

Regulatory Effects of PRF and Titanium Surfaces on Cellular Adhesion, Spread, and Cytokine Expressions of Gingival Keratinocytes

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Abstract

Dental implant material has an impact on adhesion and spreading of oral mucosal cells on its surface. Platelet-rich fibrin (PRF), a second-generation platelet concentrate, can enhance cell proliferation and adhesion. The aim was to examine the regulatory effects of PRF and titanium surfaces on cellular adhesion, spread, and cytokine expressions of gingival keratinocytes. Human gingival keratinocytes were cultured on titanium grade 4 (Ti4), titanium grade 5 (Ti5), and HA disks at 37 °C in a CO₂ incubator for 6 h and 24 h, using either elutes of titanium-PRF (T-PRF) or leukocyte and platelet rich fibrin (L-PRF), or mammalian cell culture medium (SFM-X) as growth media. Cell numbers were determined using a Cell Titer 96 assay. Interleukin (IL)-1 β , IL-1Ra, IL-8, monocyte chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) expression levels were measured using the Luminex® xMAP™ technique, and cell adhesion and spread by scanning electron microscopy. Epithelial cell adhesion and spread was most prominent to Ti5 surfaces. L-PRF stimulated cell adhesion to HA surface. Both T-PRF and L-PRF activated expressions of IL-1 β , IL-8, IL-1Ra, MCP-1, and VEGF, T-PRF being the strongest activator. Titanium surface type has a regulatory role in epithelial cell adhesion and spread, while PRF type determines the cytokine response.

Keywords: Interleukins, Keratinocytes, Microscopy electron scanning, Plasma

1. Introduction

Integrity of the soft-tissue seal around the abutment of dental implants is crucial for the clinical success (Atsuta et al. 2012; Ivanovski & Lee 2018). Titanium, either in pure (Ti1-

4) or in an alloy form, is the most common material used to fabricate implants in dentistry (Cordeiro et al. 2017). Surface characteristics like chemical composition, charge, topography, and roughness have a significant effect on the adhesion and protein expression of human oral cells to titanium (Lauer et al. 2001; Schwartz-Filho et al. 2012). Formation of a thin oxide layer on the outer surface of the titanium and titanium alloys also affects the adhesion of cells to implant surfaces (Lausmaa et al. 1990). The interaction of the titanium oxide layer, rutile surface, with the organic molecules provides further intercourse between the cell and implant (YazdanYar et al 2018). Moreover, the contamination of the titanium surfaces with saliva and bacteria is a potential risk factor to maintain the integrity of the titanium oxide layer (Delgado-Ruiz et al. 2018). Therefore, numerous surface modification methods have been described to alter and create an antibacterial effect on titanium surfaces (Chouirfa et al 2019). Recent evidence suggests that, in addition to its regulatory effects on cell adhesion, titanium may induce a strong immune response in macrophages (Li et al. 2018). There is evidence that titanium particles can be found in epithelial cells, connective tissue, macrophages, and bone tissue, and presence of these particles are associated with the initiation of the inflammatory response and expression of cytokines (Suárez-López del Amo et al. 2018). However, information on oral mucosal keratinocyte response is missing in this context.

Platelets have a stimulatory effect on wound healing and tissue hemostasis (Mosesson et al. 2001). Platelet-rich fibrin (PRF) is a natural biomaterial, and it is prepared by centrifuging the blood sample directly after drawing it. The main principle to obtain PRF membrane is the centrifugation of the freshly drawn blood in a glass/glass coated tubes at 2700-3000 rpm for 12-15 minutes, approximately 400G forces (Dohan et al. 2006). PRF is an autogenous source of growth factors, in which platelets are the main cells responsible for its biologic activity. The flexible and dense fibrin structure of the PRF membrane provides slow and long term secretion of the growth factors such as platelet derived growth factor,

transforming growth factor β , vascular endothelial growth factor, insulin like growth factor-1 which are responsible for the enhanced healing (Pierce et al. 1991). Moreover, the trapped cytokines in the gel form of the PRF membrane induce the regulation of the epithelial cell migration to wound surfaces and accelerates angiogenesis (Ratajczak et al. 2018). PRF may stimulate the proliferation and differentiation of cells of oral origin as well (Ehrenfest et al. 2009). The clinical benefits of both platelet products have been discussed recently (Schliephake et al. 2018) and their effect on enhancing the early phase of osseointegration has been pointed out (Strauss et al 2018).

All protocols to produce PRF use an immediate centrifugation; however, variations in techniques, such as using titanium tubes [for titanium platelet rich fibrin (T-PRF)] or glass tubes [for leukocyte and platelet-rich fibrin (L-PRF)], can significantly affect biological properties and clinical implications of the outcome product (Tunali et al. 2014). The histological structures of the L-PRF and T-PRF show similarities to each other, yet the fibrin network of the T-PRF was much denser than L-PRF (Tunali et al 2014). Even though there are slight differences in the centrifugation protocol of both fibrin products (2800 rpm, 12 min for T-PRF (Tunali et al 2014); 3000 rpm, 10 min for L-PRF (Dohan et al 2009)), titanium-tubes that are used in production of T-PRF are found to be the underlying reason of potent platelet activation (Tunali et al 2014). Dense fibrin structure of T-PRF membranes gives additional beneficial effects on the treatment of soft tissue treatment around the teeth and dental implants due to its elongated resorption time (Ustaoglu et al 2016).

In the present study, we hypothesized that adhesion, spread, and cytokine expression of gingival keratinocytes are affected by the grade of titanium, and this effect can be modified by PRF. Therefore, we aimed to examine the cellular adhesion of gingival keratinocytes to Ti4 and Ti5 titanium surfaces and the expression of interleukin (IL)-1 β , IL-1Ra, IL-8,

monocyte chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) in the presence or absence of T-PRF and L-PRF.

2. Material and methods

2.1 Preparation of L-PRF and T-PRF

A systemically healthy non-smoking study volunteer donated the whole blood samples. The L-PRF and T-PRF membranes were produced as described by Tunali et al. (2014), with slight modifications. A 36 ml of blood sample was collected with four non-additive test tubes (9 ml, Vacuette 455092; Grenier Bio-One GmbH, Frickenhausen, Germany). A half of the blood sample (18 ml) was quickly transferred into two separate titanium grade 4 tubes. Two non-additive and two titanium grade 4 tubes, were centrifuged (#SL8R, Thermo Fisher Scientific, Waltham, MA, USA) at 2700 rpm for 15 min at room temperature. After centrifugation, L-PRF (from non-additive tubes) and T-PRF (from two titanium grade 4 tubes) clots were taken out from the tubes by using sterile tweezers and each one was placed on discrete sterile gauze pads. In order to obtain the L-PRF and T-PRF membranes, the bases of all fibrin clots including the red corpus part were removed with sterile scissors, and serum components of the fibrin clot were removed by squeezing the clot gently between sterile gauze pads.

2.2 PRF-elute extraction

Elute extractions were performed according to Gassling et al. (2013). Briefly, L-PRF and T-PRF membranes were broken into rectangular fragments with one side length 7 mm and three pieces obtained from each membrane were placed in a 24-well cell culture plate (Costar 3524, Corning Incorporated, Corning, NY, USA). Membrane fragments were incubated with

a serum-free DMEM media for 1 h at 37 °C and 5% CO₂. The elutes, which were obtained from the pieces of the PRF membranes, were pooled and stored separately at 4 °C.

2.3 Isolation of human oral keratinocyte cells

Human oral keratinocyte (HMK) cells, which are a spontaneously transformed cell line, were isolated from a healthy human gingival biopsy sample and were received from Dr. M. Mäkelä, University of Helsinki, Finland (Mäkelä et al. 1999). The chromosome number of the cells was found to be around hypertriploid range (70-76, with a cell to cell variation). HMK cells were sustained in a 75 cm² cell culture flask (Corning Incorporated, Corning, NY, USA) with a keratinocyte-serum free media (SFM-X, Gibco, Thermo Fisher Sci. Co, Waltham, Massachusetts, USA), containing human recombinant epidermal growth factor, bovine pituitary extract and antibiotics (penicillin-streptomycin, 10.000 U/mL, Gibco, Thermo Fisher Sci. Co, Waltham, Massachusetts, USA) at 37 °C and 5% CO₂. Keratinocyte growth media were replaced with fresh SFM-X three times per week, and the HMK cells were passaged when reaching 80-90% confluence. HMK cells used for the experiments were chosen from passage number 22.

2.4 Test Discs

In all experiments, machine surfaced titanium grade 4 (Ti4, Servo-Dental, Hagen, Germany), machine surfaced titanium grade 5 (Ti5, Implance, Trabzon, Turkey), and as a control surface hydroxyapatite (HA, Clarkson Chromatography Inc., South Williamsport, PA, USA) discs were used. All discs had a diameter of 5 mm and a thickness of 2 mm.

2.5 Cell culture

Equal numbers of HMK cells (1×10^4) were seeded on Ti4, Ti5, and HA discs, using either elutes of T-PRF or L-PRF, or SFM-X as growth media. Cells were incubated at 37 °C in a

CO₂ incubator for 6 h and 24 h. All experiments were performed in quintuplicate. At the end of the incubation periods, elute and media were transferred into Eppendorf tubes and stored in -70 °C for cytokine analysis. The cells on titanium and HA surfaces were used for the cell number and SEM analyses.

2.6 Cell number analysis on test discs

Cell numbers on each test disc were determined using the Cell Titer 96 assay (Promega Corporation, Wisconsin, USA) with a plate reader at 490 nm. To convert the absorbance values to the number of living cells, HMK cells at five different densities were incubated with the cell titer assay at 37 °C in a CO₂ incubator for 1 h. After incubation, the absorbance was measured at 490 nm, a standard curve was created by plotting the absorbance against the number of incubated cells. Elute and media alone were used in determination of the background absorbance.

2.7 SEM imaging

After the incubation periods, the cells covering the surface were immediately fixed by a conventional chemical fixation with 5% glutaraldehyde in 0.16 mol 1-1 s-collidine-HCl buffer at pH 7.4 for 20 min, followed by a chemical dehydration in graded ethanol series (50%, 70%, and 98%) with 5 min embedding intervals. The discs were removed from the bottom of the Eppendorf tubes and kept at room temperature to allow air drying for overnight. Then, the discs were mounted on SEM specimen metal stubs (covered with electrically conductive material) and each one of them sputter carbon-coated for one second. The surface microtopography of the specimen was visualized by the LEO 1530Gemini scanning electron microscope (Carl Zeiss, Oberkochen, Germany). The magnification of the images taken corresponds to a Polaroid 545 print and an image size of 8.9x11.4 cm.

2.8 Cytokine concentrations

After incubating the cells for 6 and 24 h, IL-1 β , IL-1Ra, IL-8, MCP-1, and VEGF concentrations in T-PRF and L-PRF elutes, and SFM-X media were measured with the Luminex® xMAP™ technique (Luminex Corporation, Austin, TX) using the pro-human cytokine group I assays (Bio-Rad, Santa Rosa, CA). The detection limit of the assay was 0.6 pg/mL for IL-1 β , 5.5 pg/mL for IL-1Ra, 1.0 pg/mL for IL-8, 1.1 pg/mL for MCP-1, and 3.1 pg/mL for VEGF. Elute and media alone were used in determination of the background absorbance.

2.9 Statistical analysis

The IBM SPSS V24.0 software (IBM, Armonk, North Castle, New York, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) with Tukey's HSD was used for the analysis of the differences in cell proliferation and cytokine expression levels. The p value of $p < 0.05$ was accepted as statistically significant.

3. Results

Elevated numbers of HMK cells on Ti4, Ti5, and HA discs were found after 6 and 24 h incubations in the T-PRF and L-PRF groups, in comparison to the SFM-X group. In addition, the numbers of HMK cells on HA discs after 24 h incubation were higher than those on Ti4 and Ti5 discs in the L-PRF and T-PRF groups (Fig. 1).

The SEM analysis revealed that after 6 h incubation, more cell adhesion and spread had occurred in the Ti4 and Ti5 groups, in comparison to the HA group. Keratinocytes in the HA group were either round or started to be elongated with few filopodia. In the Ti4 and Ti5 groups, both filopodia and lamellipodia were visible in majority of the cells. Of the three surfaces examined, cell spread was most prominent on Ti5 surface. In the HA group, cell spread improved when the cells were incubated with the L-PRF extract while cells tend to form aggregates when they were incubated with T-PRF. A small number of platelet cells adhered to epithelial cells was observed in the PRF and T-PRF groups (Figs. 2, 3).

After 24 h incubation, the PRF extract treated cells on Ti4 and Ti5 discs formed uneven multilayers, however the number of cell layers were not even in all parts of the disc (Figs. 4, 5). Cell-to-cell junctions and cell spread were most prominent in Ti5 group. Cells in the HA group lost their initial roundness and become more elongated.

After 6 h incubation, cells on the T-PRF discs produced the highest concentrations of IL-1 β , IL-1RA, and IL-8, while those on the SMF-X discs were the lowest. No difference was observed in the MCP-1 concentrations between the tested groups (Fig. 6). After 24 h incubation, the highest concentrations of IL-1 β , IL-1RA, and IL-8 were produced by cells in the T-PRF group, and the lowest concentrations were in the SFM-X group. VEGF and MCP concentrations did not significantly differ between the T-PRF and L-PRF groups, yet their concentrations were higher than in the SFM-X group (Fig. 7).

There was no detectable concentration of IL-1 β , IL-8, VEGF, or MCP-1 in elutes of L-PRF or T-PRF, or in SFM-X growth media, whereas low concentrations of IL-1RA were measured in each growth media.

4. Discussion

To our knowledge, this is the first study to examine adhesion characteristics and cytokine production of gingival epithelial cells on two different titanium grade discs and to analyze modifying effects of PRF on these parameters. According to our results, Ti5 has superiority in epithelial cell adhesion and spread against Ti4 and HA, besides the cytokine expression by epithelial cells is strongly induced in the presence of T-PRF.

Although machined surfaces of Ti4 and Ti5 show similar characteristics in their morphology, corrosion resistance, and biocompatibility, they differ in their chemical composition (Özcan et al. 2012). The amorphous oxide layer formation and existence of small amounts of alloying elements on Ti5 (Shah et al. 2016) may explain its superiority in epithelial cell spread and adhesion over Ti4 and HA surfaces.

One interesting finding in the present study was that the application of PRF increased the cell spread only on HA disks. Growth factors in the PRF play a significant role in stimulating the proliferation of human oral cells (Dohan Ehrenfest et al. 2009). It has been shown that bone morphogenetic proteins have affinity to bind HA (Rohanizadeh et al. 2011). As PRF carries high amounts of bone morphogenetic proteins, it is possible that these proteins accumulated on HA surface stimulate the proliferation and spread of gingival keratinocytes.

Based on our results, titanium surfaces have no regulatory effect on cytokine expression of gingival keratinocytes. It was recently demonstrated that titanium ions (9 ppm) activate cytokine expression of gingival epithelial cells (Wachi et al. 2015). In the present study, we used 6 and 24 h steady incubation models, which may not necessarily induce an abrasion on titanium surfaces, and eventually titanium release and elevated cytokine expression. Both T-PRF and L-PRF stimulated cytokine expression significantly. Previous studies have shown that platelet-released growth factors stimulate the induction of IL-6 and antimicrobial

peptides in primary human keratinocytes (Bayer et al. 2016; 2018). Conversely, in a clinical study where the use of PRF membrane was compared to connective tissue graft in the treatment of localized gingival recessions, the application of PRF membrane had no effect on the gingival crevicular fluid IL-1 β levels after 10 days (Eren et al. 2016). L-PRF contains leukocytes, monocytes, and platelets, thus being a significant source of IL-1 β , IL-6, and VEGF as well, and the concentration, release dynamics, and kinetics of these growth factors have been demonstrated in vitro (Schär et al. 2015). Yet, in the present study, cytokine levels in PRF elute were nearly undetectable. Instead of PRF, we used PRF elute as a test material. This model has been applied successfully on osteoblast cultures for bone tissue engineering (Gassling et al. 2013). The purpose of the PRF elute preparation was to get a well-homogenized PRF structure to be used in a disc-culture model. It was previously shown that leukocytes and platelets are not homogeneously distributed in L-PRF and the heterogeneous distribution of growth factors and some other proteins in the fibrin network might be an explanation to the unequal release of these molecules after PRF's application (Dohan Ehrenfest et al. 2010). One limitation of the use of elute is that elute does not contain the fibrin component of PRF; thus, the cellular composition differs between PRF and PRF elute. The second limitation is that the origin of the blood and the epithelial cells used in the present study come from two different individuals. Even though the blood donor was a systemically healthy and non-smoking volunteer, it is unclear if age or gender have any effect on PRF composition. Finally, present study used monolayer culture model to evaluate the cell spread and cytokine secretion. Non-differentiating cell monolayers do not simulate gingiva, because they lack of stratification, vertical cell contacts, and differentiation (Gursoy et al., 2016). Thus, it might be beneficial to test the present study hypothesis on an organotypic- titanium cell culture model.

In conclusion, the composition of titanium has a regulatory effect on the adhesion and spread of human gingival epithelial cells. PRF proved to be a strong inducer of cytokine expression of gingival epithelial cells, but also contributed to the cell spread characteristics. Increased understanding of these mechanisms involved in the cellular process may help in an attempt to improve the wound healing in peri-implant tissues. Besides, it might be possible to improve pocket reduction and clinical attachment gain in peri-implantitis treatment with the aid of PRF treatments.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure.1

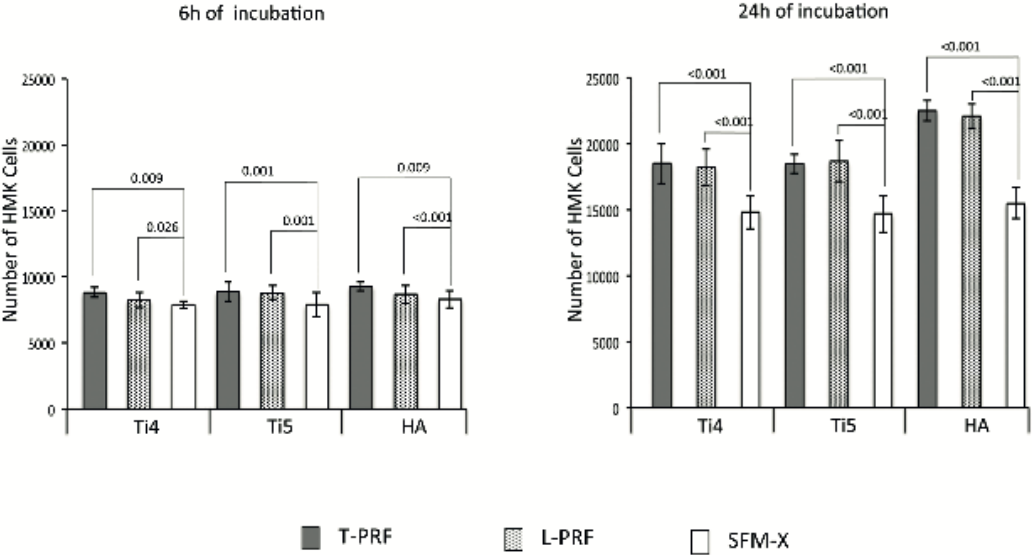


Figure.2

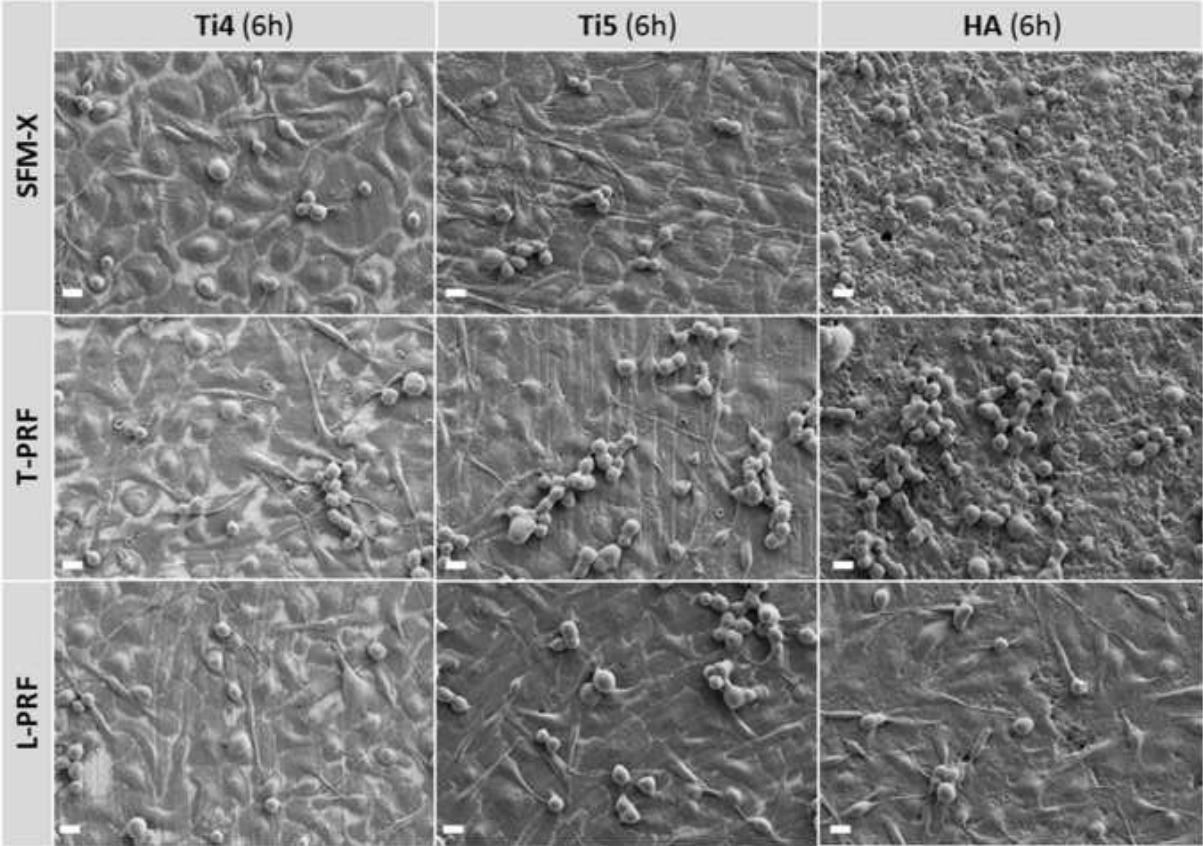


Figure.3

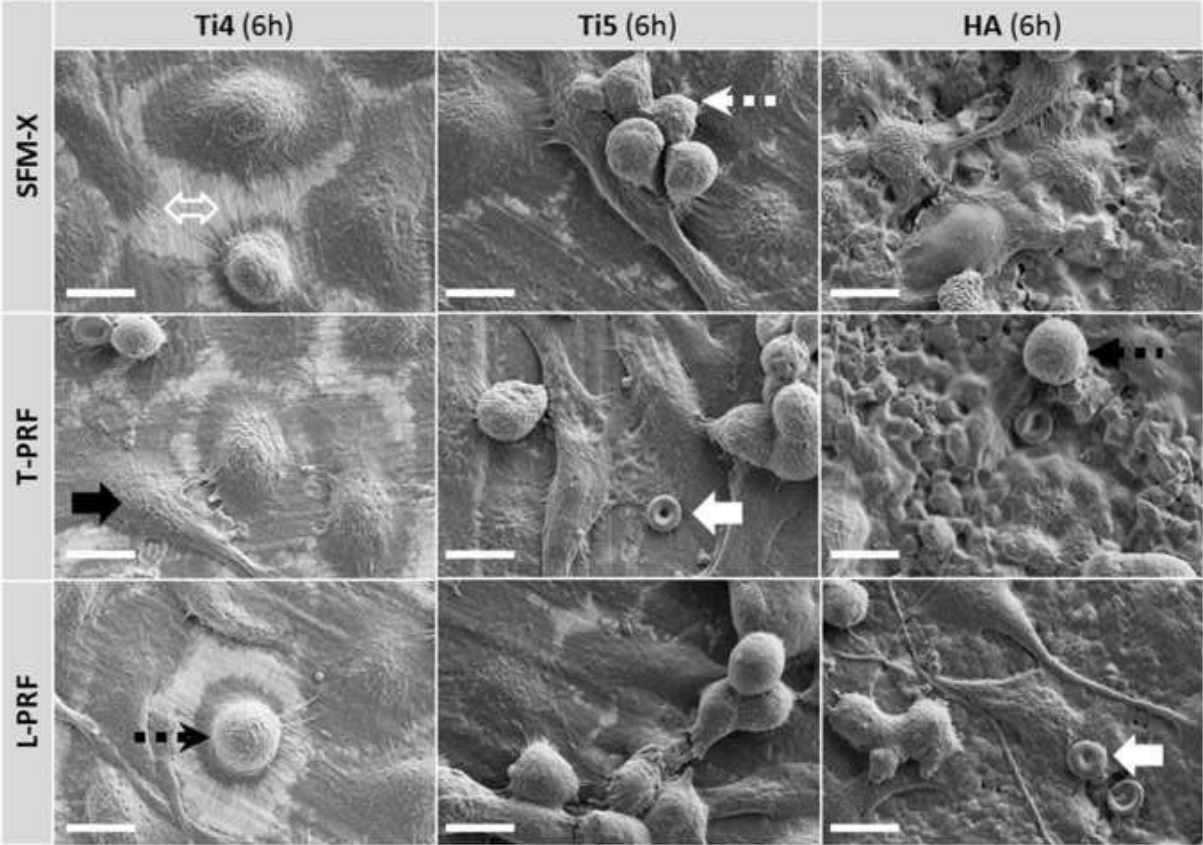


Figure.4

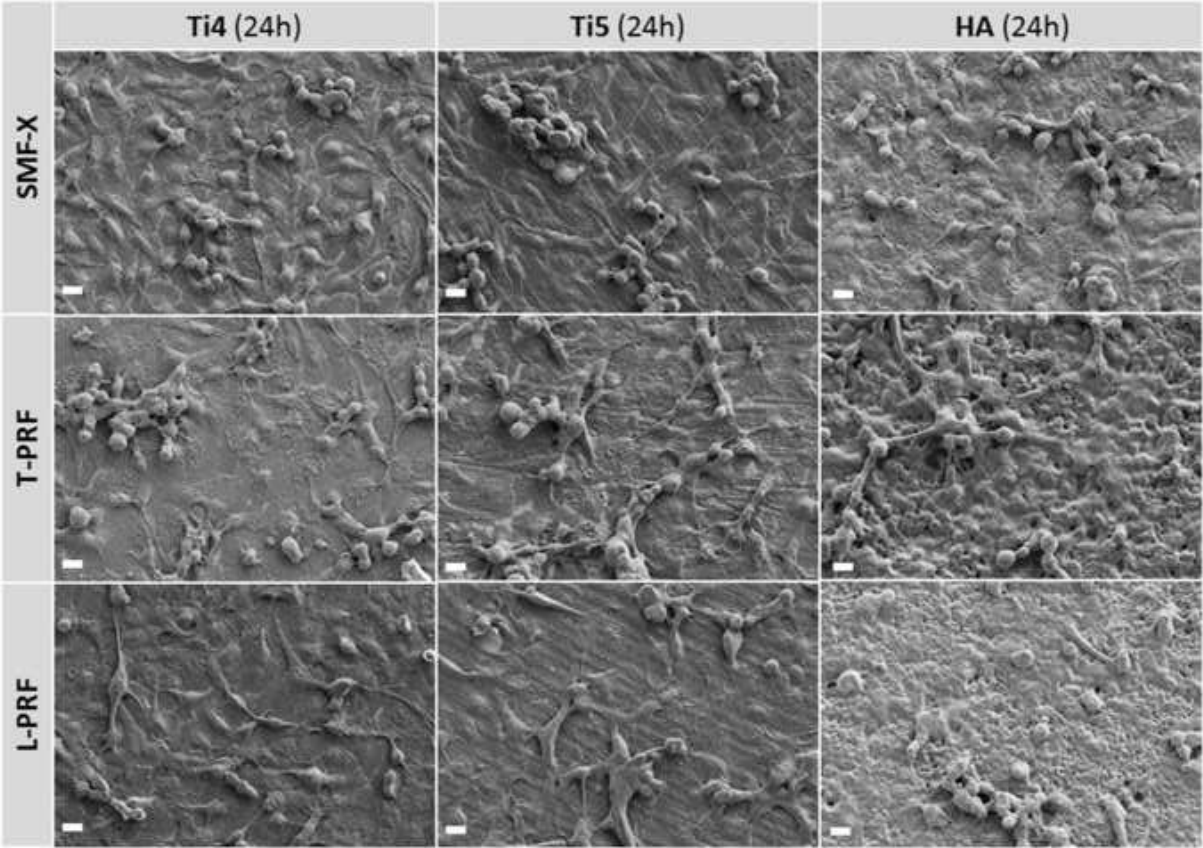


Figure.5

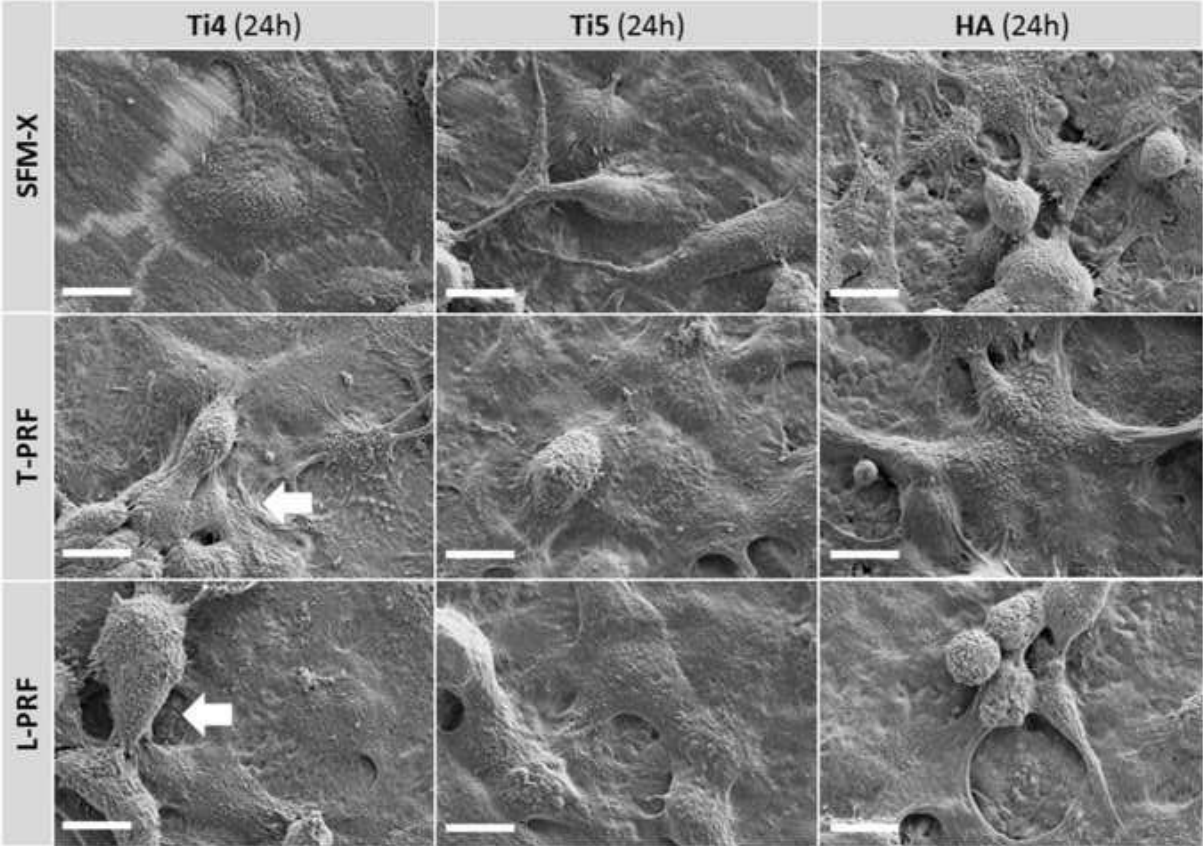


Figure.6

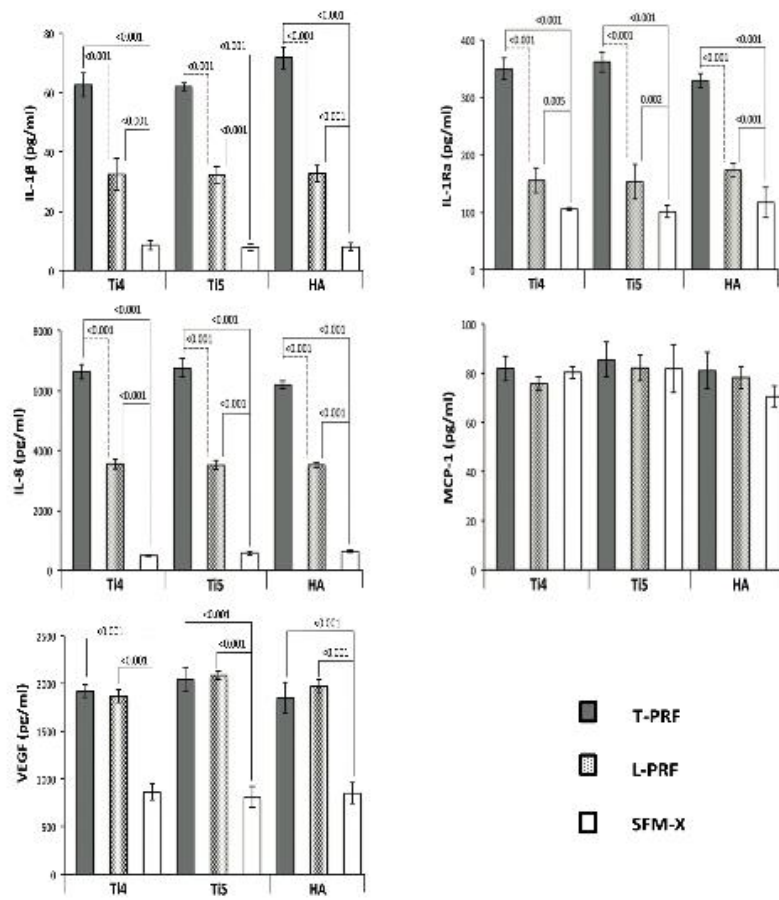


Figure.7

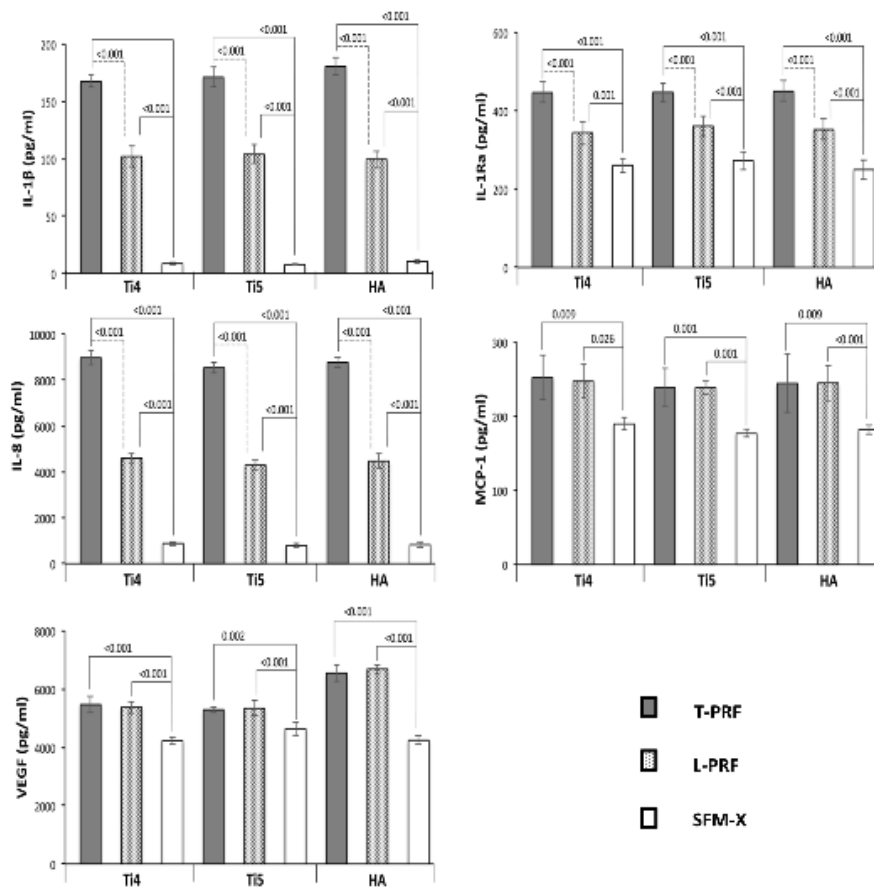


Figure legends

Figure.1 Comparison of human oral keratinocyte (HMK) cell numbers cultured on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs after 6 and 24 h of incubation.

Figure.2 SEM analysis of human oral keratinocyte (HMK) cell adhesion and spread on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs after 6 h of incubation. Scale bars indicate 10 μm .

Figure.3 SEM analysis of human oral keratinocyte (HMK) cell adhesion and spread on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs after 6 h of incubation . Two ended arrow: Gap between HMK cells. Black arrow: A spreading HMK cell with lamellopodia. Black dashed arrow: A round HMK cell with filopodia. White arrows: Platelets. White dashed arrow: HMK cell clumps. Scale bars indicate 1 μm .

Figure.4 SEM analysis of human oral keratinocyte (HMK) cell adhesion and spread on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs after 24 h of incubation. Scale bars indicate 10 μm .

Figure.5 SEM analysis of human oral keratinocyte (HMK) cell adhesion and spread on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs after 24 h of incubation. White arrows: Unevenly distributed multilayered human oral keratinocyte (HMK) cells. Scale bars are 1 μm .

Figure.6 Concentrations of interleukin (IL)-1 β , IL-1ra, IL-8, monocyte chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) in T-PRF and L-PRF elutes and SFM-X media after incubating gingival keratinocytes on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs for 6 h.

Figure.7 Concentrations of interleukin (IL)-1 β , IL-1ra, IL-8, monocyte chemoattractant protein (MCP)-1, and vascular endothelial growth factor (VEGF) in T-PRF and L-PRF elutes and SFM-X media after incubating gingival keratinocytes on grade 4 and 5 titanium (Ti4 and Ti5) and hydroxyapatite (HA) discs for 24 h.