Archives of Oral Biology 87 (2018) 204–210

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Influence on proliferation and adhesion of human gingival fibroblasts from different titanium surface decontamination treatments: An in vitro study

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ARTICLE INFO

Keywords:
Dental implant
Decontamination
Human gingival fibroblasts (HGFs)
Proliferation
Adhesion.

ABSTRACT

Objectives: To investigate the effects of different decontamination treatments on microstructure of titanium (Ti) surface as well as proliferation and adhesion of human gingival fibroblasts (HGFs).
Material and methods: Ti discs with machined (M) and sand blasted, acid etched (SAE) surfaces were treated with five different decontamination treatments: (1) stainless steel curette (SSC), ultrasonic system with (2) straight carbon fiber tip (UCF) or (3) metal tip (UM), (4) rotating Ti brush (RTB), and (5) Er:YAG laser (30 mJ/pulse at 30 Hz). Surface roughness was analyzed under optical interferometry. HGFs were cultured on each disc. Proliferation and adhesive strength were analyzed. qRT-PCR and ELISA were performed to detect the RNA and protein expression of FAK, ITGB1, COL1A1, and FN1 respectively from different Ti surfaces.
Results: Surface roughness increased on M surface. Proliferation, adhesive strength and gene expression were higher on M surface than SAE surface. Decontamination treatments affected surface parameters significantly ($P < 0.001$), making M surface less smooth while SAE surface became less rough. SSC, UCF, UM and RTB decreased proliferation on M surfaces significantly ($P < 0.05$). UCF, RTB and laser increased proliferation on SAE surface significantly ($P < 0.05$). UM decreased adhesive strength on M surface significantly and laser increased adhesive strength on SAE surface significantly ($P < 0.05$). Gene expression increased with time and was altered by decontamination treatments significantly ($P < 0.001$).
Conclusions: Decontamination treatments influence surface roughness and cell behavior of HGFs. Laser might be an optimal decontamination treatment which has the least negative effect on M surface and the most positive effect on SAE surface.

1. Introduction

Implant dentistry is developing rapidly with an increasing 5-year survival rate (Pjetursson, Asgeirsson, Zwahlen, & Sailer, 2014), while plaque induced inflammation around implant is still the main cause of implant failure (Atsuta et al., 2016). Implant surface decontamination has been suggested as an indispensable procedure to prevent and treat inflammation around the implant (Subramani & Wismeijer, 2012). The target of decontamination is usually the polished implant surface, but could also include the sand blasted, acid etched (SAE) surface exposed from the bone because of alveolar resorption. Interaction of dental implant with its surrounding cells is critical for the stability and maintenance of the implant. For cells around implant, human gingival fibroblasts (HGFs) are major cellular components of oral soft tissue (Bruckmann, Walboomers, Matsuzaka, & Jansen, 2005). HGFs are essential for maintaining oral implants in good condition through multiple functions including repairing tissue damage and sealing implants from oral microbial environment (Lekic & McCulloch, 1996; Moon, Berglundh, Abrahamsson, Linder, & Lindhe, 1999; Palaiologou, Yukna, Moses, & Lallier, 2001). All these functions require the adhesion and proliferation of HGFs on the surface of implant and overall production and turnover of the extracellular matrix (ECM) to maintain synthesis and integrity of the gingival connective tissues (Biagini, Checchi, Pelliccioni, & Solmi, 1992; Flemmig, 1999). Focal adhesion around implant is a dynamic process which involves multiple extracellular matrix linking proteins (Rustad, Wong, & Gurtner, 2013). Focal adhesion kinase (FAK) and integrin β1 (ITGB1) play critical roles in fibroblast adhesion signaling (Rustad et al., 2013). Inhibition of FAK resulted in decreased level of fibrotic response (Kim, Wen, Prowse, & Hamilton, 2015). Block of integrin reduced fibroblast attachment on different implant surfaces (Kramer, Janikkeith, Cai, Ma, & Watanabe, 2009). Collagen type I (COL1A1) and fibronectin (FN1) are

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https://doi.org/10.1016/j.archoralbio.2017.12.013
Received 15 August 2017; Received in revised form 30 October 2017; Accepted 13 December 2017

predominant cellular components of ECM in both bone (Stadlinger et al., 2012) and periodontal ligament (McCulloch, Lekic, & McKee, 2000), and could influence the stability of implants.

It has been observed that surface microstructure can influence epithelial growth and attachment of fibroblasts (Brunette & Chehroudi, 1999; Chehroudi, Gould, & Brunette, 1989, Chehroudi et al., 1990; Oates, Maller, West, & Steffensen, 2005; Rutkunas et al., 2015). Alterations in surface microstructure may have different influences on the attachment of epithelial cells and fibroblasts, thus having an impact on the maintenance or re-establishment of the soft tissue sealing around implants after decontamination treatments. The effects of various decontamination treatments on implant surface have been evaluated (Al-Hashedi, Laurenti, Benhamou, & Tamimi, 2017; Schmidt et al., 2017). However, whether the microstructural alterations of decontaminated titanium (Ti) surface affect the cellular behavior of HGFs on the implant surface require more investigations to help guide clinical decisions of choosing decontamination method.

In the present in vitro study, we investigated the effect of different decontamination treatments on surface roughness of two kinds of Ti discs (mechanically polished surface and SAE surface) as well as on proliferation, adhesive strength and gene expression of HGFs. We hypothesized that: (1) decontamination treatments of Ti discs alter surface microstructure significantly compared to non-decontaminated control. And the changes of surface microstructure vary with different decontamination treatments; (2) surface microstructure change of decontaminated Ti discs affects proliferation and adhesion of HGFs.

2. Material and methods

2.1. Discs preparation and decontamination treatments

Commercial puretitanium (purity: 99.99%, AKEC medical, China) was processed into discs with 15 mm diameter and 1 mm thickness using the same cutting technique. Disc surfaces were mechanically polished and ultrasonically cleaned by pure acetone and ethanol for one group (M), and was sand blasted (pressure: 6 atms; distance of jet: 10 cm; angle of jet: 45°; powder material: white alundum; duration: 15s) and acid etched (2%HF + 4%HNO_3 room temperature for 25 min, then H_2SO_4 +HCl (1:1) 80 °C water bath 30 min) for the other group (SAE). Surface roughness (Sa) was measured by scanning electron microscope (SEM). The polished surfaces have an average Sa of 0.012 μm ± 0.002, while the SAE surfaces have an average Sa of 2.972 μm ± 0.126.

For each group, discs were randomly and equally allocated to the following decontamination (1)-(5) and control (6) groups (Fig. 1):

(1) Stainless steel curette (SSC) (Grace, Hu-Friedy Mfg. Co., Inc. USA), with a working force of 0.25 N and an angle of 70–80°, moving in an imbricate style for 60 s
(2) Ultrasonic system with straight carbon fiber tip (UCF) (P5 Newtron®, Satelec, ACTEON, France), 25 KHz, 10 μm swing amplitude, with a working angle of 15°, moving in an imbricate style for 60 s
(3) Ultrasonic system with metal tip (UM) (P5 Newtron®, Satelec, ACTEON, France, France), 25 KHz, 30 μm swing amplitude, with a working angle of 15°, moving in an imbricate style for 60 s
(4) Rotating titanium brush (RTB) (iBrush, NeoBioteck, South Korea), with a working force of 0.25 N, 920 rpm/min, moving in an imbricate style for 60 s
(5) Er:YAG laser (Fontona, Slovenia), 30 mJ/pulse, 30 Hz, with a working angle of 15°, moving in an imbricate style for 60 s
(6) No treatment.

After treated by different decontamination methods, all the discs were washed in an ultrasonic cleaner. Deionized water (10 min × 3 times), acetone (10 min × 2 times) and 95% ethanol (10 min × 3 times) were applied successively to wash the discs to remove debris and organic solvent. Finally, all discs were rinsed by double distilled water (10 min × 3 times) and sterilized by autoclaving (121 °C, 205.8 kPa, 30 min) before being placed into 24-well plates (Lap Tek Chamber Slide, Nalge Nunc, Naperville, IL, USA).

2.2. Surface analysis

Surface roughness parameters (ISO 25178 standard) including height parameters of arithmetical mean height of a surface (Sa), height of a surface (Sq), and maximum height of a surface (Sz); hybrid parameters of root mean square gradient of a surface (Sdq) and developed area ratio (Sdr); spatial parameters of texture aspect ratio of the surface at 20% (Str20) and 37% (Str37) were evaluated by optical interferometry (MicroXAM™, USA).

2.3. Cell culture

The study protocol was reviewed and approved by the IRBPKUSS. Informed consent to collect periodontal tissue was obtained from one healthy volunteer before crown lengthening surgery. Cell culture of HGFs was referred to an established in vitro procedure (Cabral, Costa, & Fernandes, 2007). The gingival tissue was cut into minor blocks under sterile conditions, cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Grand Island, NY, USA) in a humidified 5% CO_2 atmosphere at 37 °C for two weeks. Cell morphology and expansion were observed under phase contrast microscope (Olympus, Japan). In vitro passage was performed when the confluence reached 90%. After the fourth passage, human gingival fibroblasts were digested using a trypsin-EDTA solution (Sigma, St. Louis, MO, USA), and the cell number was determined using a hemacytometer. The cell suspension was centrifuged for 5 min at 1000 r/min at room temperature and re-suspended in DMEM containing 10% FBS to a density of 2×10^5 cells/ml. 1 ml cell suspension was seeded on each Ti disc. For each processing method group, three subgroups with cell culture time of 24 h, 72 h and 168 h were set under same culture conditions.

2.4. Cell proliferation

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma- Aldrich, St Louis, MO, USA) assay was used according to the manufacturer's instructions. After incubation at different time periods, the discs were taken out from the cell culture plate and were transferred into a new plate. They were then rinsed with PBS for three times to eliminate cells adhere to the plate. 100 μl 5 mg/ml MTT solution plus 1 ml culture medium was added into each well, and the plates were incubated for 4 h (37 °C, 5% CO_2). After removing medium and rinsing with PBS for three times, formazan crystals were dissolved by 750 μl/well DMSO (Sigma, US). Optical density (OD) of the resulting solution was measured at 570 nm by a plate reader (BioTek, USA).

2.5. Adhesive strength

The adhesive strength of HGFs was evaluated by adhesion assays described in a previous study (Baltriukiene et al., 2014). The adhesive assay was carried out in two stages. Titanium discs with cells in each treatment group were divided evenly and randomly into two groups. One group was under MTT test directly to detect the baseline cell number. Another group was shaken firstly (at 200 rpm for 5 min), after rinsing the detached cells with PBS for three times, MTT test was also performed to determine the remaining cell number. We defined a new parameter- adhesion fraction to evaluate the adhesive strength of HGFs on titanium discs on each treatment group.

Adhesion fraction = Absorbance after shaking ÷ absorbance before shaking

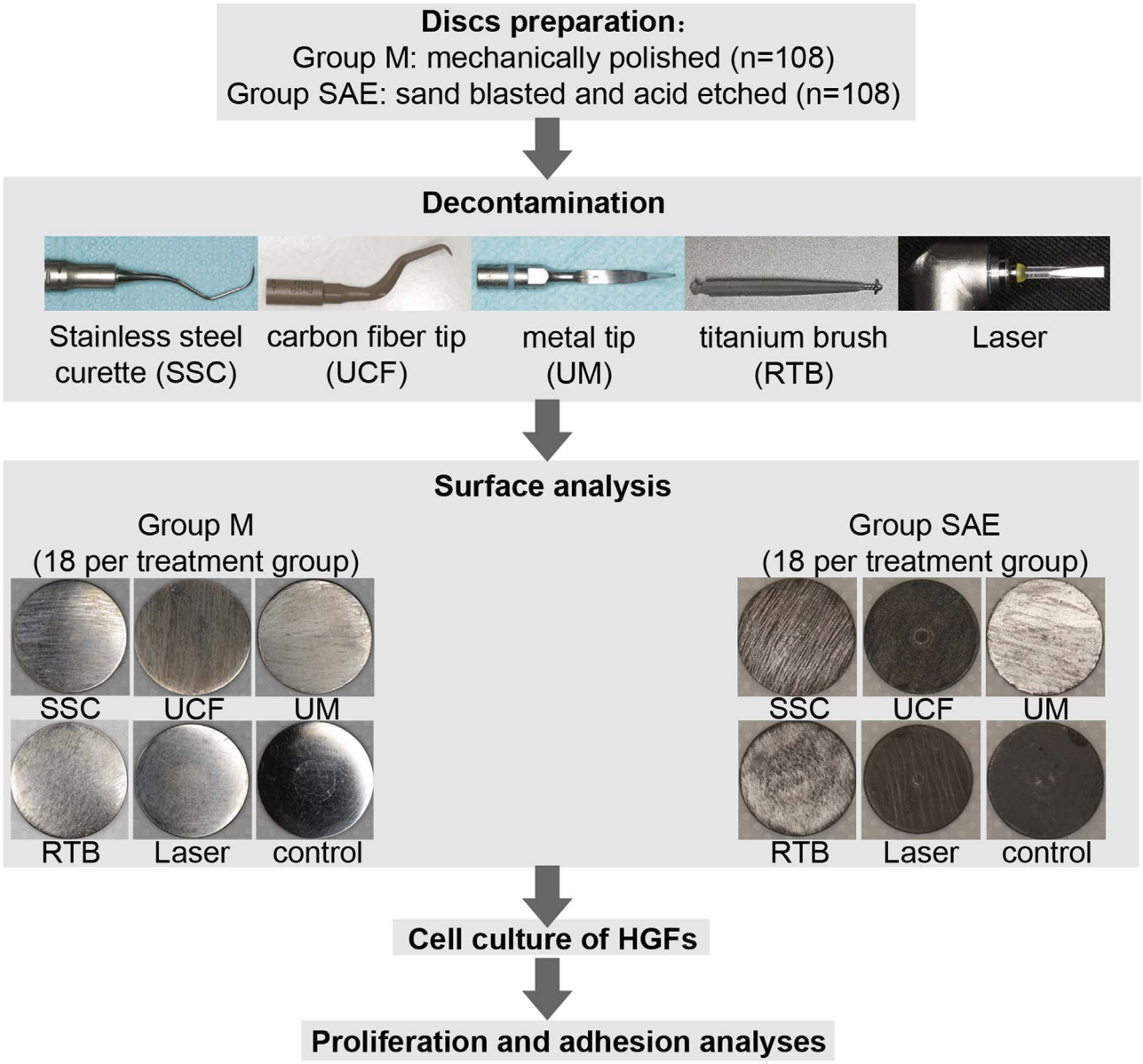

Fig. 1. General flow chart of the research.

2.6. RNA analysis

Total RNA was extracted using Trizol reagent (Invitrogen, US) following the manufacturer's instructions. The amount of RNA extracted was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). 200 ng RNA for each study group at the three culturing time period were retro-transcribed into cDNA using the Reverse Transcription Kit (Takara, Japan). Primers were designed (Table 1) using the Primer3 software (http://frodo.wi.mit.edu/primer3/) to amplify genes for adhesion (*FAK* and *ITGB1*) and genes for ECM synthesis and remodeling (*COL1A1* and *FN1*). Quantitative real-time PCR (qRT-PCR) was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen) in a Mastercycle machine (Thermo Scientific, USA). The comparative Ct method was applied to analyze the dissociation curves which is normalized by the expression levels to the housekeeping gene β-actin.

2.7. Enzyme-linked immunoabsorbent assay (ELISA)

The procedure of ELISA was derived following an established procedure (Wiesen et al., 2015). To determine the expression of FAK, ITGB1, COL1A1, and FN1, cells on each disc were lysed with 1.5 ml

Table 1
Primer pairs of amplified genes.

Gene	Forward primer	Reverse primer
FAK	5′- GTTTCCCCAGAGCTCCTCAA −3′	5′- TACTCGCTCCATTGCACCAG −3′
ITGB1	5′- ACGGACGTAAAGCTGGTCTC −3′	5′- TTGCACGGGCAGTACTCATT −3′
COL1A1	5′- GAGGGCCAAGACGAAGACATC −3′	5′- CAGATCACGTCATCGCACAAC −3′
FN1	5′- GGCCAGTCCTACAACCAGTA −3′	5′- TCTTGGCAGAGAGACATGCTT −3′

RIPA (Gibco, US) on ice. Protein supernatants were collected by centrifugation at 14,000 rpm for 10 min at 4 °C. FAK (Total) Human Monoclonal Antibody (Invitrogen,Thermofisher, US), ITGB1 Monoclonal Antibody (Invitrogen,Thermofisher, US), Fibronectin Monoclonal Antibody (Invitrogen,Thermofisher, US) and COL1A1 Propeptide Polyclonal Antibody (Invitrogen,Thermofisher, US) were used to detect the amount of protein. The absorbance at 450 nm and 550 nm was examined on a plate reader (BioTek, USA) immediately after adding 100 µl of stop solution. Subtraction of 550 nm values from 450 nm values was used to correct optical imperfections in the microplate.

2.8. Statistical analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Mean values and standard deviations were calculated for each study group. General linear model was applied to evaluate the effects of Ti surfaces and decontamination on surface parameters and adhesive strength. Repeated measures ANOVA was used to see time changes of proliferation and expression to decontamination on M and SAE Ti surfaces. Tukey's HSD was applied for post hoc tests. Differences were considered statistically significant at $P < 0.05$.

3. Results

Surface characteristics of different decontamination treatments were analyzed by optical interferometry. The means ± standard deviations of surface parameters were summarized in Table 2. Statistical analyses showed that disc surfaces and decontamination treatments significantly affected Sa, Sq, Sz, Sdq, Sdr, Str20, and Str37 ($P < 0.001$). There were also significant interactions between disc surfaces and decontamination treatments on all the surface parameters ($P < 0.05$) except Str37. Post hoc analyses indicated that on M surfaces, Sa, Sq, Sdq, and Sdr were significantly higher when treated with SSC, UM and RTB compared with control ($P < 0.05$). Sdq, Sdr were also significantly higher when treated with UCF ($P < 0.05$). Sz was significantly higher than control when treated with UM ($P < 0.001$) Laser has the least influence on M surface parameters ($P > 0.18$). However, on SAE surfaces, Sa, Sq, Sdq, and Sdr were significantly lower when having decontamination treatments compared with control ($P < 0.05$). Sz were significantly lower than control when treated with SSC ($P < 0.005$). Besides, Sz was significantly lower than control when treated with UM and RTB ($P < 0.05$).

Repeated measures ANOVA showed that proliferation increased significantly with culturing time ($P < 0.001$). Proliferation on M surfaces was significantly higher than on SAE surfaces ($P < 0.001$). When compared with control, proliferation decreased significantly when the surfaces were decontaminated with SSC, UCF, UM, and RTB on M surface ($P < 0.05$). While proliferation was significantly higher when treated with UCF, RTB and laser on SAE surface ($P < 0.05$). (Fig. 2)

The adhesive strength of HGFs on Ti surface was significantly higher on M surfaces (0.83 ± 0.05) than on SAE surfaces (0.78 ± 0.04) ($P = 0.002$). All decontamination treatments reduced adhesive strength of HGFs on M surfaces while increased adhesive strength of HGFs on SAE surfaces. (Fig. 3) There was statistically significant difference between UM and control on M surface ($P < 0.05$) and between laser and control on SAE surface ($P < 0.05$).

Both RT-qPCR and ELISA results demonstrated that the expression of FAK, ITGB1, COL1A1, and FN1 increased significantly with time ($P < 0.001$). Gene expression was significantly higher on M surfaces than on SAE surfaces ($P < 0.001$). Decontamination treatments altered gene expression significantly than control ($P < 0.001$) (Fig. 4). FAK, ITGB1, COL1A1 and FN1 showed statistically significant decreases in mRNA and protein expression when the surface was treated with SSC, UCF, UM, and RTB on M surface. FAK and COL1A1 also showed significant expression decrease when M surface was treated with laser. FN1 and ITGB1 expression at protein level didn't show significant alterations when M surface was treated with laser. While on SAE surfaces, expression of FAK, FN1, and ITGB1 increased significantly in all decontamination group except SSC. Expression of COL1A1 on SAE surface showed consistent increase when treated with laser, but demonstrated a rather fluctuated trend for other decontamination treatments.

4. Discussion

Decontamination has been applied to aid the treatment of local *peri*-implantitis (Mahato, Wu, & Wang, 2016). As an exterior force, it could alter the microstructure as well as roughness and other properties of implant surface. How such surface alterations caused by decontamination treatment influence HGFs remains unclear. This study investigated the effects of different decontamination treatments on surface microstructure of Ti discs and the proliferation and adhesion of human gingival fibroblasts (HGFs). It was found that decontamination treatments alter the surface parameters of M and SAE Ti discs and further influence the proliferation and adhesive strength of HGFs, as well as the expression of FAK, ITGB1, COL1A1, and FN1 at both mRNA and protein levels.

Surface properties have been proved to influence cellular response. And several studies, although using various cell lines and surface materials, have reached consistent conclusion that smooth surface increase proliferation and adhesion (Baharloo, Textor, & Brunette, 2005; Brunot et al., 2008; Werner et al., 2009). For example, Na et al. (An et al., 2012) compared four different implant Ti surfaces and found that a smoother and hydrophilic surface has positive influence on cell behavior. In our study, roughness parameters (e.g. Sa, Sq, Sz) and hybrid parameters (e.g. Sdq and Sdr) were higher on SAE group than M group. For laser group on M surface, not only did laser treatment has the lowest Sa, Sq, Sz but also has similar hybrid parameters, Sdq and Sdr, with control group, meaning the surface morphology was little altered. So the compatibility of implant abutment could be maintained during treatment. For SAE group roughness, the rule was UN and SSC could

Table 2
Surface parameters of different decontamination treatments on mechanically polished (M) and SLA Ti surfaces.

Disc group	Deconta-mination	Sa, µm	Sq, µm	Sz, µm	Sdq,1/nm	Sdr, %	Str20	Str37
M	SSC	0.223 ± 0.060	0.324 ± 0.080	3.734 ± 0.990	0.104 ± 0.016	0.541 ± 0.168	0.088 ± 0.008	0.070 ± 0.044
	UCF	0.206 ± 0.018	0.262 ± 0.021	2.814 ± 0.651	0.115 ± 0.008	0.658 ± 0.091	0.433 ± 0.125	0.489 ± 0.195
	UM	0.391 ± 0.083	0.539 ± 0.108	6.600 ± 1.247	0.201 ± 0.019	1.975 ± 0.345	0.206 ± 0.053	0.460 ± 0.166
	RTB	0.215 ± 0.030	0.290 ± 0.036	3.768 ± 0.341	0.122 ± 0.014	0.742 ± 0.171	0.243 ± 0.061	0.390 ± 0.130
	Laser	0.126 ± 0.008	0.169 ± 0.008	2.538 ± 0.428	0.070 ± 0.007	0.244 ± 0.049	0.564 ± 0.127	0.502 ± 0.037
	control	0.110 ± 0.029	0.149 ± 0.031	2.748 ± 1.125	0.047 ± 0.009	0.112 ± 0.039	0.386 ± 0.234	0.410 ± 0.208
SLA	SSC	1.621 ± 0.084	2.041 ± 0.100	17.813 ± 1.386	0.754 ± 0.019	24.075 ± 1.153	0.619 ± 0.116	0.650 ± 0.113
	UCF	2.677 ± 0.026	3.352 ± 0.046	22.930 ± 1.552	0.810 ± 0.010	28.550 ± 0.580	0.755 ± 0.091	0.845 ± 0.022
	UM	1.185 ± 0.186	1.579 ± 0.215	16.032 ± 0.995	0.451 ± 0.016	9.105 ± 0.589	0.664 ± 0.080	0.830 ± 0.090
	RTB	2.451 ± 0.126	3.063 ± 0.160	22.680 ± 2.457	0.694 ± 0.027	21.400 ± 1.568	0.788 ± 0.071	0.829 ± 0.051
	Laser	2.382 ± 0.084	3.017 ± 0.146	23.010 ± 2.158	0.758 ± 0.026	25.300 ± 1.643	0.774 ± 0.069	0.855 ± 0.030
	control	2.979 ± 0.104	3.759 ± 0.131	26.700 ± 1.447	1.140 ± 0.022	54.100 ± 1.874	0.747 ± 0.049	0.851 ± 0.027

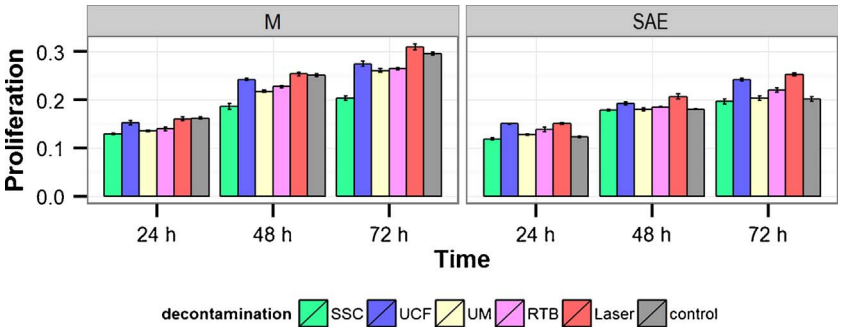

Fig. 2. Results of HGFs proliferation assay.

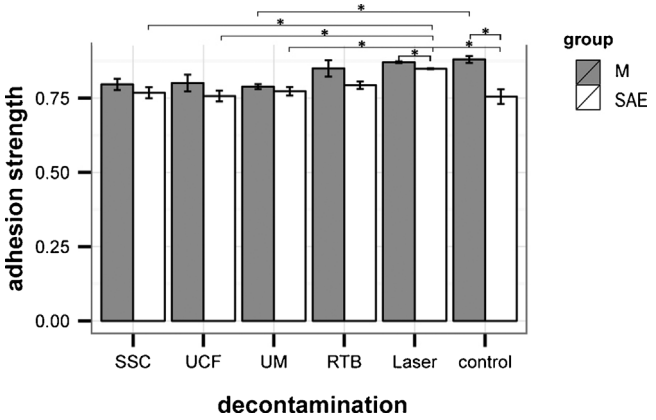

Fig. 3. Results of HGFs adhesion strength measurements.

decrease it significantly, while laser, RTB and UCF showed weaker ability in smoothing surface. Furthermore, for hybrid parameters, UM posted the strongest effect on changing surface morphology but SSC had similar surface alteration ability with laser, RTB and UCF.

Both proliferation and adhesive strength on M surfaces was higher than on SAE surfaces. These results lead to consistent conclusions that proliferation and adhesion increase as surface roughness decrease. We also observed decrease of proliferation and adhesive strength when decontamination was performed on M surface which make it rough, and increase of proliferation and adhesive strength when decontamination was performed on SAE surface which make it less rough. Among all the decontamination treatments, laser has the optimal performance that least change M surface and smooth SAE surface. Ayobian-Markazi et al. (Ayobian-Markazi, Karimi, & Safar-Hajhosseini, 2015) found that laser could reduce roughness on SAE surface and do not impair the biocompatibility of the SAE titanium surfaces. Besides, using laser on SAE surfaces seem to enhance the expression of wound healing and tissue

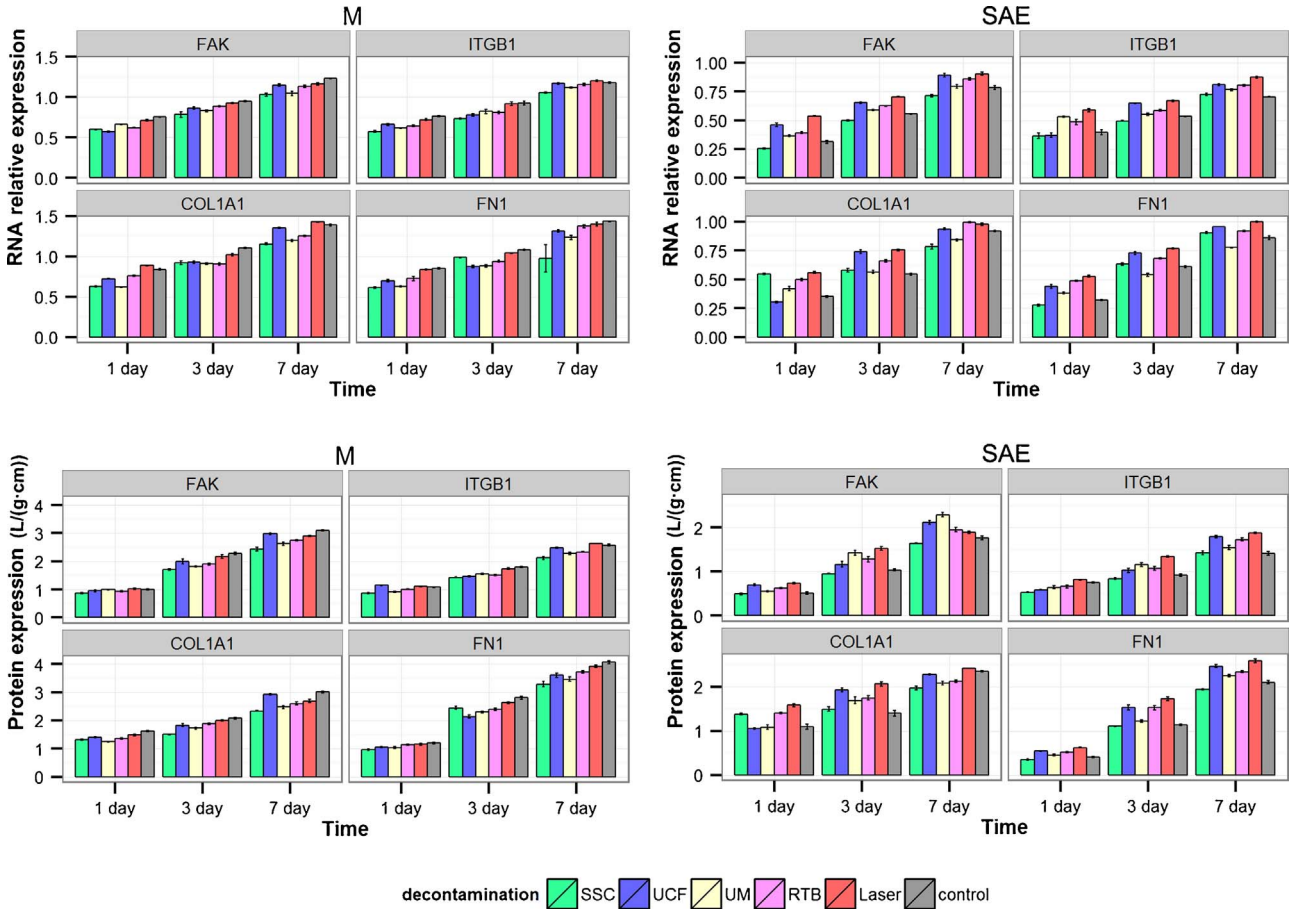

Fig. 4. RNA and proteins expression of HGFs (FAK, ITGB1, COL1A1, FN1).

sealing related gene expression. Sema et al. (Hakki & Bozkurt, 2012) studied the effect of laser on the proliferation and mRNA expression of HGFs. It was concluded that laser treatment could facilitate wound healing by regulating mRNA expression of HGFs. (Ayobian-Markazi et al., 2015) In other literatures (Kreisler, Christoffers, Willershausen, & d'Hoedt, 2003; Pourzarandian, Watanabe, Ruwanpura, Aoki, & Ishikawa, 2005; Saygun et al., 2008), laser therapy could also improve proliferation of HGFs. The results of the present study showed that laser decontamination make SAE surface less rough and increase the expression of COL1A1, FAK, FN1 and ITGB1. As a decontamination treatment, laser has been reported effective at removing biofilms on implant (Friedmann, Antic, Bernimoulin, & Purucker, 2006; Takasaki et al., 2007; Yamamoto & Tanabe, 2013). Based on these evidences, it is suggested that laser is a good choice that can both disinfect the implant surface and enhance cell behavior of HGFs. Moreover, it is worth mentioning that laser-related parameters still need to be studied to achieve the best clinical outcome (Alshehri, 2016).

There are some limitations of the present study. First, as an in vitro study, we couldn't simulate the complex oral microenvironment and validate our conclusions in vivo. Second, we didn't study the effect of various decontamination methods for disinfection but only focused on their effect on Ti disc surface and HGFs. The clinical use of decontamination treatments demands more investigations on the condition and related using parameters to improve *peri*-implant wound healing and soft-tissue sealing. Thirdly, we studied HGFs from only one patient, which could limit the extrapolation of this study due to the variation in cellular response. Last but not least, in our study, all the techniques adopted were from clinical treatments procedures, which were relatively technique-sensitive. Even though with the development of instruments, which are more user friendly and less complex of necessary techniques, individual variations (e.g. Force, angles, distance judgment et al.) still exist and may exert influence on final results. So when trying to transit our results to finally clinical effects, it should be quite careful and more in-depth experiments are still necessary.

5. Conclusions

Within the limitation of this in vitro study, it might be concluded that:

(1) Decontamination treatments on both M and SAE Ti discs alter surface microstructure and roughness.
(2) Decontamination treatments influence proliferation and adhesion of HGFs on M surface and SAE surface.
(3) Laser might be an optimal decontamination treatment which has the least negative effect on M surface and the most positive effect on SAE surface.

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