Lasers Med Sci
DOI 10.1007/s10103-016-1927-6

ORIGINAL ARTICLE

Morphology, proliferation, and gene expression of gingival fibroblasts on Laser-Lok, titanium, and zirconia surfaces

Nasrin Esfahanizadeh[1,2] · **Sara Motalebi**[1] · **Niloufar Daneshparvar**[3] · **Nasrin Akhoundi**[4] · **Shahin Bonakdar**[5]

Received: 22 October 2015 / Accepted: 18 March 2016
© Springer-Verlag London 2016

Abstract Soft tissue seal plays a critical role in long-term success of dental implants, and the effects of implant surface treatments such as laser ablation have been a topic of particular interest in this respect. Considering the existing controversy regarding soft tissue behavior in contact with implant surfaces, this study sought to assess the morphology, proliferation, and gene expression of human gingival fibroblasts (HGFs) on different abutment surfaces. In this in vitro, experimental study, HGFs were cultured on 45 discs (Laser-Lok, titanium, and zirconia). Cell morphology, proliferation rate, and interleukin 10 (IL-10), tumor necrosis factor alpha (TNFα), fibronectin, and integrin gene expressions were assessed by electron microscopy, methyl thiazol tetrazolium (MTT) assay, and real-time polymerase chain reaction (PCR), respectively. Data were analyzed using ANOVA and the Kruskal–Wallis H test. Fibroblast attachment was noted in all the three groups. Spindle-shaped cells with pseudopod-like processes were more frequently seen in the Laser-Lok group. Cell proliferation was significantly higher in the Laser-Lok group compared to those in the other groups ($P = 0.0002$). Significant differences were found in the expression of IL-10, TNFα, fibronectin, and integrin genes among the groups ($P < 0.01$). Within the limitations of this study, HGFs on Laser-Lok surfaces had a more mature morphology and greater proliferation and differentiation as compared to those on zirconia and titanium surfaces. This indicates better attachment of these cells to laser-modified surfaces, creating a more efficient soft tissue seal around dental implants.

Keywords Fibroblasts · Implant abutment · Laser-Lok · Titanium · Zirconia

✉ Niloufar Daneshparvar
nildan89@yahoo.com

[1] Department of Periodontics, Dental Branch, Islamic Azad University, Tehran, Iran

[2] Dental Implant Research Center, Tehran University of Medical Sciences, Tehran, Iran

[3] 1501 Harvey Rd, #586, College Station, TX 77840, USA

[4] Department of Mathematics, South Tehran Branch, Islamic Azad University, Tehran, Iran

[5] National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran

Introduction

A success of dental implants depends on maintaining a stable attachment and efficient seal between the implant surface and the surrounding soft tissue and crestal bone [1]. A firm connective tissue attachment to the trans-mucosal portion of implants can prevent apical migration of the epithelial tissue [2, 3]. Soft tissue attachment to the trans-mucosal portion of implant or abutment serves as a biological barrier and prevents the invasion of bacterial toxins, conferring resistance to the alveolar bone against resorption [3–5].

Several factors affect and dictate the behavior of cells exposed to implanted artificial (such as titanium) surfaces [1, 3, 6]. Evidence shows that abutment surface characteristics can affect the behavior of epithelial, fibroblast, and osteoblast cells at the implant–abutment interface [3]. Surface energy, surface topography, and surface treatment techniques are among the factors impacting the adhesion, distribution, and migration of cells at the soft tissue-implant interface. According to several human and animal studies, surface roughness

Published online: 30 March 2016

in an amount of Ra = 0.2 μ is necessarily required to achieve optimal abutment/soft tissue seal; whereas, smooth surfaces may result in unwanted adhesion and apical migration of epithelial cells [7]. Moreover, micrometer-scale grooves created on the implant surface perpendicular to the direction of migration of epithelial cells enhance the attachment of HGFs and prevent apical proliferation of epithelial cells [8, 9].

Several surface treatments have been proposed for different implant systems to improve the implant surface properties and obtain optimal soft and hard tissue response. The surfaces of prosthetic components such as abutments can be modified by the use of nanotechnology, polishing, and sandblasting [10, 11]. Hydroxyapatite coating, plasma spray with titanium oxide, and acid etching are among the commonly used techniques to modify the abutment surface [12, 13]. Laser irradiation has been recently employed to alter the microtopography of the implant and abutment surfaces. In this technique, identical three-dimensional microgrooves are created on the implant and abutment surfaces with 8- and 12-μ intervals to control the behavior and arrangement of fibroblasts and osteoblasts [6].

Studies on the behavior of fibroblasts on modified titanium surfaces have yielded controversial results. For instance, alkaline-treated surfaces showed increased fibroblast attachment in the first hour but no significant change was noted in cell adhesion at 3 h [14]. Another study demonstrated that unlike osteoblasts, nanotopography decreased the adhesion and spreading of fibroblasts and epithelial cells, and the latter cells had greater affinity for smooth or micro-grooved surfaces [15]. Nevins et al., in their human histological study, showed direct attachment of connective tissue to Laser-Lok micro-grooved implant collar [5]. In addition to the surface processing technique, type of abutment material can also impact the reaction and behavior of gingival fibroblasts (GFs) [16, 17]. Abutments are made of various materials such as titanium, base metals, gold alloy, and alumina or zirconia ceramics. In the recent years, use of zirconia abutments as an alternative to titanium abutments has increased particularly for use in the esthetic zone [18, 19]. However, aside from the esthetically superior appearance of zirconia, clinical evidence is contradictory regarding the superiority of zirconia abutments over titanium abutments, and their biological advantages remain a matter of debate [17].

Considering the variability in the behavior of GFs exposed to different surfaces and materials [5, 8, 17], the diversity in types of abutments available in the market, and the recent introduction of abutments with laser collars as well as the gap of information in this regard, this study sought to assess the adhesion, proliferation, and gene expression of HGFs on Laser-Lok, titanium, and zirconia surfaces.

Materials and methods

In this in vitro, experimental study, sterile discs (BioHorizons, USA) measuring 9 mm in diameter and 1 mm in thickness were used. A total of 45 discs with Laser-Lok (a laser-modified titanium surface has identical microgrooves with 8-μ intervals), zirconia, and titanium surfaces ($n = 15$) were used for assessment of morphology, proliferation rate, and expression of IL-10, TNFα, fibronectin, and integrin genes of HGFs using electron microscopy, MTT assay, and real-time PCR, respectively. Five discs of each group were used for each test.

Cell culture

The HGF1s (NCBI C165) were obtained from the National Cell Bank of Iran (Iran Pasteur Institute). The cells were cultured in Dulbecco's modified eagle's medium (DMEM, GIBCO, Scotland)/Ham's F12 supplemented with 10 % fetal bovine serum (FBS; Nano-Zist-Araye Co., Iran), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, USA). Before seeding cells on each sample, the HGF1 cells were harvested using 0.25 % trypsin-EDTA solution (Sigma) in phosphate-buffered saline (PBS; pH 7.4). The cells were incubated at 37 °C with 5 % CO_2 in a humidified atmosphere [8].

Cell seeding

Discs intended for biological assessments including microscopic examination, MTT assay, and real-time PCR were placed in a 12-well plate. A total of 5×10^3 cells in 0.1 mL of culture medium were seeded on each sample and incubated at 37 °C for 3 h. Afterwards, 1 mL of culture medium was added to each well to cover the sample surfaces. Tissue culture plate was considered as control for each test.

After 3 days of culture, specimens were removed from the culture medium and gently rinsed with PBS to wash out the unattached or loosely attached cells from the surface. The cells firmly attached to the disc surface were fixed by immersion in 4 % glutaraldehyde at room temperature for 20 min. Dehydration was then performed using graded alcohol (50, 60, 70, 80, 90, 95, and 100 %, respectively) [2].

Discs were gold-coated (thickness of 5 nm) using a sputter coater (EM Technologies Ltd., England) under vacuum conditions with 15 V voltage for 5 min at the Razi Metallurgy Research Center. The samples were observed by VEGA electron microscope (Tescan, Czech Republic). The images were captured under vacuum conditions at a voltage of 15 kV to

evaluate the surface topography, cell morphology, and attachment of cells to disc surfaces at ×1000 to ×5000 magnifications [20].

Cell proliferation assay

Direct cell proliferation assay was performed according to ISO 10993–5. Fifteen discs ($n=5$ of each group) were seeded with cells and incubated at 37 °C. Three days after the incubation, the overlaying culture medium was discarded, and 500 μL of the MTT solution was added to each well. Incubation was performed at 37 °C for 4 hours in an incubator containing CO_2. During incubation, MTT is reduced by the action of succinate dehydrogenase present in cell mitochondria. Reduction of this enzyme results in the production of insoluble purple formazan crystals in the cytoplasm of cells, which are easily detectable under a microscope. Then, 1 mL of isopropyl alcohol was added to each well. Alcohol lyses cell cytoplasm and extracts the insoluble formazan. It dissolves the purple crystals and gives a purple color to the medium. The absorbance (optical density) of the solution was read at 570-nm wavelength using an ELISA microplate reader (Statfax 2100, USA) [21].

Gene expression analysis

Cell-seeded samples were incubated, and the medium was exchanged every 4 days. After 4 days, total RNA was extracted using RNA extraction kit (Takara, Japan), and cDNA was synthesized using reverse transcription kit (Takara, Japan). The concentration and purity of RNA sample were determined by reading the optical density at 260 and 280 nm wavelengths. For this purpose, a NanoDrop® spectrophotometer was used to assess the purity of extracted RNA [8].

Real-time PCR

StepOne™ real-time PCR system (ABI) and Master Mix kit (ABI) were used for PCR. In all the control and experimental cells (seeded on discs), two replicates of each sample were amplified for each gene to ensure accuracy. For each reaction, 10 μL of Master Mix, 1 μL of primers (forwards and reverse primers), and 6 μL of water were used. For each gene, one test tube containing primer, water, and Master Mix was employed. Next, 18 μL of the mixture was transferred to test tubes, and finally, 2 μL of the synthesized cDNA was added to each reaction tube. StepOne™ real-time PCR system was adjusted in such a way that the temperature first remained at 95 °C for 15 min. This step was necessary for the activation of DNA polymerase. Next, 40 cycles were performed with the following thermal changes of 15 s at 95 °C and 60 s at 60 °C per each cycle. The reference gene in this study was glyceraldehyde 3-phosphate dehydrogenase. The primers are listed in Table 1.

Surface roughness assessment

Surface roughness was assessed at the Dental Research Center of Tehran University of Medical Sciences using a contact surface profilometer (TR200, Germany) measuring surface roughness (Ra) in a range of 0.01–40 μm. The samples were fixed in a wax mold to prevent their mobility while the stylus scanned across their surface and measured their surface topography. The profilometer linearly scanned the surface of specimens with a crosshead speed of 0.5 mm/s. Tip radius of the stylus was 5 μ. Each disc was analyzed in triplicate in different areas in such a way that the lines did not overlap each other. The mean of the obtained values was calculated and used as the surface roughness value of the respective specimen [22].

Statistical analysis

Data were analyzed using the Kolmogorov–Smirnov, the ANOVA, the Kruskal–Wallis H, and the Bonferroni multiple comparison test. According to the results of the Kolmogorov–Smirnov test, all quantitative variables had normal distribution. Since the assumption of homogeneity of variances was hold for the three groups in the expression of IL-10, fibronectin, and integrin genes; they were analyzed using ANOVA. For the remaining two quantitative variables namely cell proliferation (MTT assay) and TNFα gene expression, this assumption was not hold for the three groups; thus, they were analyzed by the Kruskal–Wallis H test. SPSS ver:22 software was used. The level of significance was established at $\alpha=0.05$, for the overall comparisons, $\alpha=0.016$ in the individual comparisons between two groups.

Table 1 Primers list

Primer	Sequence	Length
GAPDH	F: 5′- GAGTCCACTGGCGTCTTCA -3′ R: 5′- TCTTGAGGCTGTTGTCATACTTC -3′	148
Fibronectin	F: 5′- GCAACTCTGTCAACGAAGGCT -3′ R: 5′- TCCCACTCATCTCCAACGG -3′	94
Integrin α2	F: 5′- CCAGAGGAAGGAAAAGGAGGA -3′ R: 5′- AGGGAGGAGGAAGGTTGCC -3′	141
IL-10	F: 5′- TCCATTCCAAGCCTGACCA -3′ R: 5′- CCCAAGCCCAGAGACAAGATA -3′	84
TNF	F: 5′- TGCTCCTCACCCACACCAT -3′ R: 5′- CCCAAAGTAGACCTGCCCAG -3′	239

Results

Cell morphology

Assessment of cell morphology using electron microscopy revealed cell adhesion to the surfaces of the all discs. In the Laser-Lok group, the frequency of spindle cells was higher than that of round cells. Elongated spindle cells had pseudopod-like processes, indicative of their ability to attach to disc surfaces. Cells had specific arrangements and were well adhered to certain areas. Elongated cells were more frequently observed in the superficial grooves than over prominences (Figs. 1 and 2). In the zirconia group, the majority of cells were round, and spindle cells with pseudopod-like processes were not seen (Figs. 3 and 4). In the titanium group, round and spindle cells with no specific arrangement were found in almost equal amounts. Spindle cells in the titanium group were less elongated compared to those in the Laser-Lok group (Figs. 5 and 6).

Cell viability

Cell viability/proliferation was 1.27 ± 0.222 in the Laser-Lok, 0.73 ± 0.008 in the zirconia, and 0.72 ± 0.007 in the titanium group; the difference in this regard was statistically significant among the three groups ($P = 0.002$) (Table 2, Fig. 7).

Pairwise comparison of the groups in terms of cell proliferation revealed significant differences between the Laser-Lok and zirconia, and Laser-Lok and titanium groups ($P = 0.0001$); however, the difference between the zirconia and titanium groups was not significant ($P = 1.000$).

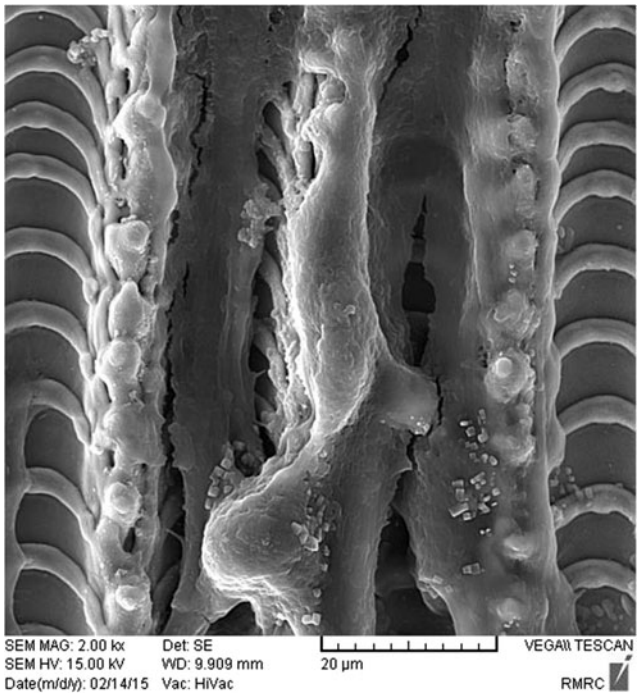

Fig. 2 Electron microscopic images of fibroblasts attached to the surface of a disc in the Laser-Lok group at ×2000

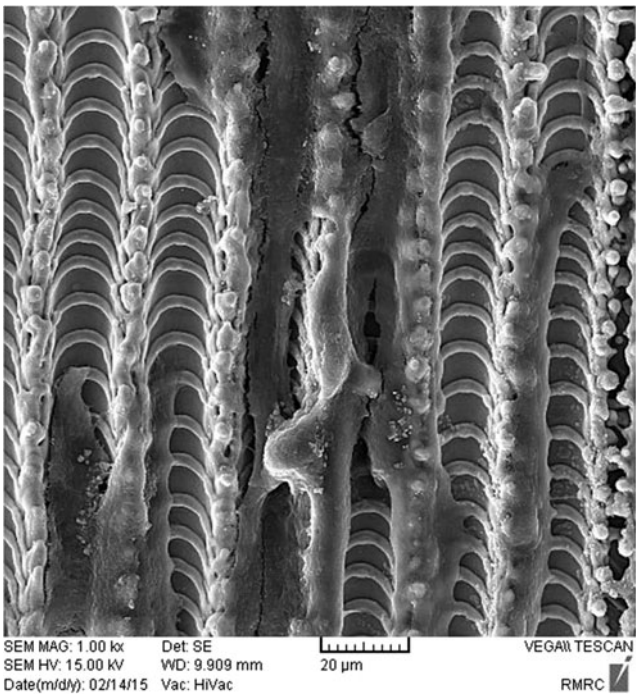

Fig. 1 Electron microscopic images of fibroblasts attached to the surface of a disc in the Laser-Lok group at ×1000



Fig. 3 Electron microscopic images of fibroblasts attached to the surface of a disc in the zirconia group at ×1000

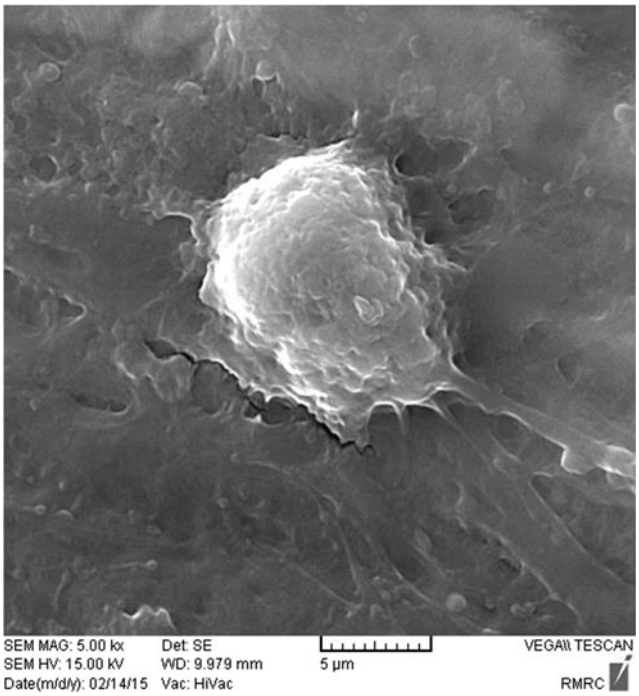

Fig. 4 Electron microscopic images of fibroblasts attached to the surface of a disc in the zirconia group at ×5000

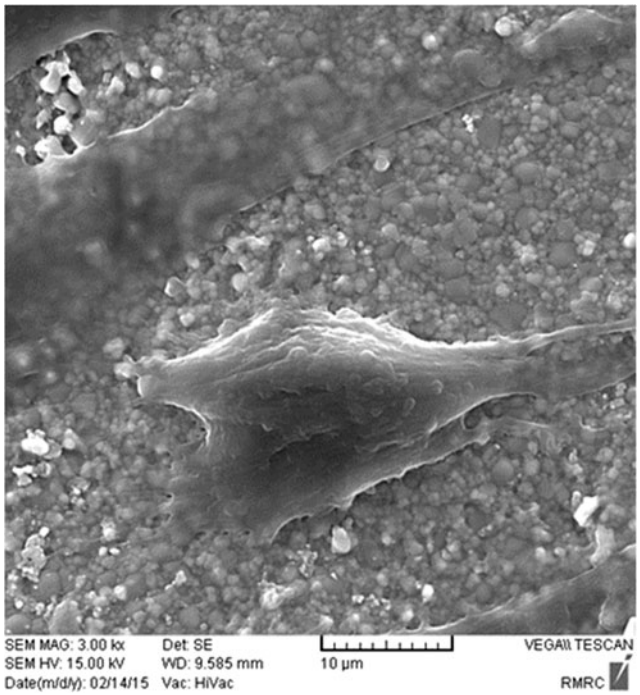

Fig. 6 Electron microscopic images of fibroblasts attached to the surface of a disc in the titanium group at ×3000

Gene expression

Expression of fibronectin gene in the Laser-Lok, zirconia, and titanium groups was 16.82 ± 0.10, 17.12 ± 0.01, and 16.65 ± 0.96, respectively. Expression of integrin gene in the Laser-Lok, zirconia, and titanium groups was 22.04 ± 0.03, 22.10 ± 0.01, and 21.26 ± 0.01, respectively. Expression of IL-10 gene in the Laser-Lok, zirconia, and titanium groups was 28.93 ± 0.10, 28.90 ± 0.07, and 28.01 ± 0.10, respectively. Expression of TNFα gene in the Laser-Lok, zirconia, and titanium groups was 32.03 ± 0.31, 31.69 ± 0.04, and 29.96 ± 0.37, respectively. The difference in this regard among the study groups was statistically significant (Table 3, Fig. 8).

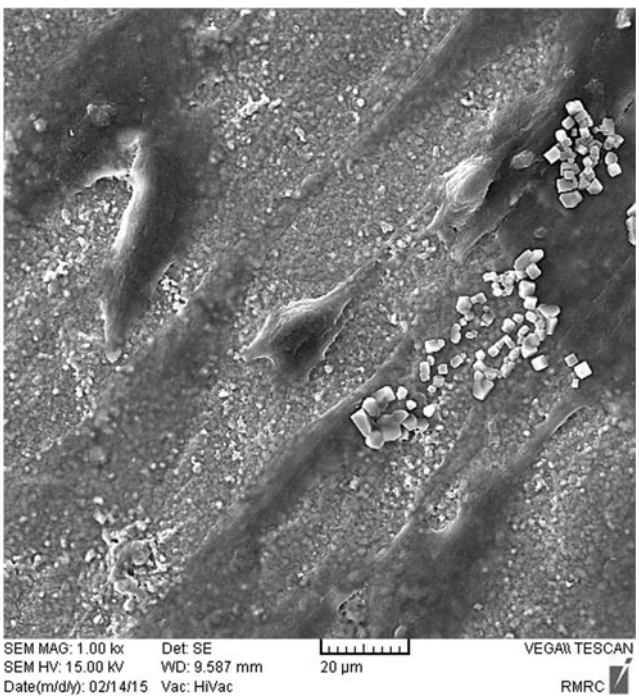

Fig. 5 Electron microscopic images of fibroblasts attached to the surface of a disc in the titanium group at ×1000

Pairwise comparison of the groups in terms of expression of IL-10 gene revealed significant differences between the Laser-Lok and titanium, and zirconia and titanium groups ($P = 0.0001$).

In terms of expression of fibronectin gene, the difference between the Laser-Lok and zirconia and also zirconia and titanium groups was statistically significant ($P = 0.0001$).

In terms of expression of integrin gene, significant differences were noted between the Laser-Lok and titanium, zirconia and titanium ($P = 0.0001$), and Laser-Lok and zirconia groups ($P = 0.002$).

Pairwise comparison of the groups in terms of expression of TNFα gene was significantly different between the titanium and zirconia, and Laser-Lok and titanium ($P = 0.0001$).

Table 2 Proliferation of fibroblasts in the three study groups

Study groups/MTT assay	Mean ± Sd	*P* value
Laser-Lok	1.27 ± 0.222	
Zirconia	0.73 ± 0.008	0.002
Titanium	0.72 ± 0.007	

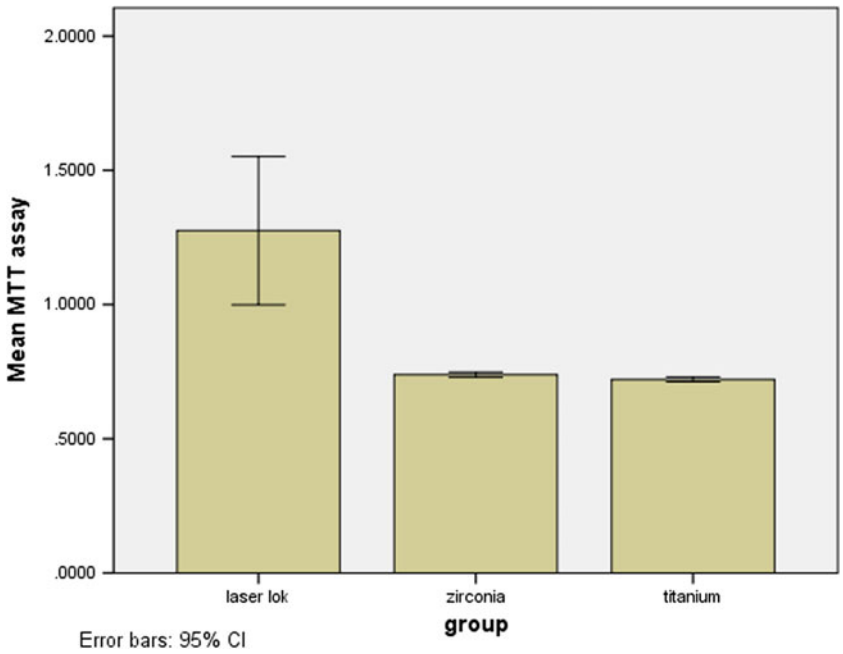

Fig. 7 Proliferation of fibroblasts in the three study groups

Surface roughness

The surface roughness value was 1.33 ± 0.03, 0.40 ± 0.05, and 0.20 ± 0.08 μm in the Laser-Lok, zirconia, and titanium groups, respectively (Table 4, Fig. 9). The difference in this regard was statistically significant among the three groups ($P = 0.0001$).

Pairwise comparison of the groups in terms of surface roughness revealed significant differences between the Laser-Lok and zirconia, Laser-Lok and titanium, and zirconia and titanium groups ($P = 0.0001$).

Discussion

Long-term prognosis of dental implants depends not only on the quality of osseointegration but also on the quality of mucosal seal between the gingiva and the abutment and implant [1, 2, 4]. In the recent years, implant manufacturers have modified the surface texture of dental implants from smooth to rough with the purpose of enhancing osseointegration. This modification is random on dental implants and increases the contact area between the bone cells and implant, with no significant effect on the soft tissue [23]. Considering the importance of soft tissue seal, increasing attention has been recently paid to the surface modifications of dental implants in regions in contact with the soft tissue (abutment). The mechanical substrate underneath the biological tissues has a significant effect on cell proliferation and differentiation [24]. In vitro studies have demonstrated variations in the morphology of fibroblasts attached to moderately rough surfaces compared to those adhered to polished surfaces [25]. Creation of microgrooves results in adhesion of fibroblasts to the underlying surface and subsequent connective tissue attachment while

Table 3 Expression of IL-10, TNFα, fibronectin, and integrin genes in the Laser-Lok, zirconia, and titanium groups

Groups/genes	Mean ± SD			
	IL-10	TNFα	Fibronectin	Integrin
Laser-Lok (I1)	28.93 ± 0.10	32.03 ± 0.31	16.82 ± 0.10	22.04 ± 0.03
Zirconia (I2)	28.90 ± 0.07	31.69 ± 0.04	17.12 ± 0.01	22.10 ± 0.01
Titanium (I3)	28.01 ± 0.10	29.96 ± 0.37	16.65 ± 0.96	21.26 ± 0.01
P value	0.0001	0.002	0.0001	0.0001
95 % CI[a]				
I1-I2)	(−0.145–0.20)	(0.257–0.409)	(−0.441–(−0.151))	(−0.107–(−0.026))
(I1-I3)	(0.738–1.084)	(1.76–3)	(0.026–0.317)	(0.733–0.814)
(I2-I3)	(0.711–1.056)	(1.43–2.67)	(0.323–0.613)	(0.80–0.881)

[a] 95 % confidence interval mean difference: (I1-I2), (I1-I3), and (I2-I3), respectively

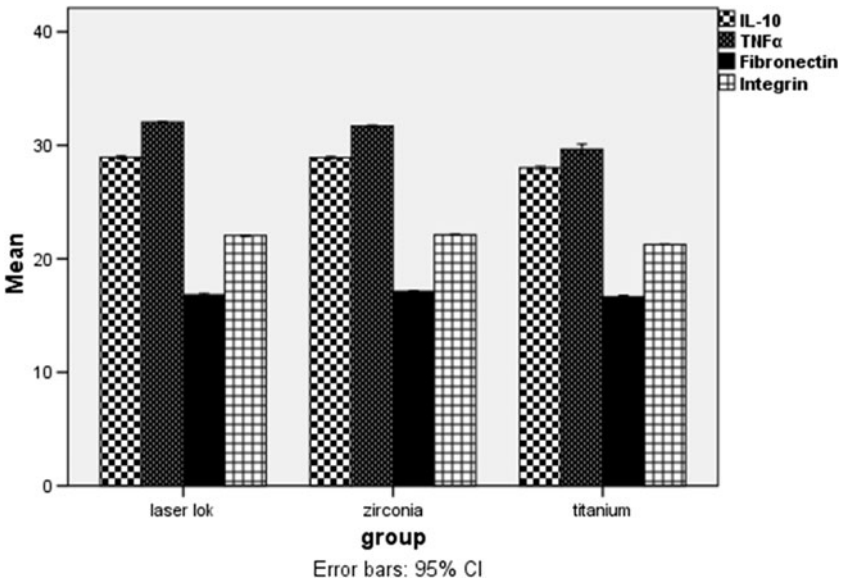

Fig. 8 Expression of IL-10α, fibronectin, and integrin genes in the three groups of Laser-Lok, zirconia, and titanium

preventing the apical migration of epithelial cells [20, 26]. Evidence shows organized arrangement of gingival fibroblasts proliferated on surfaces with irregular microgrooves; whereas, fibroblasts often show random arrangements on surfaces with other forms of modifications such as sandblasted substrates [16, 25]. Coarse or regularly roughened surfaces with specific dimensions can selectively manipulate the activity of osteoblasts and fibroblasts and alter their proliferation pattern via contact guidance [27].

In the current study, the morphology of cells (observed under an electron microscope) attached to Laser-Lok surfaces was significantly different from that on zirconia and titanium surfaces, which is in agreement with the findings of Miura et al., [12] Groβner-schreiber et al., [1], and Al Mustafa et al. [14]. They showed that various titanium surface modifications caused changes in the morphology of fibroblasts. Assessment of cell morphology is important in that it can determine the cell-surface affinity. Cells with an elongated morphology can more strongly attach to titanium surfaces due to having cytoplasmic pseudopods as compared to round cells [28]. Electron microscopic micrographs in our study revealed that the majority of cells in the Laser-Lok group were elongated and had pseudopods. In the zirconia group, most cells were round, and in the titanium group, a combination of elongated and round cells was observed on the surfaces.

Table 4 Surface roughness of the Laser-Lok, zirconia, and titanium groups

Surface roughness/groups	Mean ± SD	*P* value	95 % CI[a]
Laser-Lok (I1)	1.33 ± 0.03		(0.83–1.02)
Zirconia (I2)	0.40 ± 0.05	0.0001	(1.03–1.23)
Titanium (I3)	0.20 ± 0.08		(0.104–0.301)

[a] 95 % confidence interval mean difference: (I1-I2), (I1-I3), and (I2-I3), respectively

Creation of microgrooves is a type of surface modification performed on dental implants. Laser-Lok micro-channels include a series of cell-sized circumferential channels meticulously created by employing laser ablation technology. Using this novel technology, uniform micro-channels with an optimal size for attachment and organized arrangement of osteoblasts and fibroblasts are created. Moreover, the Laser-Lok microstructure includes a repeating nano-structure that increases the contact area and enables the integration of cell pseudopods and collagen micro-fibrils with the Laser-Lok surface [29, 30]. The elongated morphology of fibroblasts observed in the Laser-Lok group in our study further confirms the above-mentioned observations.

Zhu et al. [22] stated that changing the topography of the substrate increases cell spreading and migration and decreases focal contacts among cells. This promotes the rate of mitosis and cell proliferation and enhances cell adhesion to the substrate. Round cells have larger focal contacts, which suppresses mitosis and decreases the available surface area for adhesion. Flat and elongated cells with pseudopods can increase the quantity and quality of bone adjacent to the implant collar [31]. Heinrich et al. showed that the surface roughness created by laser irradiation increased the adhesion of fibroblasts [32].

In the current study, MTT assay was used to determine cell proliferation and viability. This assay is a direct method for measurement of the activity of cellular succinate dehydrogenase [4]. This test has been used for assessment of cell viability in many previous investigations; however, other techniques such as cell counting and measurement of the level of lactate dehydrogenase (LDH) have also been employed in some previous studies [8, 17].

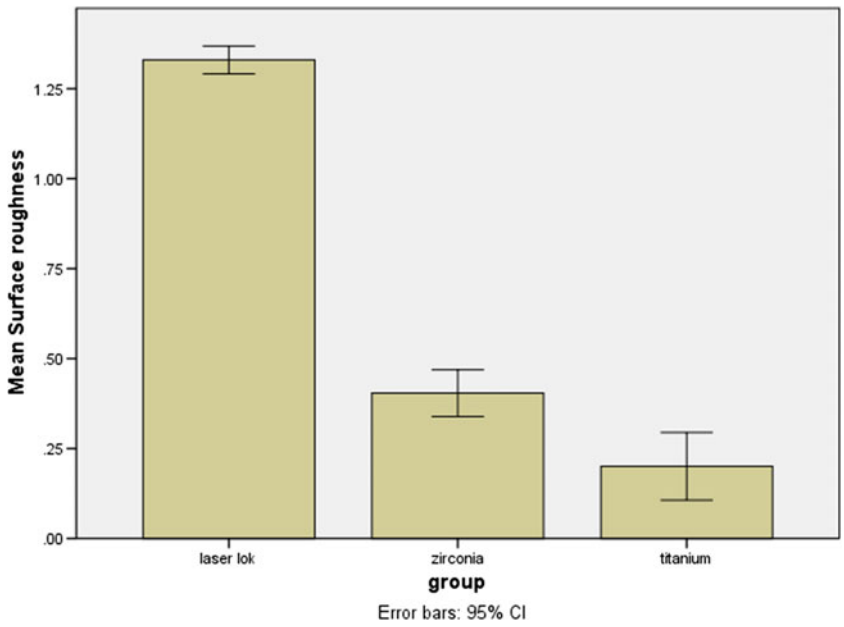

Fig. 9 Surface roughness of the Laser-Lok, zirconia, and titanium groups

In the current study, the highest rate of cell proliferation was noted in the Laser-Lok group, which is in contrast to the findings of Baltriukiene et al. [4]. They showed that modified titanium surfaces had variable effects on the proliferation of gingival fibroblasts and the highest proliferation was noted on polished titanium surfaces. The difference between their results and ours may be attributed to the difference in the technique of laser treatment of discs employed in the two studies.

Nothdurft et al. [17] stated that substrate chemistry plays an important role in proliferation of fibroblasts, which is in line with our findings. However, they reported higher proliferation of fibroblasts on machined and polished surfaces than on rough (sandblasted) surfaces. Such a difference in results may be due to the method of assessment of cell proliferation since they used cell counting and fluorescent imaging in their study while we performed the MTT assay, which exclusively measures viable cells. Moreover, different techniques of surface preparations may yield different results.

For the assessment of gene expression using real-time PCR, two genes coding for inflammatory cytokines namely IL-10 and TNFα and two genes coding for adhesion markers namely fibronectin and integrin were chosen in the current study to evaluate the effect of type of surface treatment on different cell processes. Real-time PCR has revolutionized the assessment of genes due to having high sensitivity and specificity, performing a quantitative reaction and enabling absolute quantification of genome. Fast conduction, no need for the post PCR product detection, live observation of reaction, and the ability to stop the reaction at any time are among the other advantages of this technique. In this method, instead of detecting the product by electrophoresis, the reaction product is detected using markers [33].

Assessment of the above-mentioned genes in the current study revealed significant differences in the expression of IL-10 and TNFα genes in the Laser-Lok group compared to that in the titanium group. In our study, the highest expression of IL-10 at 72 h was noted in the Laser-Lok group followed by the zirconia and titanium groups, respectively. This finding is in contrast to the results of Schwartz-Filho et al. They assessed cytokines such as IL-10 on modified titanium surfaces at 24 and 48 h and found no trace of IL-10 [25]. They used ELISA for assessment of gene expression at 1 and 2 days after culture; whereas, we used real-time PCR after 72 h. Different techniques and time points of assessment in the two studies can explain the contradictory results. Higher expression of IL-10 in the Laser-Lok compared to those in the other groups in our study may indicate greater compatibility of fibroblasts with Laser-Lok surfaces.

IL-10 is an anti-inflammatory cytokine [8, 23] and is known as a cytokine synthesis inhibitory factor. This cytokine stimulates B cell proliferation and maturation and increases the production of antibodies; it also enhances the process of tissue healing after inflammation. TNFα is a pro-inflammatory mediator that regulates a wide range of biological processes such as cell proliferation, differentiation, and apoptosis [34]. TNFα stimulates the release of matrix metalloproteinases (MMPs) and is secreted by the resident cells such as osteoblasts, gingival fibroblasts, and immune cells. It is responsible for early responses to microbial plaque accumulation, attracts other inflammatory cells, and is a key regulator of the formation of osteoclasts [35].

Our findings regarding the expression of TNFα gene were in contrast to those of Francova et al. [6]. They did not find significant levels of TNFα on titanium surfaces subjected to

different treatments after 72 h. This result may be due to the use of ELISA in their study versus real-time PCR in our study since the latter is more accurate. Expression of TNFα in the titanium group was significantly lower than those in the other two groups, which may be explained by the lower roughness of titanium compared to other surfaces.

In the current study, the three groups were significantly different in terms of the expression of fibronectin gene. The expression of fibronectin gene was significantly higher in the zirconia group. This expression was also higher in the Laser-Lok compared to that in the titanium group, but not significantly. Fibronectin is a glycoprotein of the extracellular matrix with high molecular weight. It bonds to the membrane protein receptors known as integrins. Its insoluble type, which is an important component of the extracellular matrix, is secreted by different cells and particularly fibroblasts. Fibronectin plays an important role in adhesion, proliferation, migration, and differentiation of cells [36].

Integrins are trans-membrane receptors involved in cell–cell and cell–extracellular matrix adhesion [24, 37]. These molecules have α2, α8, and β3 subtypes, all of which play key roles in cell adhesion [8]. In conjunction with some other molecules such as the cadherins and selectins, they participate in cell interactions as well. The ligands for integrin include fibronectin, vitronectin, collagen, and laminin [38]. Expression of integrin was significantly different among our study groups, and the highest expression was noted in the zirconia followed by the Laser-Lok group. The difference between the Laser-Lok and titanium groups was statistically significant. This finding confirms the results of Lee et al. [2]. They reported higher expression of integrin, which is a key molecule in cell adhesion, on laser-modified surfaces compared to polished titanium surfaces. Increased expression of integrin and fibronectin in the zirconia group in our study is probably attributed to the dominance of round morphology of cells in this group. Cell–cell and cell–extracellular matrix attachments of round cells are mainly based on focal adhesion.

In most previous studies, titanium surfaces were modified by creating grooves using laser irradiation, sandblasting, or alkaline treatment; however, in the current study, the authors were provided with discs fabricated by the manufacturers (BioHorizons) with standards similar to those of abutments available in the market.

Significant differences existed in terms of surface roughness among the three groups in our study. Cell proliferation on Laser-Lok surfaces with a mean surface roughness of 1.33 μ was higher than that in the other two groups, which contradicts the findings of Kunzler et al. They analyzed the behavior of fibroblasts on surfaces with variable roughness and found that increasing the surface roughness decreased the proliferation of fibroblasts [13]. Such a controversy in results may be due to the extremely high surface roughness value (Ra = 6.1 μm) in their study compared to ours.

A dental implant with a machined collar does not allow connective tissue attachment, and thus, gingival tissue seal will not be achieved [39]. Evidence shows that a surface roughness value of Ra = 0.2 μm is necessary to achieve a soft tissue seal around an abutment; whereas, smooth and machined surfaces result in unwanted adhesion and apical migration of epithelial cells [7]. Despite all the above, optimal value of surface roughness for soft tissue adhesion is still a matter of debate. Moreover, variability in the methods employed to measure the surface topography and the different variables reported (Ra, Sa, and St) in studies make the comparison of results difficult, if not impossible [40].

Our study showed higher count of viable cells on Laser-Lok surfaces despite having a higher surface roughness value (1.33 μm), and the morphology of the cells justified their greater adhesion. This finding can be explained by the microstructure of Laser-Lok surfaces, which includes a repeating nano-structure, maximizing the contact area and enhancing the integration of pseudopods of cells and collagen micro-fibrils with the surface [14, 27, 41].

Laser treatment can affect the physicochemical properties of surfaces such as surface wettability, which is an influential factor in cell response to the surface [42]; however, assessment of this factor was out of the scope of this study.

The limitation with the use of micrometer-scale surface topography to guide the attachment and arrangement of fibroblasts is that increasing the surface roughness may enhance bacterial accumulation [7]. However, Di Giulio et al., in a recent study, showed that biofilm formation by Porphyromonas gingivalis was significantly lower on laser-treated surfaces compared to that on sandblasted and machined surfaces [43].

Simulating the intraoral conditions and assessing the confounding effects of saliva and intraoral bacteria increase the generalizability of the results. Thus, variables must be necessarily evaluated in vivo in order to assess their possible role in cell behavior adjacent to dental implants.

Within the limitations of this study, it may be concluded that surface characteristics of materials used in dental implant abutments can affect the morphology and proliferation of fibroblasts as well as the expression of IL-10, TNFα, fibronectin, and integrin genes. Laser-Lok surfaces are more biocompatible with fibroblasts compared to titanium and zirconia surfaces. Better adhesion of fibroblasts to laser-modified

surfaces can result in more favorable soft tissue seal around dental implants.

Acknowledgment The authors would like to express their gratitude to the Zarin Iran Company and BioHorizons, USA, for providing us with the discs.

References

1. Groβner-Schreiber B, Herzog M, Hedderich J, Duck A, Hannig M, Griepentrog M (2006) Focal adhesion contact formation by fibroblast cultured on surface-modified dental implants: an in vitro study. Clin Oral Implants Res 17(6):736–745
2. Lee DW, Kim J-G, Kim MK, Ansari S, Moshavernia A et al (2015) Effect of laser-dimpled titanium surfaces on attachment of epithelial-like cells and fibroblasts. J Adv Prosthodont 7(2):138–145
3. Nevins M, Kim DM, Jun SH, Schupbach P, Guze K, Schupbach P, Nevins ML (2010) Histologic evidence of a connective tissue attachment to laser microgrooved abutments: a canine study. Int J Periodontics Restorative Dent 30(3):245–255
4. Baltriukiene D, Sabaliuskas V, Balciusas E, Melninkaitis A, Liufkelskene E, Bukelskiene V (2014) The effect of laser-treated titanium surface on human gingival fibroblast behavior. J Biomed Mater Res A 102(3):713–720
5. Nevins M, Nevins ML, Camelo M, Boyesen JL, Kim DM (2008) Human histologic evidence of a connective tissue attachment to a dental implant. Int J Periodontics Restorative Dent 28:111–121
6. Frankova J, Pivodova V, Ruzicka F, Tomankova K, Vrlkova J (2013) Comparing biocompatibility of gingival fibroblast and bacterial strains on a different modified titanium discs. J Biomed Mater Res A 101(10):2915–2924
7. Kearns VR, Williams RL, Mirvakily F, Doberty PJ, Martin N (2013) Guided gingival fibroblast attachment to titanium surface: an in vitro study. J Clin Periodontol 40(1):99–108
8. Gómez-Florit M, Ramis JM, Xing R, Taxt-Lamolle S, Haugen HJ, Lyngstadaas SP, Monjo M (2014) Differential response of human gingival fibroblast to titanium- and titanium-zirconium-modified surfaces. J Periodontal Res 49(4):425–436
9. Pecora GE, Ceccarelli R, Bonelli M, Alexander H, Ricci JL (2009) Clinical evaluation of lasermicrotexturing for soft tissue and bone attachment to dental implants. Implant Dent 18(1):57–66
10. Nevins M, Nevins ML, Gobbato L, Lee HJ, Wang CW, Kim DM (2013) Maintaining inter-implant crestal bone height via a combined platform-switched, Laser-Lok® implant/abutment system: a proof-of-principle canine study. Int J Periodontics Restorative Dent 33(3):261–267
11. Geurs NC, Vassilopoulos PJ, Reddy MS (2011) Histologic evidence of connective tissue integration on laser microgrooved abutments in humans. Clin Adv Periodontics 1:29–33
12. Miura S, Takebe J (2012) Biological behavior of fibroblast-like cells cultured on anodized-hydrothermally treated titanium with a nanotopographic surface structure. J Prosthodont Res 56(3):178–186
13. Kunzler TP, Drobek T, Schuler M, Spencer ND (2007) Systematic study of osteoblast and fibroblast response to roughness by means of surface-morphology gradients. Biomaterials 28(13):2175–2182
14. Al Mustafa M, Agis H, Muller H, Watzck G, Gruber R (2015) In vitro adhesion of fibroblast cells to titanium allay discs treated with sodium hydroxide. Clin Oral Impl Res 26(1):15–19
15. Thoma DS, Jones AA, Dard M, Grize L, Obrecht M, Cochran DL (2011) Tissue integration of a new titanium-zirconium dental implant: a comparative histologic and radiographic study in the canine. J Periodontol 82(10):1453–1461
16. Rutkunas V, Bukelskiene V, Sabaliauskas V, Balciunas E, Malinauskas M, Baltriukiene D (2015) Assessment of human gingival fibroblast interaction with dental implant abutment abutment materials. J Mater Sci Mater Med 26(4):169
17. Nothdurft FP, Fontana D, Ruppenthal S, May A, Aktas C, Mehraein Y (2014) Differential behavior of fibroblasts and epithelial cells on structured implant abutment materials: a comparison of materials and surface topographies. Clin Imp Dent and Related Res 16:640–648
18. Fenner N, Hämmerle CH, Sailer I, Jung RE (2015) Long-term clinical, technical, and esthetic outcomes of all-ceramic vs. titanium abutments on implant supporting single-tooth reconstructions after at least 5 years. Clin Oral Implants Res. doi:10.1111/clr.12654
19. Traini T, Pettinicchio M, Murmura G, Varvara G, Di Lullo N, Sinjari B, Caputi S (2011) Esthetic outcome of an immediately placed maxillary anterior single-tooth implant restored with a custom-made zirconia-ceramic abutment and crown: a staged treatment. Quintessence Int 42(2):103–108
20. Ketabi M, Deporter D (2013) The effect of laser microgrooves on hard and soft tissue attachment to implant collar surfaces: a literature review and interpretation. Int J Periodontics & Restorative Dentistry 33:e145–e152
21. Bonakdar S et al (2010) Preparation and characterization of polyvinyl alcohol hydrogels crosslinked by biodegradable polyurethane for tissue engineering of cartilage. Mater Sci Eng C 30(4):636–643
22. Zhu X, Chen J, Scheideler L, Reichl R, Geis-Gerstorfer J (2004) Effects of topography and composition of titanium surface oxides on osteoblast responses. Biomaterials 25(18):4087–4103
23. Berglundh T, Lindhe J, Ericsson I et al (1991) The soft tissue barrier at implants and teeth. Clin Oral Implants Res 2:81–90
24. Brunette DM (1998) The effect of implant surface topography on the behavior of cells. Int J Oral Maxillofac Implants 3:231–246
25. Schartz-Filho HD, Morandini ACF, Ramos-Juniur ES, Santos CF, Marcantonio JRE, Wennerberg A (2012) Titanium with nanotopography modulate cytokine production in cultured human gingival fibroblast. J Biomed Mater Res A 100(10):2629–2636
26. Grew JC, Ricci JL, Alexander H (2008) Connective-tissue responses to defined biomaterial surfaces. II. Behavior of rat and mouse fibroblasts cultured on microgrooved substrates. J Biomed Mater Res A 85(2):326–335
27. Ricci JL, Grew JC, Alexander H (2008) Connective-tissue responses to defined biomaterial surfaces. I. Growth of rat fibroblast and bone marrow cell colonies on microgrooved substrates. J Biomed Mater Res 85(2):313–325
28. Trylovich DJ, Cobb CM, Pippin DJ, Spencer P, Killoy WJ (1992) The effect of the Nd:YAG laser on in vitro fibroblast attachment to endotoxin-treated root surfaces. J Periodontol 63:626–632
29. Canullo L, Penarrocha-Oltra D, Marchionni S, Bagan L, Penarrocha- Diago M, Micarelli C (2014) Soft tissue cell adhesion to titanium abutments after different cleaning procedures: preliminary results of a randomized clinical trial. Med Oral Patol Pral Cir Bucal 19(2):e177–e183
30. Weiner S, Simon J, Ehrenberg DS, Zweig B, Ricci JL (2008) The effects of laser microtextured collars upon crestal bone levels of dental implants. Implant Dent 17(2):217–228
31. Meyer U, Joos U, Mythill J et al (2004) Ultrastructural characterization of the implant/bone interface of immediately loaded dental implants. Biomaterials 25:1959–1967
32. Heinrich A, Dengler K, Koerner T, Haczek C, Deppe H, Stritzker B (2008) Laser-modified titanium implants for improved cell adhesion. Lasers Med Sci 23(1):55–58
33. Mackay IM, Arden KE, Nitsche A (2002) Survey and summary: real-time PCR in virology. Nucleic Acid Res 30(6):1292–1305

34. Ouyong W, Rutz S, Valdez PA, Hymowitz SG (2011) Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol 29:71–109
35. Newman MG, Takei HH, Klokkevold PP, Carranza FA (2012) Carranza's clinical periodontology. Elsevier Saunders, St. Louis
36. Pankov R, Yamada KM (2002) Fibronectin at a glance. J Cell Sci 115:3861–3863
37. Atsuta I, Ayukawa Y, Furuhashi A, Ogino Y, Moriyama Y, Tsukiyama Y (2014) In vivo and in vitro studies of epithelial cell behavior around titanium implants with machined and rough surfaces. Clin Imp Dent and Related Res 16: 772–780
38. Alberts B, Johnson A, Lewis J et al (2002) Molecular biology of the cell. Garland science, New York
39. Cochran DL, Hermann JS, Schenk RK, Higginbottom FL, Buser D (1977) Biologic width around titanium implants. A histometric analysis of the implanto-gingival junction around unloaded and loaded nonsubmerged implants in the canine mandible. J Periodontol 68:186–198
40. Bachle M, Kohal RJ (2004) A systematic review of the influence of different titanium surfaces on proliferation, differentiation and protein synthesis of osteoblast-like MG63 cells. Clin Oral Impl Res 15(6):683–692
41. Gomes-Florit M, Xing R, Ramis JM, Taxt-Lamolle S, Haugen HJ, Lingstadaas SP, Monjo M (2014) Human gingival fibroblast function is stimulated on machined hydride titanium zirconium dental implants. J Dent 42(1):30–38
42. Khosroshahi ME, Mohmoodi M, Saeedinasab H (2009) In vitro and in vivo studies of osteoblast cell response to a titanium-6 aluminium-4 vanadium surface modified by neodymium:yttrium-aluminium-garnet laser and silicon carbide paper. Lasers Med Sci 24:925–939
43. Di Giulio M et al (2015) Porphyromonasì gingivalis biofilm formation in different titanium surfaces, an in vitro study. Clin Oral Implants Res. doi:10.1111/clr.12659