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ORIGINAL ARTICLE

Physicochemical, morphological, and biological analyses of Ti-15Mo alloy surface modified by laser beam irradiation

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Abstract

Perform a physicochemical and morphological characterization of a Ti-15Mo alloy surface modified by laser beam irradiation and to evaluate in vitro the morphological response and proliferation of osteoblastic cells seeded onto this alloy. Disks were made of two different metals, Ti-15Mo alloy and cpTi, used as control. A total of four groups were evaluated: polished cpTi (cpTi-pol), laser-irradiated cpTi (cpTi-L), polished Ti-15Mo alloy (Ti-15Mo-pol), and laser-irradiated Ti-15Mo alloy (Ti-15Mo-L). Before and after laser irradiation of the surfaces, physicochemical and morphological analyses were performed: scanning electron microscopy (FEG-SEM), energy-dispersive spectroscopy (EDX), and X-ray diffraction (XRD). The wettability of the samples was evaluated by contact angle measurement. Murine preosteoblastic cells MC3T3-E1 were cultured onto the experimental disks for cell proliferation, morphology, and spreading analyses. Laser groups presented irregular-shaped cavities on its surface and a typical microstructured surface with large depressions (FEG-SEM). The contact angle for both laser groups was 0°, whereas for the polished groups was ≈ 77 and ≈ 78 for cpTi-pol and Ti-15Mo-pol, respectively. Cell proliferation analysis demonstrated a higher metabolic activity in the laser groups ($p < 0.05$). From the fluorescence microscopy, Ti-15Mo-L surface seems to induce greater cellular differentiation compared to the cpTi-L surface. The preliminary biological in vitro analyses suggested possible advantages of laser surface treatment in the Ti-15Mo alloy regarding cell proliferation and maturation.

Keywords Dental implants · Surface modification · Titanium alloys · Cell culture · SEM

Introduction

Over the last decades, technological advances in the field of dental implants have led to a success rate of 97% of the installed implants [1], resulting in popularization of their use. With the high success rate and advances in this field, the current research has focused on understanding the mechanisms of the interaction between the implant surface and the surrounding tissues and on the development of implants with different characteristics that favor or stimulate osseointegration in areas with limited bone or in patients with impaired reparative potential [1, 2].

Currently, the most used metals in dental implants are commercially pure titanium (cpTi) and titanium 6-aluminum 4-vanadium (Ti-6Al-4V) alloy [3–5]. Since titanium is a biocompatible metal, the addiction of other elements such as aluminum, vanadium, or molybdenum is intended to improve the biomechanics and/or the biochemical properties of the implants. However, concerns about Ti-6Al-4V alloy application are the cytotoxicity of vanadium (V) and the possible occurrence of Alzheimer's disease related to the presence of aluminum (Al) ions [6, 7], factors that suggest the necessity of replacement of these alloys for biomedical applications such as dental and orthopedic implants [5, 8, 9].

Therefore, growing interest has been recently observed in the development of materials prepared with nontoxic elements such as niobium (Nb), tantalum (Ta), zirconium (Zr), and

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molybdenum (Mo) [3–5, 8, 9]. Attempts were made to develop Ti alloys of different compositions to achieve better performance in terms of biomechanical compatibility (by reducing Young's modulus) and biochemical compatibility (by excluding toxic elements) [4, 5, 8–11]. Mo is a nontoxic element able to stabilize the β phase of titanium [3, 5, 8]. Previous studies have shown, from X-ray diffraction (XRD) analysis, that the crystal structure of Ti-Mo alloys is sensitive to the Mo concentration; the α phase is observed almost exclusively when the concentration of Mo added to the Ti reaches 6%. A significant retention of the β phase is observed for the alloy containing 10% Mo (α+β Ti-Mo alloys), while at higher Mo concentrations (15 and 20%), retention of only β phase is verified [3, 4, 12]. The β Ti alloys have properties such as low elastic modulus, increased mechanical strength, corrosion resistance, and improvement in biological tissues response compared to α+β alloys [3, 5].

Electrochemical behavior of cpTi and Ti-Mo alloys was investigated as a function of immersion time in electrolyte simulating physiological media [4, 12]. These electrochemical results suggest that the Ti-Mo alloys are promising to be applied in biomedical devices, particularly in the case of Ti-15Mo since electrochemical stability is directly associated with biocompatibility and is a necessary condition for applying a material as biomaterial [4, 9, 12].

Besides the material properties, another important aspect to be considered for implant success is the physicochemical properties of its surface treatment. The physicochemical properties of implant surface can increase the percentage of bone/implant contact and thus interfere in the biological responses and consequent repair of bone/implant interface [13–15].

Since this is an essential factor to the success and survival of implants, many studies have evaluated different types of surfaces, development of new surfaces, in vitro cellular behavior, and in vivo models to investigate tissue responses to different surface treatments [2, 16]. The surface modifications alter the topography and modify the physicochemical properties of the metal. While the mechanism of action of these changes on the living organism is unclear, it is known that they are important for a better biological response [2, 16–18].

By comparing with several implant surface modification processes currently on the market, such as mechanical processes (machining and abrasive sandblasting), chemical processes (acid etching and oxidation), and thermal processes (plasma spray), it has been observed that the resulting surface by laser beam irradiation shows similar characteristics without the occurrence of contaminations since it is a clean and reproducible process. Furthermore, it enables a better control of the variables involved in such process [9, 14, 15, 19–21].

The irradiation of the surface by high-power laser beam leads to a nanostructured and tridimensional surface, enhancement of roughness, resistance to corrosion and wear, absence of contaminants on the surface, and an increase in Ti oxide layer by ablation phenomenon, in addition to being able to be carried out in a controlled and reproducible manner [9, 14, 15, 20–22].

Therefore, the aim of this study was to perform a physicochemical and morphological characterization of a Ti-15Mo alloy surface modified by laser beam irradiation and to evaluate in vitro the morphological response and proliferation of osteoblastic cells seeded onto this new developed biomedical alloy.

Materials and methods

Sample preparation

Disks with 10 mm diameter × 2 mm thick were made of two different metals, Ti-15Mo alloy (developed by the Biomaterials Group, Institute of Chemistry of Araraquara, UNESP) and cpTi grade 2 (Titanews, Brazil), used as control.

The disks were ground with 320, 400, and 600 grit silicon carbide papers; ultrasonically and progressively cleaned in distilled water, acetone, and ethanol for 10 min; and then air-dried at room temperature. Half of the disks had their surface treated with laser beam irradiation using a pulsed laser Yb:YAG (Pulsed Ytterbium Fiber Laser, Ominitek Tecnologia Ltda, Brazil). The following laser beam parameters were used: fluency (density power inside irradiated surface) 1.9 J/cm^2, scanning speed 0–200 mm/s, pulse frequency 20–35 kHz, and average exposure area 14 mm^2. Differently of other surface modification processes, with laser beam irradiation, it is possible to obtain different surface compositions (e.g., Ti oxides and nitrides) by changing the applied fluency [9, 19]. After the surface modification, the samples were ultrasonically cleaned as described previously.

A total of four groups were evaluated: (1) polished cpTi (cpTi-pol), (2) laser-irradiated cpTi (cpTi-L), (3) polished Ti-15Mo alloy (Ti-15Mo-pol), and (4) laser-irradiated Ti-15Mo alloy (Ti-15Mo-L).

Physicochemical and morphological characterization

The morphology of the studied surfaces was analyzed by high-resolution scanning electron microscopy (FEG-SEM; JEOL, model 7500F), and the energy-dispersive spectroscopy (EDX) was used to check the chemical composition or detect any contamination of the materials. The microstructures of the sample surfaces before and after laser irradiation were evaluated by X-ray diffraction (XRD) using a Siemens D5000 X-ray diffractometer (Siemens, Germany), angular scanning 20–60° with 0.02 pitch (2θ), and the pitch time was 10 s for each sample, on the Bragg-Brentano buildup, using Cu (k α1) radiation.

The wettability of the samples was evaluated by contact angle measurement using a contact angle tester OCA-15 (Dataphysics, Germany). Drops of distilled water were delivered onto the specimen surface by a syringe giving the same drop size. The contact angle was measured after 20 s and repeated three times for each sample.

Cell culture

All samples used for cell culture were sterilized by gamma radiation with 25-kGy dosage. Murine preosteoblastic cells MC3T3-E1 (ATCC, USA) were cultured in alpha-minimum essential medium (α-MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA), 10 mM β-glycerophosphate (Acros Organics, Belgium), and 5 μg/mL ascorbic acid (Sigma, USA) under 37 °C, 5% CO_2 environment. 2×10^4 cells were seeded onto the experimental disks in a 50-μL drop on a 24-well polystyrene plate. After 4 h, the final volume of the well was completed (1 mL). Culture media were changed every 3 days.

Cell proliferation assay

Cell proliferation was assessed using the alamarBlue kit (Life Technologies, USA). Cells seeded in polystyrene plate were used as positive control. Briefly, at 1, 3, 5, 7, 10, and 14 days after seeding, the medium was aspirated and 1 mL of dye solution (10% alamarBlue and 90% culture medium) was added to each well, and cells were incubated at 37 °C for 4 h. Subsequently, 100 μL of this solubilization solution was added into 96-well plates (TPP) and the absorbance of each well was measured at 570-nm and 600-nm wavelengths with an automatic microplate (ELISA) reader (Molecular Devices, USA). The percentage difference in the reduction of alamarBlue between tests and control cells was calculated using the formula suggested by the kit manufacturer. The statistical analysis of these data was performed applying the one-way ANOVA with post hoc Tukey test using a significant level of 5% ($p < 0.05$).

Cell morphology

Cell morphology and spreading were evaluated by FEG-SEM and direct fluorescence. After 3 days, the medium was removed and the cells were fixed, dehydrated in gradual series of alcohol, and air vacuum dried for observation in FEG-SEM. For direct fluorescence, after 1, 3, and 10 days in culture, cells were stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, USA) which labels ubiquitous actin cytoskeleton and 40,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, USA) for nuclear stain, using the protocol suggested by the manufacturer. A descriptive analysis was made for SEM and direct fluorescence.

Results

Physicochemical and morphological characterization

The surface morphology of the four groups (cpTi-pol, cpTi-L, Ti-15Mo-pol, and Ti-15Mo-L) was analyzed by FEG-SEM (Fig. 1a–d). The microscale was observed at a magnification of × 1000. Clear differences in the surface topography between polished and laser-irradiated surfaces were observed, independently of the material of the implant (cpTi or Ti-15Mo). The polished groups (cpTi-pol and Ti-15Mo-pol) showed a smooth surface with grooves compatible with the sandpaper used for polishing (Fig. 1a, b), while samples from the laser-irradiated groups (cpTi-L and Ti-15Mo-L) presented irregular-shaped cavities on its surface and a typical microstructured surface with large depressions (Fig. 1c, d). The EDX analysis showed no contamination after laser irradiation process in both cpTi and Ti-15Mo alloy groups (Fig. 2a–d).

A peak of oxygen was observed in the Ti-15Mo-L samples in EDX analysis (Fig. 2b), and TiO_2 was detected in the same samples by XDR analysis (Fig. 3b). Also in the XDR analysis, only the β phase of titanium was observed in both Ti-15Mo alloy groups (Fig. 3a, b).

The results of contact angle assay showed a higher wettability for both laser-irradiated groups: cpTi-L = 0°, cpTi-pol ≈ 77, Ti-15Mo-L = 0°, Ti-15Mo-pol ≈ 78. The laser groups demonstrated complete wetting in 20 s.

Cell proliferation assay

The alamarBlue test represents the percentage of alamarBlue reduction in the culture medium in comparison to positive control—cells seeded in the plastic plate. This assay analyzes the metabolic activity and growth of cells. In the inter-group comparison, a higher metabolic activity was demonstrated in the laser groups compared to the polished groups, mainly between 5 and 10 days ($p < 0.05$). A tendency to greater response was observed in the Ti-15Mo-L compared to the cpTi-L group at 5, 7, and 10 days, but without statistical significance (Fig. 4).

In the intra-group analysis, the Ti-15Mo-L group demonstrated an increased percentage of reduction along the periods with statistical significance at 7 days. In contrast, the cpTi-pol showed a decrease along the period, with significance at 10 days (Fig. 5).

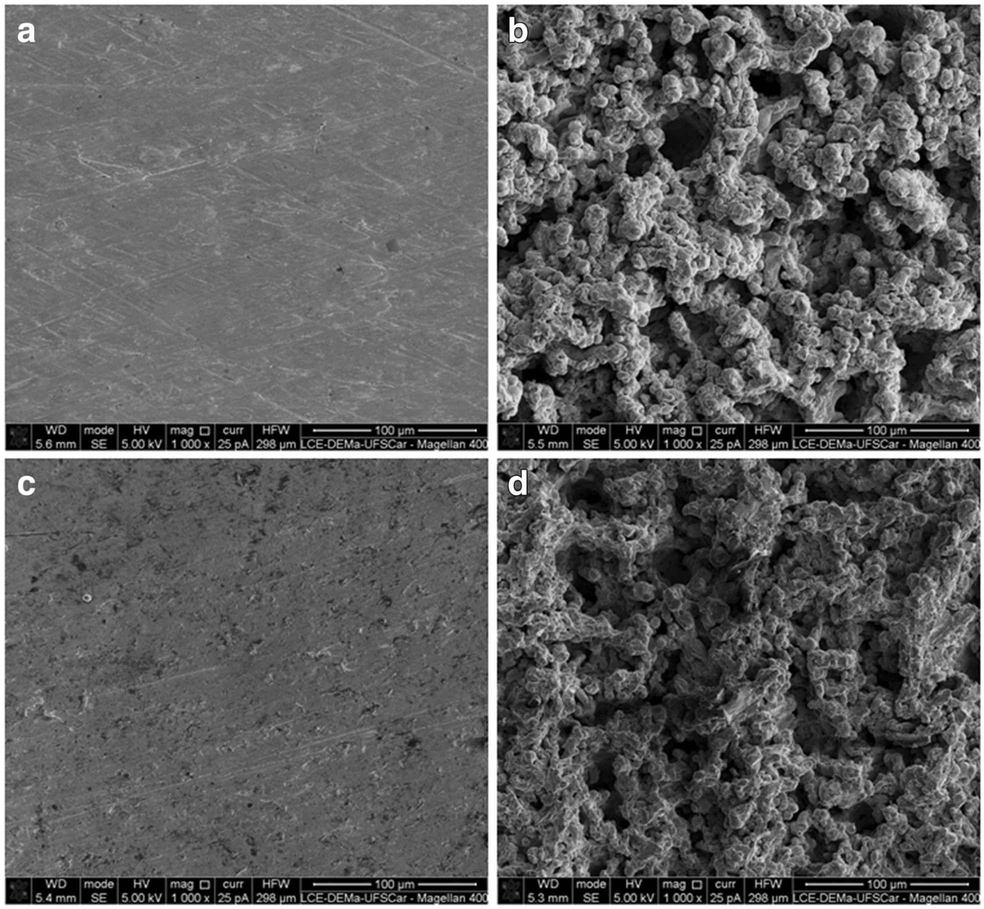


Fig. 1 FEG-SEM of cpTi and Ti-15Mo samples (× 1000). **a** cpTi-pol, **b** cpTi-L, **c** Ti-15Mo-pol, and **d** Ti-15Mo-L

Cell morphology—FEG-SEM

At the FEG-SEM analysis, osteoblasts had spread well with observation of filopodias in all groups (Fig. 6), confirming cell maturation at 2 days. Interestingly, in the polished groups, filopodias are projected in a linear way following the polishing grooves at the surface, while in the laser samples, the emission of filopodias occurred in a 3D manner as demonstrated in Fig. 7.

Cell morphology—direct fluorescence

The morphology of cytoskeleton was also analyzed by fluorescence (Fig. 8). At day 1 of culture, the cells in the polished groups (cpTi and Ti-15Mo) were more elongated while in laser groups, small filopodias were present, suggesting a lower degree of differentiation in laser groups. In addition, the polished groups presented an almost complete covering of the surface by cells. When comparing the different materials (cpTi and Ti-15Mo), the Ti-15Mo seems to induce greater differentiation compared to the cpTi for laser-irradiated surfaces.

After 3 days, the same pattern was observed for the cytoskeleton in polished groups with full surface coverage by the cells and an apparent increase in the morphological differentiation for laser groups. The difference between Ti-15Mo-L and cpTi-L groups also remained within 3 days with a better response to Ti-15Mo-L group.

After 10 days, the groups were similar without differences for morphological aspects and degree of cell surface coverage. It is important to note that the fluorescence analysis was performed in a bi-dimensional way. A 3D analysis would better describe the results observed in the laser-irradiated surfaces.

Discussion

Physicochemical and morphological characterization

The laser parameters used in this study were efficient to produce a 3D surface on both cpTi and Ti-15Mo alloy, similar to those found for cpTi obtained by Braga et al. [19] and Heinrich et al. [23] and for Ti-15Mo alloy by Oliveira et al. [9].

Several previous studies have used laser to modify titanium and titanium alloy surface with good results in their physicochemical properties and wear and corrosion resistance [9, 14, 15, 20, 21, 24]. In the laser groups from this study, the

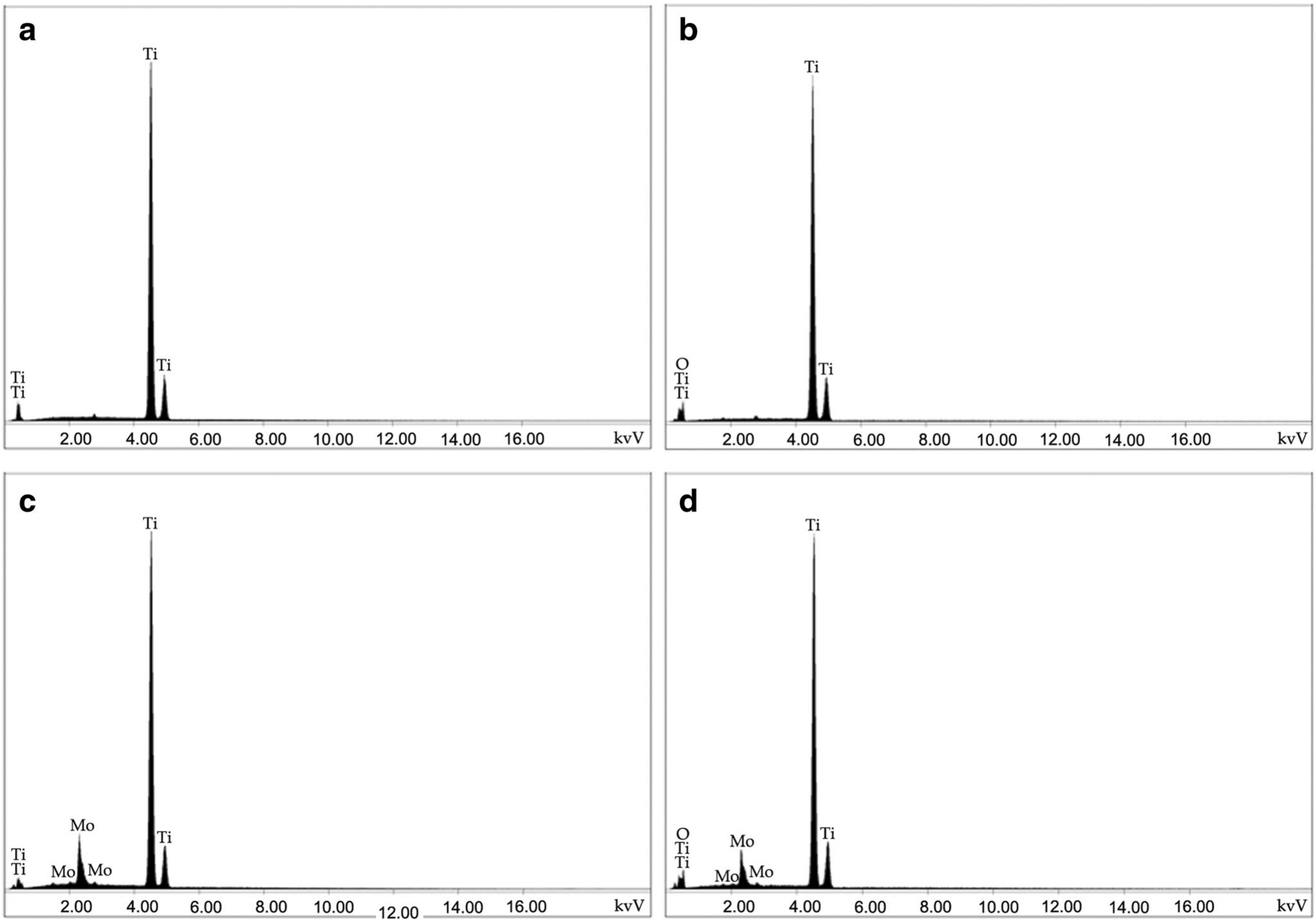


Fig. 2 EDX of cpTi and Ti-15Mo samples. **a** cpTi-pol, **b** cpTi-L, **c** Ti-15Mo-pol, and **d** Ti-15Mo-L

morphology of surface appears rougher than the previously described for laser Nd:YAG treatment on different titanium implant materials [24], indicating that the roughness of the surface is sensible to the laser application parameters [19].

Considering the EDX analysis, after laser beam irradiation in both cpTi and Ti-15Mo alloy groups, similar findings were observed by Bini et al. [20] and Filho et al. [21] after laser irradiation on cpTi surface and Oliveira et al. [9] after laser irradiation on Ti-15Mo alloy surface.

During the laser irradiation of the Ti-15Mo samples at room temperature, an ablation process occurs, which is directly correlated with the energy density used, leading to oxygen diffusion through the molten metal and oxidation of the alloy surface. Ablation process is characterized by a quick melting (melted metal zone formation) and solidification process on metal surface [20, 21]. The melted metal zone created by ablation process can produce morphologies with different roughness degrees and with different oxide formations at

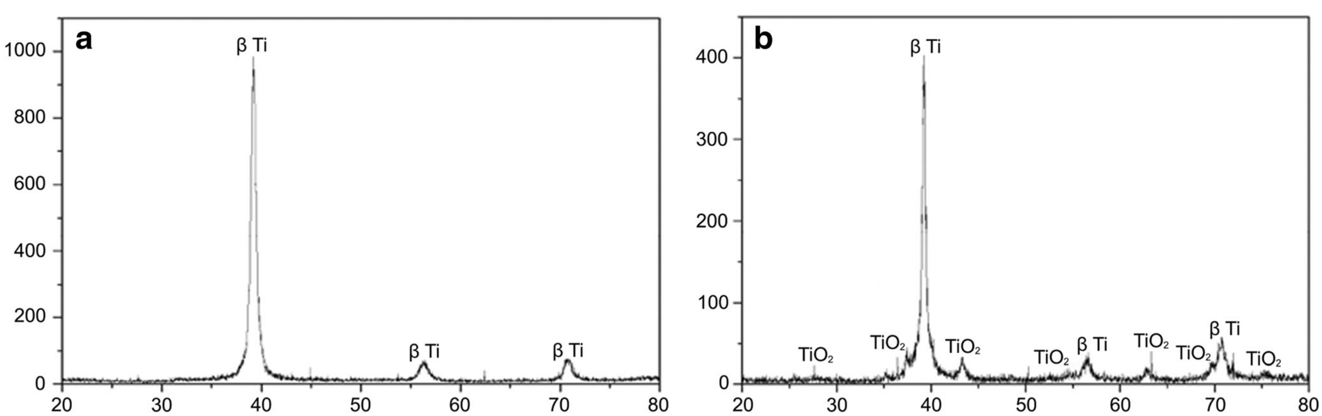


Fig. 3 XRD of Ti-15Mo samples. **a** Ti-15Mo-pol and **b** Ti-15Mo-L

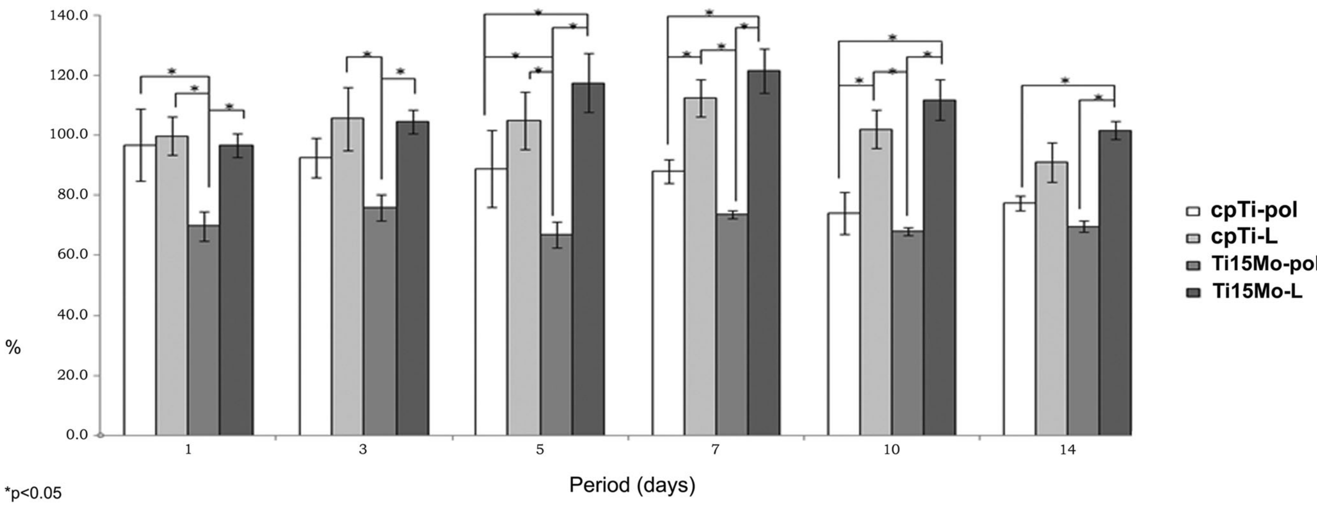


Fig. 4 alamarBlue®—percentage difference in reduction related to the positive control (%)—inter-group comparison (*$p < 0.05$)

several concentrations. Roughness is an important characteristic for dental implants. In the literature, it has also been noted that the cellular adhesion, the proliferation, and detachment strength are sensitive to surface roughness [20, 21].

The peak of oxygen observed in the Ti-15Mo-L samples in EDX analysis and TiO_2 detected in the same samples by XDR analysis demonstrate that the laser irradiation enhanced the oxide surface of the samples. TiO_2 has been shown to increase calcium interaction which is important for protein and subsequent osteoblast adhesion [24].

Also, only the β phase of titanium was observed in both Ti-15Mo alloy groups, from the XRD analysis. The β phase of titanium alloys exhibits lower Young's modulus (Ti-15Mo = 75–78 GPa) (ASTM F2066-08) [25] closer to the natural bone (10–30 GPa) compared with cpTi (approximately 110 GPa) [5, 9, 26], which may favor bone response against the dissipation of mechanical loads.

Regarding the results of the contact angle assay, a complete wetting for the laser groups was shown. Hydrophilicity is considered an important factor to early bone response [27,

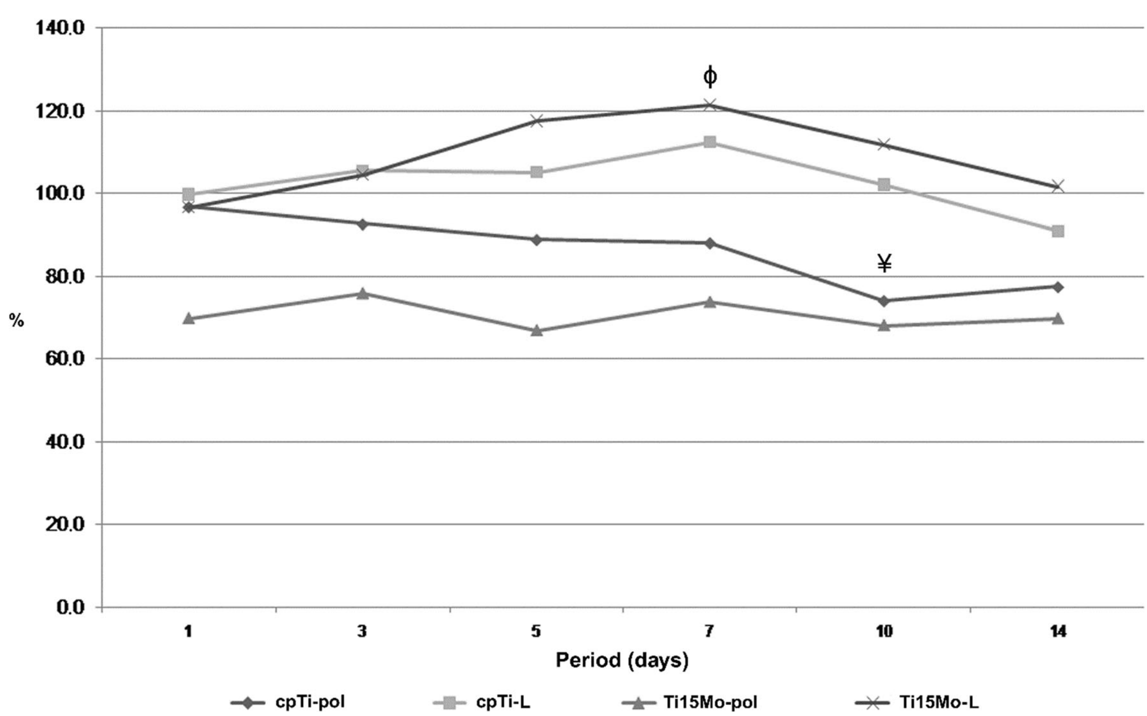


Fig. 5 alamarBlue®—percentage difference in reduction related to the positive control (%)—intra-group comparison. $^{\phi}p < 0.05$ in Ti-15Mo-L between 1 and 7 days; $^{¥}p < 0.05$ in cpTi-pol between 1 and 10 days

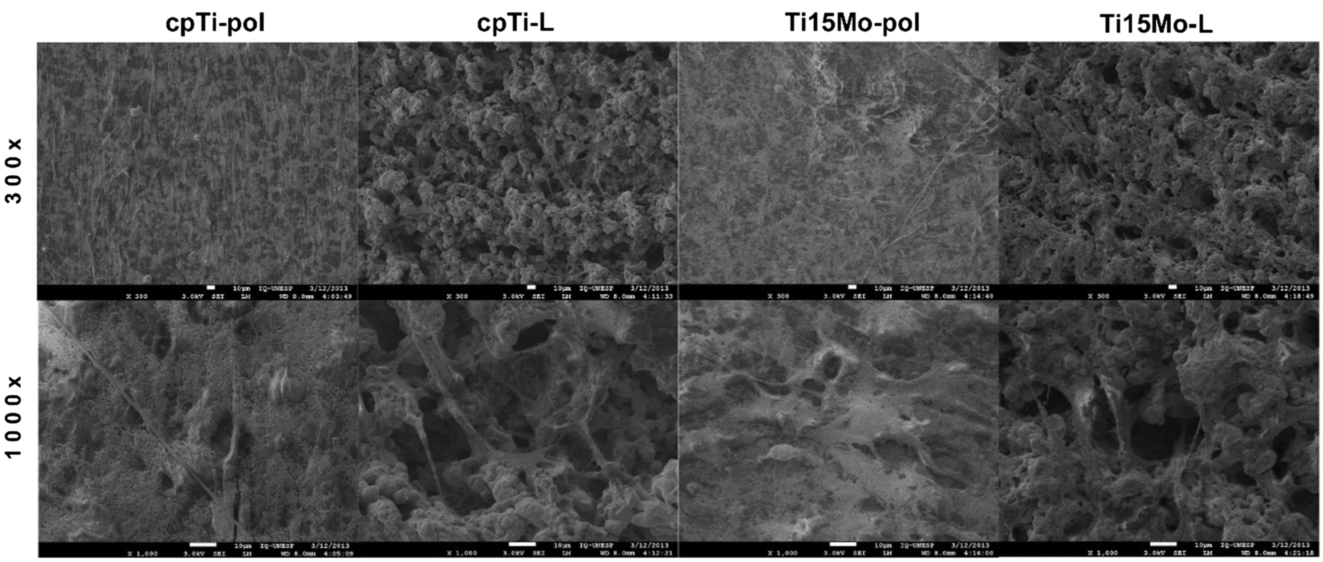


Fig. 6 Cell morphology observed by FEG-SEM (× 300 and × 1000) at 2 days

28], which can lead to faster healing process and consequently early loading [29]. In the past years, to predictably improve the osseointegration of Ti implants, close attention has been paid to a higher surface energy and increased wettability since both properties have been shown to enhance the interactions between a surface and its biologic environment [30].

The initial interaction between living bone and tissue and implant surface occurs as the surface of the implant biomaterial is exposed to tissue fluids. This produces a layer of macromolecules and fluids, which influences the behavior of cells when they encounter the implant surface. Then, wettability may be one of the surface factors to be considered when selecting dental and orthopedic implant biomaterials [31]. Furthermore, osteoblast cultures exhibit better adhesion and growth on hydrophilic surfaces [32].

Cell proliferation assay

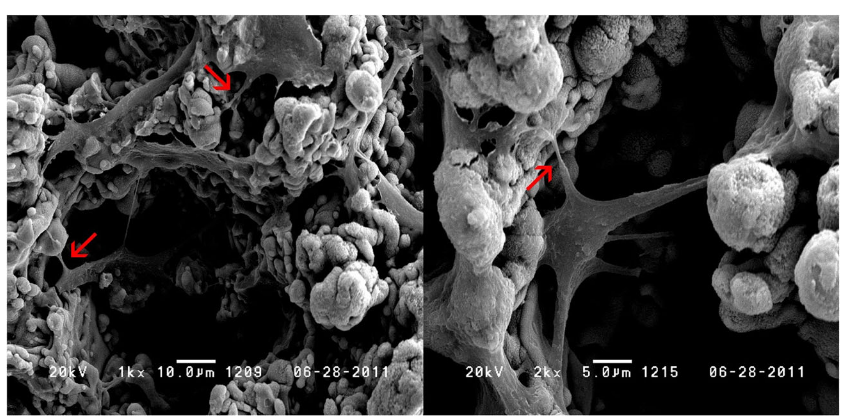
The results obtained from both inter-group and intra-group analyses suggest a stimulatory effect of the laser surface treatment on cell proliferation. One possible explanation for this response is the high wettability observed on surface treatment by laser. It is known that osteoblast culture exhibits better adhesion and growth on hydrophilic surfaces [32] and wettability influences the osteoblastic response in vitro [33].

Great attention has been given to study, in vitro, the cellular and molecular activities on different substrates and to extrapolate the results to the actual interfacing between implant and living bone tissue. Based on results from in vitro studies, the surface influences the initial sequences of protein adsorption, platelet adhesion and hemostasis, complement activation, inflammation, and osteogenic cell response [32–34].

Cell morphology—direct fluorescence

From a clinical point of view, the main purpose for the development of implant surface modifications is to promote osseointegration, with faster and stronger bone formation. This will likely confer better stability during the healing process, which, preferentially, will improve the

Fig. 7 FEG-SEM observation of osteoblast behavior in the 3D laser surfaces at 2 days (× 1000 and × 2000). Filopodias are indicated by arrows



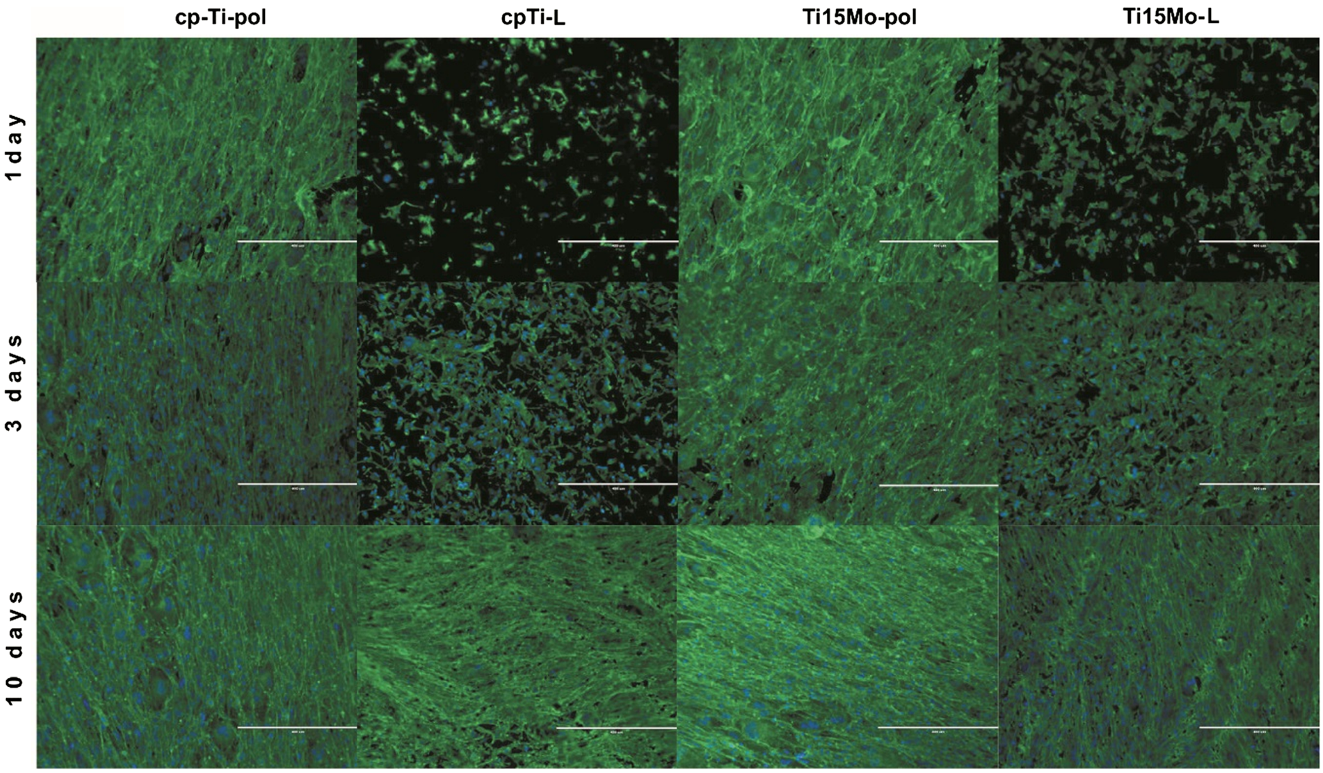


Fig. 8 Direct fluorescence to observe cell morphology at 1, 3, and 10 days

clinical performance in poor bone quality and quantity [34]. Therefore, the better response of the laser-irradiated groups at 3 days (early time point) may influence the bone response in the laser-irradiated groups in vivo, shortening the healing time.

Analyzing together, results from fluorescence and proliferation assay seem divergent. However, the 3D aspects of laser-irradiated surface may have impaired cell visualization by the bi-dimensional fluorescence analysis. Therefore, the images of this analysis need to be interpreted with caution, and additional three-dimensional analysis needs to be performed in the future to confirm proliferation results from the alamarBlue assay.

The increased area of metal achieved by laser-irradiated surface together with the three-dimensional and rough aspects of these surfaces justifies the better proliferation results observed by the alamarBlue assay. In addition, fluorescence images suggest an advanced differentiation of cells seeded over laser-irradiated surfaces.

Osseointegration of dental implants depends on a direct contact between bone and metal surface. In vitro cellular responses in the short term (migration and anchorage) and long term (differentiation and expression of the matrix) play a role for the understanding of cell expression at titanium and titanium alloy surfaces. Histologically, the initial cell adhesion and growth are important factors in this process. Properties like physical, chemical, mechanical, and topography of the implant surface strongly affect cellular responses, including adhesion and growth.

Conclusion

The surface modification by laser beam irradiation allowed obtaining suitable physical-chemical properties and morphology for the use as dental implants. Furthermore, the applied laser treatment enables a better control of the variables involved in such process where the implant surface does not interact with any other materials for its modification and improves the implant surface with significant properties which may influence the osseointegration phenomenon, such as morphology and surface roughness. However, the determining factor for osseointegration is the formation of some compounds that facilitate the wetting and the physicochemical properties of the surface.

The preliminary biological in vitro analyses suggested possible advantages of laser surface treatment in the Ti-15Mo alloy regarding cell proliferation and maturation. Therefore, additional in vitro analyses (e.g., quantitative gene expression levels for mineralized tissue-associated proteins—OCN, BSP, ALP—and transcription factors—Runx2) and in vivo studies are necessary to evaluate bone response to confirm the beneficial effects of this new alloy and surface treatment in the field of dental implants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Not required.

Informed consent Not required.

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