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