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INFLUENCE OF CO2 LASER IRRADIATION STRATEGY ON TITANIUM SURFACE MODIFICATION AND BACTERIAL BIOFILM CONTAMINATION: AN IN VITRO STUDY

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ABSTRACT

BACKGROUND: This study investigates the effects of CO_2 laser surface treatment on grade V commercially pure titanium (CP Ti) to improve its antibacterial properties. It evaluates the impact of this process on the implant's surface characteristics, including topography, and hydrophilicity.

METHODS: A total of 22 Grade V CP Ti discs were CNC-machined into 8mm × 3mm, polished, and divided into two groups: Ti/control and Ti/laser (n=11 per group). The Ti/laser samples were subjected to CO_2 laser treatment at a 1064 nm wavelength with a power of 6 watts. Surface properties were examined using a wettability test and atomic force microscopy (AFM). To assess bacterial adhesion, biofilms of S. epidermidis, S. aureus, E. coli, and P. aeruginosa were grown on the titanium surfaces of both groups. Dead bacteria were stained with Propidium Iodide (PI) fluorescent dye, visualized under a fluorescent scanning microscope, and quantified using ImageJ software. The obtained data were analyzed statistically.

RESULTS: The mean surface roughness (Ra) of the laser-treated samples was measured at 229.97 nm, which was significantly greater than the 72.09 nm observed in the control group. Contact angle measurements indicated values of 83.19° for Ti/control and 41.39° for Ti/laser, demonstrating a substantial increase in hydrophilicity for the laser-treated surfaces. Fluorescent imaging of dead bacteria revealed a significantly higher count on Ti/laser surfaces (338.18) compared to the control group (6.92) ($P < 0.0001$*).

CONCLUSION: The CO_2 laser effectively modified CP Ti surfaces, producing nano-textured, hydrophilic surfaces with antibacterial properties. This enhancement reduced biofilm formation.

KEYWORDS: CO2 laser, Titanium implants, Bacterial biofilm

RUNNING TITLE: CO2 Laser Effects on Titanium Surface & Biofilm: In Vitro Study

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INTRODUCTION

Lasers stand as a major technological revolution in the 20th century, progressively gaining dominance in various daily life applications, particularly within the medical field. Their unique optical radiation modifies surfaces from micro to nano scales, altering adhesion properties for surface characteristics customization. This benefits medical equipment, like dental implants, by surface engineering to prevent biofilm development [1].

Implants provide a reliable solution for tooth loss, boasting a 90% success rate among fully or partially edentulous patients. However, they are prone to inflammatory issues [2]. Titanium, the gold standard for dental implants, offers remarkable mechanical properties, corrosion resistance, higher elastic modulus than bone, exceptional fracture resilience, and superior tissue compatibility compared to alternative metals [3]. Despite these qualities, laser irradiation systems have been developed to enhance titanium surfaces [4], optimizing the osteogenic response for improved osseointegration by modifying surface features, and wetting capacity [5, 6]. Laser light induces surface oxidation, aiding in oxygen diffusion within the molten metal, creating morphologies like laser-induced periodic surface structures (LIPSSs) [7].

High-energy lasers in dentistry modify titanium surfaces through fusion, vaporization, ablation, and solidification, creating diverse textures. Laser wavelength and mode significantly impact surface features. Pulsed lasers like Yttrium Aluminum Garnet (YAG) and CO2 yield energy peaks exceeding 10^6 W/cm^2 [5, 8]. Studies stress evaluating laboratory setups before clinical use, considering parameters like wavelength, pulse duration, repetition rate, fluence, intensity, and treatment duration [9].

Commercially Ti6Al4V alloys lack inherent antibacterial properties, indicating a persistent risk of bacterial colonization [10]. In recent decades, enhancing implant surface antimicrobial properties has become crucial in combating bacterial infections. Coatings containing agents like Cu, Ag, etc., are employed using different techniques to deter bacterial attachment and biofilm formation. Thin coatings are vulnerable to damage, reducing antibacterial efficacy, these coatings release antibacterial agents for a limited duration, addressing early post-surgical infections. Enhancing the durability of antibacterial coatings remains a pressing need [11]. Topography plays a significant role in bacterial adherence, micro- or nano-topographical patterns on titanium surfaces offer a promising strategy to impart antimicrobial properties [12]. Interest in developing new antimicrobial therapies is increasing due to the potential escalation of drug resistance [13].

A biofilm is a well-organized community structure consisting of bacteria adhered to diverse solid surfaces, accompanied by the generation of extracellular polysaccharides (EPSs) [14], matrix proteins, and extracellular DNA (eDNA) [15-18]. Accordingly, bacteria develop heightened infectivity and resistance to drugs. The majority of These infections are predominantly instigated by S. aureus and S. epidermidis, along with Gram-negative bacilli [15, 17, 19], E. coli, and P. aeruginosa [18, 20]. The typical biofilm life cycle is attachment, maturation, and dispersal. Lasers create precise patterns on metal surfaces, enhancing titanium implants' biocompatibility. However, there is Limited research on microbial colonization of laser-treated surfaces [12].

From this perspective, this research aims to evaluate the efficacy of the CO_2 laser strategy used for engineering Grade V commercially pure titanium (CP Ti) surfaces, with the creation of antibacterial surface properties against bacterial biofilms. The null hypothesis stated that CO_2 laser irradiation will not alter titanium surface properties.

MATERIALS AND METHODS

2.1. Specimen preparation

A Grade V commercially pure (CP) titanium alloy block (KERA TI 5-DISC) was used to manufacture titanium discs measuring 8mm × 3mm (N=22) [21]. The disc design was created using Auto-cut software (Version 6.0160115, China) and fabricated with a Computer Numerical Control (CNC) electron discharge machine (DK7740, China). A single operator polished the discs sequentially with silicon carbide abrasive papers of 180, 320, and 1200 grits [21, 22]. To eliminate potential contaminants, the discs underwent ultrasonic cleaning (T-14, L&R manufacturer, USA) with distilled water and acetone for 15 minutes [23], followed by air drying. The samples were then randomly assigned to two groups (n=11 each): the Ti/control group, consisting of unmodified titanium discs, and the Ti/laser group, which featured CO_2 laser-engineered surfaces. The sample size was determined assuming a 5% alpha error and 80% study power, and calculated by a software program (G*Power 3.1.9.7). The mean [±SD] percent surface coverage of biofilm was found to be 68.71 [±9.50] for untreated titanium surfaces and 52.53 [±14.34] for laser-treated titanium surfaces [12].

2.2. Laser treatment

The surfaces of the titanium discs were modified using a continuous-wave CO_2 fiber laser (ML025-CA, USA) with a wavelength of 1064 nm, an energy output of 120 millijoules, a power setting of 6 watts, and a frequency of 50 hertz. A single clinician performed the treatment, applying three consecutive repetitions. Each session lasted 15 seconds and employed a perpendicular linear scanning mode, with a spot size of 400 microns. [23, 24].

2.3. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) imaging was performed at room temperature using contact mode [22]. Surface analysis was conducted with a scanning probe microsco pe (SPM-9600, Japan) fitted with a silicon cantilever (NCHR-20; Nano World AG, Neuchâtel, Switzerland). Surface roughness (Ra) was measured in nanometers through scans covering an area of 40µm × 40µm, with the results visualized as colored 3D images [25].

2.4. Contact angle test

For the surface wettability assessment, a Rame-hart contact angle goniometer (Model 190, USA) equipped with DROP image CA v2.5 software was used. A micro-syringe dispensed 2 µL of distilled water onto the titanium disc surfaces [26]. Contact angle measurements were recorded as the average of three readings taken at different locations within 20 seconds of water droplet placement [27].

2.5. Bacterial biofilm preparation

For bacterial adhesion and biofilm formation analysis, five bacterial species were cultured: Gram-positive *Staphylococcus epidermidis* (12228) *Staphylococcus aureus* (29213), along with Gram-negative *Escherichia coli* (25922) and *Pseudomonas aeruginosa* (27853). The bacteria were grown overnight for 20 hours in Müller Hinton Broth (MHB; Oxoid) at 37°C, with continuous shaking at 100 rpm in a gyratory incubator. After incubation, the bacterial cultures were standardized to an optical density (OD) of 0.3 at 550 nm and further diluted 1:50 in fresh sterile MHB, producing an inoculum of approximately 1×10^7 Colony Forming Units (CFU)/ml. Titanium discs were then submerged in the prepared bacterial suspension within a sterile 6-well plate and incubated for 24 hours at 37°C under continuous agitation at 100 rpm. This procedure was designed to evaluate bacterial interactions with the titanium surfaces [28-31]

2.6. Fluorescent scanning microscope analysis

For antimicrobial analysis of the biofilm, titanium discs from both the control and laser-treated groups were removed from the bacterial inoculum and washed with sterile Phosphate Buffered Saline (PBS) [32]. Following this, the discs were stained with a fluorescent dead cell marker, Propidium Iodide (PI), for 30 minutes at 37°C while shielded from light [28]. The stained biofilms were then analyzed using confocal fluorescence microscopy (Leica TCS SPE, Germany) equipped with imaging software (Leica LASX, Germany). Dead cells, indicated by red fluorescence, were selectively stained due to their compromised membranes. The extent of bacterial cell damage was quantified using ImageJ software (NIH, U.S.), which measured the red fluorescence-covered area [33]. This analysis was performed for each titanium disc, and biofilm coverage was calculated based on fluorescence intensity patterns.

2.7. Statistical analysis

Normality was assessed using the Shapiro-Wilk test and Q-Q plots. Surface roughness and surface wettability were normally distributed while the count of dead bacteria was not normally distributed. Group comparison was conducted using an independent t-test and Mann-Whitney U test. All tests were conducted with two tails, and the significance level was set at a p-value of ≤0.05. Data analysis was conducted using IBM SPSS, version 23 for Windows, Armonk, NY, USA.

RESULTS

3.1. Atomic force microscopy (AFM)

The evaluation of surface nano-roughness using atomic force microscopy (AFM) indicated that the control titanium discs had an average Ra value of 72.09 nm with a standard deviation of ±1.83. In comparison, the laser-treated discs demonstrated a significantly higher Ra value, averaging 229.97 nm with a standard deviation of ±44.30 ($P < 0.0001$) (Table 1). These findings were further supported by AFM-generated 2D profiles and 3D topographical images. The Ti/control samples (Figure 1a) displayed a relatively smooth surface, whereas the Ti/laser samples (Figure 1b) exhibited a distinct spiky texture, emphasizing the alterations in surface morphology due to laser treatment.

Table 1: Comparison of surface nano-roughness parameter (Ra) as measured by AFM between Ti/control and Ti/laser groups

Ra values in nanometers	**Ti/control (n=11)**	**Ti/laser (n=11)**	***p* value†**
Mean ±SD	72.09 ±1.83	229.97 ±44.30	<0.0001*
Median	73.54	234.89	
Min – Max	70.00 – 73.83	169.00 – 292.80	

*Statistically significant different at *p* value≤0.05, †Independent t test

3.2. Contact angle test

The Ti/laser discs demonstrated significantly enhanced hydrophilicity, with a mean contact angle of 41.39° (SD ±1.40), compared to the Ti/control specimens, which had a mean contact angle of 83.19° (SD ±0.35) ($P < 0.0001$) (Table 2). This difference is visually represented in Figure 2(a, b), where the results of the Rame-hart contact angle tests for both Ti/control and Ti/laser samples are depicted.

Table 2: Comparison of surface wettability between Ti/control surfaces and Ti/laser ones

Contact angle	**Ti/control (n=11)**	**Ti/laser (n=11)**	***p* value†**
Mean ±SD	83.19 ±0.35	41.39 ±1.40	<0.0001*
Median	83.00	40.50	
Min – Max	82.90 – 83.90	40.30 – 43.40	

*Statistically significant different at *p* value≤0.05, †Independent t test

3.3. Fluorescent scanning microscope analysis

For microbial biofilm analysis, Table 3 presents the mean values of dead-stained bacteria for both control and laser-treated titanium samples. The Ti/control discs had a mean value of 6.92 (SD ±1.24), whereas the Ti/laser specimens showed a significant increase, reaching 338.18 (SD ±148.84) (P < 0.0001). Fluorescence microscope images (Figure 3a, b) provide a visual representation of red fluorescence staining, which indicates dead bacterial cells and helps quantify biofilm coverage. A substantial rise in red fluorescence-stained dead bacteria was observed on Ti/laser surfaces (Figure 3b) in contrast to Ti/control surfaces (Figure 3a).

Table 3: Comparison of dead bacterial number between Ti/control and Ti/laser groups

Dead bacterial number	**Ti/control (n=11)**	**Ti/laser (n=11)**	***p* value†**
Mean ±SD	6.92 ±1.24	338.18 ±148.84	<0.0001*
Median	6.50	327.00	
Min – Max	6.00 – 10.00	140.00 – 650.00	

*Statistically significant different at *p* value≤0.05, †Mann Whitney U test

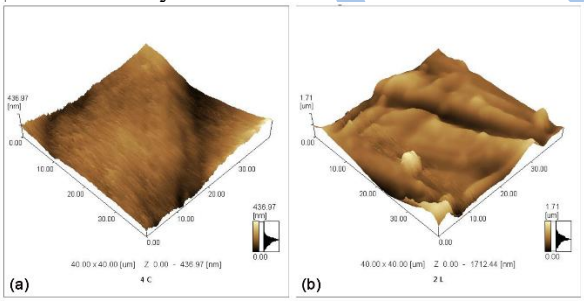

Figure 1. The atomic force microscope displays 3D surface nano-roughness maps and 2D profiles of titanium discs, where (a) corresponds to Ti/control specimens, and (b) illustrates Ti/laser-treated surfaces.

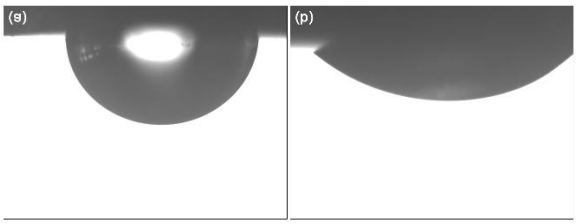

Figure 2. Contact angle images from the Rame-Hart test on Ti/control (a) and Ti/laser (b) specimens show that all surfaces display hydrophilic properties, with contact angles measuring below 90°.

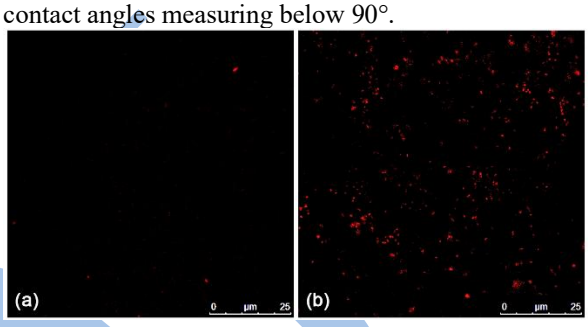

Figure 3. Confocal fluorescence microscopy images: (a) shows dead-stained bacteria on a Ti/control surface, while (b) depicts the Ti/laser surface. A significant increase in dead bacteria is observed on laser-treated surfaces compared to the control.

DISCUSSION

Titanium treated with a laser improves the success of dental implants by altering surface characteristics. To optimize implant surfaces, it is crucial to understand these modifications and their effects on bacterial interactions. The CO2 laser was selected due to its wavelengths, which have low absorption in titanium, preventing excessive heat generation [34]. Surface modifications by lasers depend on factors such as energy density, focal position, power, frequency, and the number of scanning passes [5]. Laser parameters can either smooth or texture surfaces, with high frequency and power leading to polishing, while low frequency and power create roughness [35]. The depth of a laser scan increases progressively with each additional pass due to greater energy absorption[36]. This study utilized a CO_2 laser with a 1064 nm wavelength, 120 mJ energy, 6 W power, and 50 Hz frequency. The laser was applied in a perpendicular linear scanning mode, with three passes lasting 15 seconds each and a spot size of 400 microns. Atomic force microscopy measurements revealed a significant increase in surface roughness compared to the control group (P < 0.0001*), with laser-treated surfaces exhibiting a mean Ra value of 229.97 nm ± 44.30. The surface morphology exhibited a wavy pattern with a spiky texture [37]. A study reported that low laser power combined with a high pulse repetition rate generates smoother surfaces but may cause micro-

cracks due to rapid cooling [35]. Similarly, Ma C et al. [38] observed that laser treatment creates a polished surface with visible melting pathways. In contrast, the findings of this study revealed that, high laser power and a low pulse repetition rate result in rougher surfaces. This occurs due to increased pulse energy, which shifts the process from polishing to laser surface ablation.

In this study, the contact angle measurements for the control and laser-treated titanium were recorded as 83.19 ± 0.35 and 41.39 ± 1.40, respectively ($P < 0.0001$*). In contrast, a separate investigation on titanium alloys reported different results, revealing distinct surface patterns and reduced roughness. These outcomes were achieved using nanosecond laser processing, which led to highly hydrophilic surfaces. This effect is mainly attributed to an increase in surface oxides and a decrease in surface impurities [39], While Yang C-J et al. [40] documented a rise in contact angle values, wettability is affected by multiple factors beyond laser processing, such as elemental composition and surface characteristics. Conversely, this study revealed that CO_2 laser treatment on titanium surfaces lowered contact angle measurements while increasing nano-scale roughness, ultimately enhancing hydrophilic properties.

To analyze bacterial biofilms, titanium discs from both groups were cultured with different bacterial strains. These included gram-positive species such as S. epidermidis and S. aureus, along with gram-negative strains like E. coli and P. aeruginosa, which are commonly utilized for toxicity assessment. [30]. Implant-related bacterial infections, which result in peri-implantitis, may lead to failure due to poor fitting, biofilm development, and insufficient antimicrobial properties. Ensuring strong antimicrobial efficacy is essential to prevent colonization and infection. [32]. Fluorescent staining of control titanium revealed a mean value of 6.92 (SD ±1.24), which increased significantly to 338.18 (SD ±148.84) following laser treatment. This indicates a substantial rise in the number of dead bacteria on Ti/laser surfaces ($P < 0.0001$). Scheuerman TR et al. [41] stated that bacteria are believed to preferentially adhere to rougher surfaces for three main reasons: (i) a larger surface area available for attachment, (ii) increased protection from shear forces, and (iii) chemical alterations that facilitate favorable physicochemical interactions. However, according to Yang K et al. [42] surface patterns approximately 1 µm in size exhibit anti-adhesive properties. Moreover, Perera-Costa et al. [43] demonstrated that the investigated microtopographies led to a significant decrease (approximately 30–45%) in bacterial adhesion compared to smooth control samples. This reduction, attributed to spatially organized micro/nanotopographic surface patterns, supports the findings of the current study by highlighting an effective strategy for minimizing bacterial adhesion and preventing biofilm formation. These findings indicate that CO_2 laser irradiation generates nano-roughness on titanium surfaces, which inhibits bacterial biofilm colonization and lowers the risk of implant failure, thereby rejecting the null hypothesis.

CONCLUSION

The study revealed several key insights. CO2 laser irradiation significantly enhances the nano-topographical roughness of titanium disc surfaces, leading to improved hydrophilicity. Notably, this laser-based surface modification imparts antibacterial properties against both gram-positive bacteria (S. epidermidis and S. aureus) and gram-negative bacteria (E. coli and P. aeruginosa). As a result, it inhibits bacterial biofilm formation, thereby lowering the risk of implant failure.

Data availability

Data are available upon request from the corresponding author.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

This in-vitro study was approved by the scientific research ethics committee at the faculty of dentistry, Alexandria University, it was submitted on 22/5/2023, under international no: IORG0008839, and Ethics committee no: 0688-05/2023

Authors' contributions

Mohamed Ibrahim Sherif. Conceptualization, Methodology, Investigation, Funding acquisition, Formal analysis, visualization, Resources, Writing - original draft; Rewaa Gaber AboElhassan. Conceptualization, Investigation, supervision; Dawlat Mostafa. Conceptualization, investigation, Methodology, visualization, Data curation, Writing - review & editing, Supervision; and Yousreya Shalaby. Conceptualization, Validation, Data curation, Writing - review & editing, Supervision.

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