with a LED light source with their study, even though the power output of the LED device in the present study was 200mW and a 60 second irradiation time was also used.

In another study, Nielsen HK et al. (43) concluded that the combination of toluidine/red light has an excellent antimicrobial effect compared to riboflavin/blue light. Similarly, Umeda M et al. (183) reported a good bactericidal effect when using a light emitting diode in combination with methylene blue or toluidine blue.

There are also studies that question the effectiveness of using a LED light for aPDT treatment. De Angelis et al. (184) in their clinical study treated 40 patients with mechanical debridement by hand, ultrasonic or piezoelectric scalers in combination with a LED light source and 40 patients with the same protocol without aPDT. According to their results, after 4 months there was no significant difference for any of the clinical parameters, such as pocket depth reduction, bleeding on probing (BOP) and clinical attachment levels (CAL) between the groups.

In the present study, our results showed a reduction of only 68.7% in the total count of bacteria. Regarding the number of bacteria separately, the efficacy of the PDT3 ranged from 50.1% reduction of *A.actinomycetemcomitans* to 91.4% reduction of *P.gingivalis.* However, statistically none of these results was significantly different when compared to the NC.

We assume that the difference in results between PDT3 and the other two study groups (PDT1 and PDT2) might be due to the differences in power density. Power density is dependent on the power output of the device and the light beam diameter. Since the device used for this research was a LED curing light and the light beam diameter was larger than that in PDT1 and PDT2, it might be the reason why PDT3 was less effective than PDT1and PDT2.

Another goal of the present study was to evaluate if different photosensitizers react differently with the bacteria used in our study. The most common photosensitizers used for aPDT treatments are phenothiazine derivatives. They are also the most effective photosensitizers for eradicating oral microorganisms (43). However ,comparing photosensitizers in *in vitro* conditions is very difficult due to the differences in absorption by the photosensitizer and bacteria (10). Moreover, some bacteria have the capability of producing endogenous photosensitizers (f.e. *Porphyromonas gingivalis*), a property that further proves the difficulty in comparing photosensitizers in *in vitro*

conditions (185). In the present study we did not find any differences between the groups (PDT1 and PDT2) that were treated by using different photosensitizers (toluidine blue and phenothiazine chloride) in combination with a light source regarding the total bacterial count.

The use of only diode laser light without the application of photosensitizer has already been shown to be less effective compared to the effect of aPDT (11). In the present study, the use of a photosensitizer without the application of a light source (TB group) was evaluated. Based on the obtained results, it turned out to be the least effective study group in the present study. There was no significant difference between this group and the negative control group, in terms of the total bacterial count or in terms of each bacterial species, separately. As in many other studies, this further proves that in order to have an effective photodynamic therapy there must be an interaction between the light source and the photosensitizer. The use of the photosensitizer or light alone are not effective and are not recommended as a treatment option (21, 28, 29).

5.2 The effect of aPDT on zirconia implants

There are many studies that evaluate the effect of aPDT *in vitro* on titanium implant surfaces, animal studies and clinical studies. However, to the best of our knowledge, there are no published studies evaluating the antimicrobial effect of aPDT on zirconia implant surfaces.

In the present study the results showed that, even though there are differences among the study groups, they all had significantly lower bacterial counts compared to NC. This huge difference when compared to the NC group might suggest that the bacteria were not strongly attached to the implant surface after 72h of incubation. According to other studies, the affinity of bacteria to attach to zirconia is significantly lower than titanium surfaces due to their surface properties, such as surface roughness and surface free energy (146,186). Scarano et al. (146) in their study placed titanium and zirconium oxide discs in the mouths of 10 patients for 24h in order to evaluate the adhesion of bacteria in both surfaces. According to their results, zirconium oxide surfaces showed significantly less bacteria compared to titanium discs. Al-Radha et al. (186) in their study showed similar results. The zirconia material and titanium blasted with zirconia surface showed superior effect to titanium material in reducing the adhesion of bacteria, especially after coating with

saliva pellicle. In addition to the weak attachment of bacteria on the implant surface, it could be assumed that the rinsing of the photosensitizer might have caused an additional detachment of bacteria from the implant surface.

When comparing the study groups among themselves as expected, the most effective were PDT1, PDT2 and PDT3. In addition to NC, they differed significantly from the TB group also. When compared to PC, even though they had lower bacterial counts, there was no difference among them. It is worth noting that the results obtained from the PDT3 group are comparable to PDT1 and PDT2, which can suggest that with alternative light sources, such as light-emitting diodes, an effective antibacterial effect could be achieved. There are conflicting results regarding the antimicrobial effect of using light-emitting-diode as a light source. There are studies that report good results after the use of LED lights as a light source (43,183), however there are also studies that report that the effect of using LED light for photodynamic therapy does not significantly improve the treatment outcomes (184). However, it is difficult to compare our results with any of these studies due to the differences in the study protocol and due to the lack of studies conducted on zirconia implant surfaces.

Similarly, with regard to titanium implants, in terms of the use of different photosensitizers among PDT1, PDT2 and PDT3, in our study we could not find any differences as it was expected.

In contrast to the results obtained for the titanium implants, every group in the zirconia implants had a lower number of bacteria. This was expected, as in many studies the zirconia implants are shown to attract less bacteria to their surface due to surface roughness and surface free energy. The largest difference between titanium and zirconia implants was seen in the PDT3 group which was very effective for the zirconia implants. Mellinghoff (187) in his review of literature on peri-implant soft tissues concluded that zirconia implants and abutments provide a very good soft tissue interface and irritation free attachment. Reduced plaque adhesion, better healing response and less inflammatory infiltrate around zirconia implants when compared to titanium are reported in many *in vitro* and *in vivo* studies (146,147,188).

Similarly to the results obtained for the titanium implants, there were no significant differences among different photosensitizers used when aPDT was applied on zirconia implants.

The least effective among the study groups was the TB group. A similar result was also obtained for the titanium implants in the present study.

In regard to the effect of aPDT on zirconia surfaces, further *in vitro* and clinical studies are needed, especially on the potential efficacy of LED light as a light source for aPDT treatment.

5.3 The effect of aPDT on implant surfaces

In addition to the antimicrobial effect of PDT, the aim of the present study was to examine if PDT causes physical alterations on implant surfaces. In the present study the use of two different diode lasers and one LED light was evaluated and compared to sterile and unopened (in original package), titanium and zirconia implants under scanning electron microscope.

Laser decontamination of implant surfaces has been shown to be effective in many studies. However, it was shown that some lasers cause damage to the implant surface. Kreisler et al. (169) in their study concluded that Nd:YAG and Ho:YAG lasers significantly damage the surfaces studied at any power settings which makes them unsuitable for the decontamination of implant surfaces. Er:YAG and CO2 lasers damage the implant surfaces at specific settings and should be used at a limited power output. Miranda et al. (189) evaluated the effect of Er,Cr:YSGG (Eribium, Cromium: yttrium-scandium-gallium-garnet) laser with 1.5 W/20 Hz, air-water cooling proportion of 80 %/25 %, on zirconia and titanium implants. They concluded that the application of Er,Cr:YSGG alters the surface roughness of both zirconia and titanium implant surfaces.

In contrast, the use of diode lasers in many studies has been shown to be safe. Castro et al. (190) in their study evaluated whether 980-nm diode laser irradiation causes damage on implant surfaces. They reported that the use of this laser does not damage titanium surfaces, and seems to be safe irrespective of power output for use on titanium surfaces. Similar results were reported in a study conducted by Romanos et al. (191). According to their results, the use of diode laser with a wavelength of 980nm and power settings of 5.0, 10.0, and 15.0 W respectively, in continous mode does not cause any damage to titanium plasma-sprayed (TPS), and hydroxyapatite (HA) coated implant surfaces.

In the present study we did not observe any structural changes on the implant surfaces. The use of two types of diode lasers with 100mW power for 60 seconds, and the use of LED lamp with 200mW power for 60 seconds, did not cause visible damage on either titanium or zirconia at a magnification of 1:250.

Our findings are in accordance with a previous study conducted by Haas R. et al. (9), in which they examined the titanium implant surfaces after the treatment with aPDT and compared their findings with sterile implants. They reported that aPDT is a safe method that does not cause any surface alterations on titanium surface.

To the best of our knowledge, the present study is the first of its kind regarding the evaluation of the different types of aPDT on zirconia implant surfaces. This further proves that PDT can be safely used for the decontamination of implant surfaces without concerns regarding potential damage to the implant surfaces both on titanium and zirconia implant surfaces.

6. Conclusion

Considering the limitations of this *in vitro* study on the efficacy of different types of photodynamic therapy and its effect on implant surfaces, the following conclusions can be drawn:

1. Antimicrobial photodynamic therapy using diode lasers (PDT1 and PDT2) significantly decreases the bacterial count when compared to negative control on titanium implants.

2. The effect of antimicrobial photodynamic therapy using diode lasers as a light source (PDT1 and PDT2) is not significantly different from conventional decontamination with 0.2% chlorhexidine solution on titanium implants.

3. Antimicrobial photodynamic therapy using light-emitting diode as a light source (PDT3) is the least effective method of decontamination among the three aPDT groups without significant difference to the NC for titanium implants.

4. The use of toluidine blue photosensitizer only without the application of light is the least effective method of decontamination for titanium implants.

5. All the study groups significantly lowered the bacterial count on zirconia implants compared to the NC group.

6. Antimicrobial photodynamic therapy using diode lasers (PDT1 and PDT2) and light emitting diode (PDT3) is effective on zirconia implants, significantly reducing the bacterial count when compared to the NC and TB groups.

7. The effect of antimicrobial photodynamic therapy (PDT1, PDT2 and PDT3) on zirconia implants is comparable to conventional decontamination with 0.2% chlorhexidine without significant differences among these groups.

8. There was no surface damage or alteration either on titanium or zirconia implants after using antimicrobial photodynamic therapy.

7. Literature

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8. Curriculum Vitae

Bleron Azizi was born on October 12th 1989 in Ohrid, Macedonia. He has graduated at the Faculty of Medicine, Department of Dentistry, University of Prishtina "Hasan Prishtina", Republic of Kosova in 2013 with an average grade of 9.36.

Since March 2014 he has been employed in the private dental clinic Krajkodent in Kichevo, Macedonia.

In October 2014 he started his PhD studies at the School of Dental Medicine, University of Zagreb, Croatia.

In October 2016 he started his residency in Oral Surgery at the University Dental Clinical Centre "St Pantelejmon", in Skopje, Macedonia.

He has actively participated in numerous international scientific conferences and is the author and co-author of several scientific posters and papers.

He is a member of the International Team for Implantology, European Association for Osseointegration, Dental Chamber of Macedonia and Dental Chamber of Kosova.

List of published articles:

Scientific articles:

1. **Bleron Azizi**, Ana Budimir, Ivona Bago, Blerim Mehmeti, Suzana Jakovljevic, Jeta Kelmendi, Aleksandra Presecki Stanko, Dragana Gabric. Antimicrobial efficacy of photodynamic therapy and light-activated disinfection on contaminated zirconia implants: an in vitro study. Photodiagnosis and Photodynamic Therapy 2018;21:328-333

2. **Bleron Azizi**, Ana Budimir, Blerim Mehmeti, Suzana Jakovljevic, Ivona Bago, Elizabeta Gjorgievska, Dragana Gabric. Antimicrobial efficacy of photodynamic therapy and light-activated disinfection against bacteria species on titanium dental implants. The International Journal of Oral & Maxillofacial Implants (Accepted for publication on December 27th 2017**)**

3. Blerim Mehmeti , Željko Alar, Matija Sakoman , **Bleron Azizi,** Jeta Kelmendi, Donika Iljazi-Shahiqi, Sandra Anić-Milošević. Comparison of shear bond strength of metal and ceramic orthodontic brackets bonded to zirconium crowns. Acta Stomatologica Croatica 2017;51(1):165-173

4. Blerim Mehmeti, Fehim Haliti, **Bleron Azizi**, Jeta Kelmendi, Donika Iljazi-Shahiqi, Suzana Jakovljević, Sandra Anić-Milosević. Influence of different orthodontic brackets and chemical preparations of ceramic crowns on shear bond strength. Australasian Medical Journal (Accepted Article in Press)

Abstracts published in international journals:

1. **Bleron Azizi,** Ana Budimir, Blerim Mehmeti , Jeta Kelmendi, Dragana Gabric. The effect of antimicrobial photodynamic therapy (aPDT) and light-activated disinfection (LAD) on contaminated titanium and zirconia implants - in vitro study. Acta stomatol Croat. 2017;51(4):350-365 (Oral Presentation)

2. Igor Smojver, Mato Suši c, Elizabeta Gjorgievska, Vanja Vuievi Boras , **Bleron Azizi**, Dragana Gabric. Densitometric analysis of prf vs xenograft for sinus augmentation procedures 5 years follow- up. Clinical Oral Implants Research. 2017; 510-510. (Poster).

3. **Bleron Azizi**, Ana Budimir, Blerim Mehmeti, Dragana Gabric. The antimicrobial effect ofphotodynamic therapy on the surfacesof dental implants – in vitro pilot study. Lasers in Surgery and Medicine. 2017;49(4):417–67). (ePosters)

4. Jana Barić , **Bleron Azizi** , Igor Smojver , Dragana Gabrić. Minimally invasive esthetic and reconstructive mucogingival surgery after excision of epulis - case report. Acta stomatol Croat. 2017;51(1):75-85 (Poster)

5. Nena Matulić , Vanja Vučićević Boras , Vlaho Brailo , **Bleron Azizi** , Dragana Gabrić Comparison of efficacy of digitally controlled er:yag laser and isotretinoin for treatment of oral precancerous lesions. Acta stomatol Croat. 2017;51(1):75-85 (Poster)

6. **Bleron Azizi,** Blerim Mehmeti, Nena Matulić, Dragana Gabrić. Retrieval of fractured implant screws using ultrasonic scaler - a case report. Acta stomatol Croat. 2017;51(1):75-85 (Poster)

7. Blerim Mehmeti, **Bleron Azizi**, Donika Ilijazi, Jeta Kelmendi, Granita Muhaxheri. Orthodontic prosthetic management of endomaxilla with hypodontia of most of maxillary teeth; a case report. Acta stomatol Croat. 2015;49(2):167-185. (Poster)

8. Donika Ilijazi-Shahiqi, Jeta Kelmendi, Blerim Mehmeti, **Bleron Azizi**, Blerim Kamberi. Evaluation of dental needs among patients in the University Dental Clinical Center of Kosova. Acta stomatol Croat. 2015;49(2):167-185. (Poster)

9. **Bleron Azizi**, Blerim Mehmeti, Donika Ilijazi-Shahiqi, Blerina Azizi. Immediate implant placement in post extraction socket without grafting; two stage surgery protocol. Acta stomatol Croat. 2015;49(4):351-365. (Poster)