

Osteogenic potential of laser modified and conditioned titanium zirconium surfaces

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Abstract

Statement of Problem: The osseointegration of dental implant is related to their composition and surface treatment. Titanium zirconium (TiZr) has been introduced as an alternative to the commercially pure titanium and its alloys as dental implant material, which is attributed to its superior mechanical and biological properties. Surface treatments of TiZr have been introduced to enhance their osseointegration ability; however, reliable, easy to use surface modification technique has not been established. **Purpose:** The purpose of this study was to evaluate and compare the effect of neodymium-doped yttrium aluminum garnet (Nd-YAG) laser surface treatment of TiZr implant alloy on their osteogenic potential. **Materials and Methods:** Twenty disc-shaped samples of 5 mm diameter and 2 mm height were milled from the TiZr alloy ingot. The polished discs were ultrasonically cleaned in distilled water. Ten samples each were randomly selected as Group A control samples and Group B consisted of Nd-YAG laser surface etched and conditioned test samples. These were evaluated for cellular response. Cellular adhesion and proliferation were quantified, and the results were statistically analyzed using nonparametric analysis. Cellular morphology was observed using electron and epifluorescence microscopy. **Results:** Nd-YAG laser surface modified and conditioned TiZr samples increased the osteogenic potential. **Conclusion:** Nd-YAG laser surface modification of TiZr, improves the cellular activity, surface roughness, and wettability, thereby increasing the osteogenic potential.

Key Words: Cell culture, neodymium-doped yttrium aluminum garnet laser modification, osteogenesis, titanium zirconium

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INTRODUCTION

Commercially pure titanium (cpTi) has been the material of choice for dental implants due to their inert property to osseointegrate.^[1] However, the mechanical properties of cpTi proved to be insufficient. These properties are to be considered while the edentulous ridge is narrow and only

small diameter implants of ≤ 3.5 mm could be used.^[2-5] The use of a small diameter implant is prone to increased chances of fatigue fracture.^[4-8] Hence, it was necessary to develop an implant material which improved the mechanical strength while retaining the necessary biological qualities of existing titanium alloys. The biocompatibility and the mechanical

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strength of titanium zirconium (TiZr)-based alloys proved to be better in comparison with cpTi.^[9] The binary TiZr alloy has increased mechanical stability with respect to elongation and fatigue strength. In addition, the corrosion resistance of TiZr is better and improved than cpTi. The α -phase crystal structure of TiZr alloy ensures that the surface treatments that have been successful on Ti implants can still be implemented on it. Further, the strength of TiZr implant is found to be greater than the strength of cold-worked, Grade IV Ti, which makes it a great candidate for small diameter implants, even in high loading situations.^[10]

When considering the mechanical properties, the biological property of the alloy should also be equally evaluated so that it has no negative influence on the desired mechanism. Many metals are known to strongly inhibit growth of osteoblasts (e.g., vanadium [V], niobium [Nb]) whereas Ti and Zr have not shown to inhibit osteogenesis with preferred osseointegration capabilities.^[11]

The osseointegration capability of an implant depends not only on the type of implant material but also to do with type of surface topography that aids in enhancing an excellent contact with the bone. Key events of biological activity such as early fibrin clot formation, platelet activation, creating a three-dimensional (3D) environment that supports bone formation, increased production of bone-related growth factors, and reduced osteoclastic activity are achieved by the micro-topography of dental implant surfaces. It is reported in the literature that there is an increased synthesis of extracellular matrix with subsequent mineralization on the rougher surfaces.^[12] However, many studies did not accept to the cell response mechanism in dental implants. Thus, there is no agreement about the importance of the degree of roughness (Ra) and its relationship to surface wettability, adhesion, and cellular proliferation.^[13-16]

Lasers have been reported to create 3D complex surface irregularities in a nanoscale level with no contamination. There is no report in the literature on modifying the laser-treated surface by inducing the hydrophilic property.^[17,18] The aim of the study was to evaluate the surface characteristics, Ra, and wettability of TiZr implant alloy samples following surface modification using neodymium-doped yttrium aluminum garnet (Nd-YAG) laser and the Osteoblastic cell response.

MATERIALS AND METHODS

For the purpose of the study, the TiZr implant alloy samples were separated into two separate groups as Group A and Group B. Group A was untreated and kept as control group whereas Group B samples were laser modified and subjected to

evaluation of surface Ra and evaluation of surface wettability. Following the surface analysis, the same samples were subjected to evaluation of cells adhesion, evaluation of cell proliferation, and mineralized bone-like nodule formation. Finally, the results were obtained microscopically statistically.

Preparation of samples

Disc samples of 2 mm thickness and 5 mm diameter were milled from TiZr ingot. These discs were subjected to silicon carbide abrasive polishing and rinsed with distilled water, later autoclaved, and air-dried. The samples were randomly grouped as A and B of 10 samples each. Group A ($n = 10$) polished untreated samples were used as control [Figure 1] and Group B ($n = 10$) were surface treated with Nd-YAG laser [Figure 2].

Laser surface modification

Group B ($n = 10$) samples were subjected to laser surface modification using Nd: YAG laser (Fotona Fidelis plus III, Slovenia). The glass fiber of the Nd: YAG laser was moved over the samples in a linear motion with 8 W power, 300 mJ/pulse energy, and 50-kHz pulse frequency with 1064 nm wavelength. Following surface treatment, all specimens were subjected to cleaning with water steam spray and ultrasonic cleaning using distilled water for 10 min at 80°C, and then rinsed in distilled water. The excess water was removed by air syringe at room temperature, and all specimens were air dried, followed



Figure 1: Polished titanium zirconium samples



Figure 2: Laser surface-modified and conditioned titanium zirconium samples

by conditioning with 78–85% nitrogen gas and stored in 0.9% NaCl solution to maintain the clean oxide layer with its hydrophilic properties.

The uniformity in surface irregularities was assessed using scanning electron microscope (SEM) and epifluorescence microscope (alizarin red [AR] stain) (Olympus BX51) [Figure 3a and b]. The microstructural analysis of surface-modified samples was performed with an SEM (Carl Zeiss Pvt., Ltd., UK., EVO MA 15, magnification range $\times 20$ –200,000).

Evaluation of surface roughness

Roughness (Ra) was measured using a Surtronic 3 (Taylor Hobson) profilometer with a cutoff of 0.25 mm from three different directions 120° apart. The mean of two sets of values was reported as Ra value of the tested samples.

Evaluation of surface wettability

Following the surface treatment, five samples from each experimental group were subjected for wettability testing. An adjustable volume digital micropipette (sigma), positioned perpendicular, was used to deposit 0.25 ml of saline solution onto the surface of the samples. For standardization of the values, the angle changes were monitored at 1 s, 30 s, and 60 s.

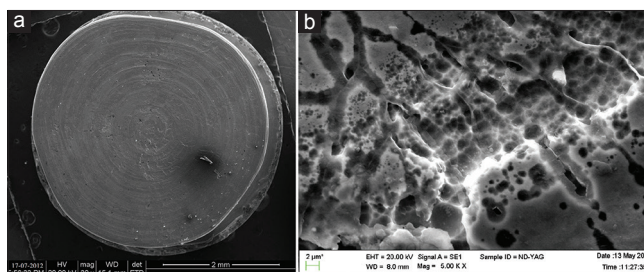


Figure 3: (a) Scanning electron microscope view of polished titanium zirconium surface. (b) Scanning electron microscope view of laser-modified and conditioned titanium zirconium surfaces

Evaluation of cells adhesion

Following gamma sterilization, the samples of both groups were plated with commercially available human calvarial osteoblastic cells (Grace Scientific co.) with a cell density of 1×10^4 cells/cm² per well on a 24-well plate. Ten discs from each experimental group were used and three samples were randomly selected from each group for cell morphology analysis.

Evaluation of cell proliferation

Cell proliferation was evaluated by determining the number of cells that adhered onto the samples at 24 h, 48 h, and 72 h after plating in triplicate. Twenty wells were counted and the number of viable cells collected was obtained using a hemocytometer and the trypan blue exclusion test.

The total numbers of cells were calculated as total counted number of cells \times dilution $\times 10^4$ /number of hemocytometers. Following which the viable cell population was found by dividing the number of viable cells and multiplying the result by hundred.

Mineralized bone-like nodule formation

One sample from each experimental group was fixed using 4% formaldehyde in phosphate buffer, pH 7, for 2 h at room temperature. Postfixation was accomplished with 1% osmium tetroxide with the same buffer. The samples were then dehydrated using a graded series of ethanols, immersed in hexamethyldisilazane for 30 min and air dried. Following which the samples were stained with 2% AR (sigma), pH 4.2, for 8 min at room temperature. The stained areas (AR) were evaluated by epifluorescence microscope (Olympus BX51). The percentage of cells occupied by AR-stained nodules was determined using image tool software (Image-Pro plus AMS).

Statistical analysis

The existence of significant differences between the surface-modified samples was identified using a nonparametric analysis which calculated the averages of ranks and quartiles. Data were analyzed by Student's *t*-test. All values were compared for their significance at a range of $P < 0.05$.

RESULTS

Roughness

The mean Ra of polished samples (control group) in comparison with the laser-treated and modified samples (test group) were lower [Table 1]. However, the ten polished samples had an average value of 0.03–0.09 μm , with an acceptable value for a mechanically polished sample ($P = 0.01$) suggesting that the laser modification has a positive effect on altering the structure of the TiZr.

Wettability

There was a low level of contact angle obtained on the samples treated with modified laser treatment, and this surface was characterized by more of hydrophilic and polar behavior in comparison with the control group [Table 1]. The rate of dispersion was found to be low for both the samples.

Cell adhesion

The human calvarial osteoblastic cells responded differently, depending on the chemical composition and the surface topography of the exposed surfaces [Table 1]. There was a significant difference in level of adhesion of cells on the surface-treated samples after 24 h. There was even more pronounced adhesion of cells onto the laser modified surface.

Cell proliferation

There was a similarity in cellular behavior for both cell proliferation and cell adhesion. There were no statistically significant differences between the control and the modified laser samples at the end of 24 h, but there was a significant difference in cell proliferation on modified laser samples at the end of 72 h [Table 1].

Cell morphology

There was a variation in the morphology of Osteoblastic cells according to the surface pattern. Due to the low Ra on the polished samples, the cells were found to be with scattered contact regions between them and failed to follow an orientation parallel to polishing lines. There were many focal adhesion areas present on the modified laser-treated surface with dendritic projections and filopodia resulting from the 3D rough surfaces and also the cells were found to be very close to one another.

The AR staining is usually done to confirm the osteoblastic cell adhesion. Depending on the intensity of the AR-stained positive

areas, the samples were qualitatively assed under epifluorescence microscope. Microscopic inspection revealed more dotted reddish areas on laser-treated discs than polished samples which lacked uniformity in cell adhesion [Figure 4a and b]. Qualitative analysis of this study showed increased amount of AR-stained regions with uniform distribution of cells forming mineralized nodules on modified laser discs with 95% density in comparison with polished samples with a grading of 40% density on both epifluorescence and Scanning electron microscopic evaluation [Figure 5a and b].

DISCUSSION

Topographical surface alterations of titanium and its alloys on a particular scale proved to enhance the osseointegration capability of the dental implant. This was achieved because of the surface Ra, changes in chemical composition and surface free energy.^[19,20] However, the mechanical properties of cpTi prove to be insufficient in many situations.^[2-5] The risk in using a small diameter implant is the increase chances of fatigue fracture.^[4-8] Hence, it was more important to develop a small diameter implant material which can improve the mechanical strength as well retains the necessary qualities of existing titanium alloys.

The biocompatibility and the mechanical strength of TiZr-based alloys proved to be better in comparison with cpTi.^[9] Hence, this study was focused on modification of TiZr implant alloy surface with a laser source. The laser source of choice was Nd:YAG as it can create 3D structure at micrometer and nanometer level on the implant surfaces whereas other lasers can only be used for decontamination of implant surface. This technique is more supportive in creating complex surface

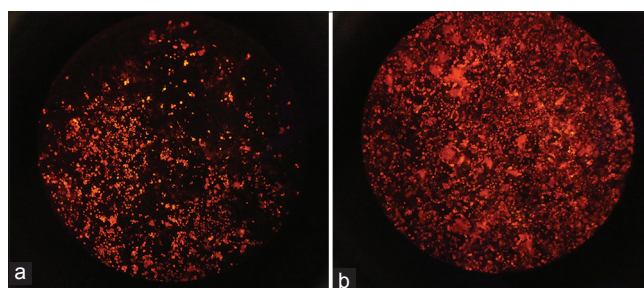


Figure 4: (a) Epifluorescence microscopic view of cells adhering onto polished titanium zirconium surfaces. (b) Epifluorescence microscopic view of cells adhering onto laser-modified and conditioned titanium zirconium surfaces

Table 1: Evaluated surface parameters on the experimental and control discs

Samples	Adhesion		Proliferation		Contact angle (°)		Roughness (μm)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group A (polished)	35.789	7.645	201.000	75.618	15.02	0.31	0.03	0.00
Group B (laser modified)	83.485	23.088	182.458	68.329	48.85	0.34	0.06	0.03

Adhesion was evaluated for 24 h and proliferation 72 h after seeding. SD: Standard deviation

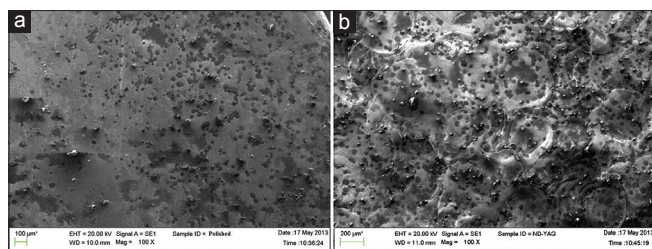


Figure 5: (a) Scanning electron microscope view of mineralized bone-like nodules onto polished titanium zirconium surfaces. (b) Scanning electron microscope view of mineralized bone-like nodules onto laser-modified and conditioned titanium zirconium surfaces

geometries. In this method, short pulse of light is focused on a single spot and moved in a linear motion with generation of heat without altering the nature of the metal. Hence, the technique can be done quicker with contamination free complex microstructures with high resolution. These factors have made the technique interesting for fabricating a geometrically complex biomedical implant surface.^[21] To achieve an enhanced hydrophilic surface, the Nd-YAG laser-modified samples were also rinsed in nitrogen atmosphere and stored in saline solution to aid in reducing the amount of carbon contamination and for improving the hydrophilicity.

The surface Ra was analyzed using contact profilometry which has its scale resolution is in microns. Even then the Ra values of polished TiZr samples and Nd-YAG modified samples had significant differences.

The surface with highest Ra value ($R_a - 0.18 \mu\text{m}$) showed the lowest liquid-titanium contact angles. This result is related to Ra of the surface and the regularity of the formed pattern, both of which reduced the possibility of retaining any contaminants in the micro deformations of the modified surface. Previous studies on surface characteristics feature the way osteoblasts adhere to and proliferate on titanium mostly to wettability^[13,14] and adding on to it hydrophilic surfaces proved better conditions for cell adhesion in comparison with hydrophobic surfaces.^[22] Nevertheless, a close relationship is observed between TiZr Ra and wettability and in the present study, the surface modification by laser followed by conditioning showed a reduction in contact angle. This confirms that a surface containing better wettability will exhibit better cell adhesion. Thus, laser etching and conditioning proved to be an effective technique for modifying a TiZr implant alloy surface in terms of increased Ra and increased wettability.

In vitro analysis using cell culture is usually done to evaluate the potential for osseointegration of biomaterials as stated the models used in this study create a well-controlled and accessible micro environment for providing a consistent data for analysis^[19,20] and also provided a better understanding of

the early stages of osseointegration and various cell interaction onto the modified surfaces.

In a controlled osteogenic environment, there is an uninterrupted clonal expansion of osteoprogenitors followed by their entry into the osteoblast differentiation sequence.^[23-26] The osteoprogenitors are the ones which are responsible for the bone formation rate, replication rate, and they determine the lifespan and number of active osteoblast.^[27,28] These osteoprogenitors play an important role in stimulation of osteoblastic activity and mineralized bone matrix formation on treated or modified implant surfaces.

Surface wettability plays an important role in enhancing the osteogenic activity on surface modified metals.^[29] Protein adsorption, cell adhesion, cell distribution, and enhanced osteoblastic activity can get altered if there is a change in surface wettability and surface energy.^[21,30-32] Hence, to achieve surface wettability, it is necessary for the implant surface to be hydrophilic to induces rapid osseointegration.^[33,34]

In the present study, Group B samples were subjected to hydrophilic treatment following a laser surface modification. The surface topography of modified laser-treated samples revealed nanopits of $24 \pm 5 \text{ nm}$ in diameter which can influence the immediate and equilibrium contact angles to decrease and thereby converting the hydrophobic surface into an extremely hydrophilic surface. This increased the rate of mineralized bone matrix formation on the laser-treated surfaces.

Considering the above factors, it was found that surface modification with modified Nd-YAG laser on TiZr implant alloy showed increased mineralized bone-like nodule formation with uniformity in comparison with polished nontreated samples. This is similar to the results of the studies conducted on CpTi using the laser sources.^[17,18] A limitation of the present study is that the results were based on the laboratory findings and yet to be confirmed with the clinical study.

CONCLUSION

Within the limitation of this study, the following conclusion was made. Nd-YAG laser surface treatment on TiZr had positive effects on the early cellular events leading to cell adhesion and proliferation. Surface wettability was greater in the test group while maintaining biocompatibility characteristics.

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Conflicts of interest

There are no conflicts of interest.

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