HOST RESPONSES IN PERI
IMPLANT TISSUE IN COMPARISON TO PERIODONTAL TISSUE

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การตอบสนองของโฮสต์ในเนื้อเยื่อรอบรากเทียมอาจแตกต่างจากเนื้อเยื่อปริทันต์ในคนที่มีสุขภาพดี วัตถุประสงค์ของการศึกษาครั้งนี้คือเพื่อเปรียบเทียบความแตกต่างของการแสดงออกของ cytokines ใน สารคัดหลั่งในร่องเหงือก จาก implant ที่บูรณะด้วย titanium และ UCLA abutment เมื่อเทียบกับเนื้อเยื่อปริทันต์ที่มีสุขภาพดี นอกจากนี้ยังได้ศึกษาผลของ cytokines อักเสบเหล่านี้ต่อการแสดงออกของ FAK และ RANKL gene

มีผู้เข้าร่วมการวิจัยจำนวน 19 คนตามเกณฑ์การคัดเลือกและการยกเว้น สารคัดหลั่งในร่องเหงือกจากรอบรากเทียมและฟันธรรมชาตินำมาเก็บโดยใช้ paper point ระดับการแสดงออกของ cytokines ได้แก่ IL-1α, IL-1β, IL-6, IL-8, TNF-α, IFN-y ได้รับการประเมินโดยใช้วิธี enzyme-linked immunosorbent assay (ELISA) มีการเปรียบเทียบการแสดงออกของ FAK และ RANKL geneโดยการใช้ PICF ที่นำมาจากรอบรากเทียมที่บูรณะด้วย 2 วัสดุมาเพาะเลี้ยงกับเซลล์ fibroblast จากเนื้อเยื่อเหงือกมนุษย์โดยวิธี qPCR

การแสดงออกของ IL-6, TNF-αและ IFN-y ไม่แตกต่างจาก GCF ในกลุ่มไททาเนียมและ UCLA abutment อย่างไรก็ตามระดับ IL-1αใน PICF จากรากฟันเทียมที่ได้รับการบูรณะโดย UCLA abutment สูงกว่า GCF อย่างมีนัยสำคัญ (p- value = 0.030) นอกจากนี้ระดับของ IL-1βใน PICF จากรากฟันเทียมที่ได้รับการบูรณะด้วยไทเทเนี่ยมสูงกว่า GCF อย่างมีนัยสำคัญ (p-value = 0.032)การแสดงออกของ IL-8 ใน PICF จากรากฟันเทียมที่ได้รับการบูรณะด้วย UCLA สูงกว่าไทเทเนี่ยมอย่างมีนัยสำคัญ (p-value 0 .003)นอกจากนี้ยังพบว่าการแสดง ออกของ RANKL และ FAK gene ในลักษณะที่ขึ้นกับความเข้มข้นในทั้งสองกลุ่ม abutment

สาขาวิชา	ทันตกรรมบูรณะเพื่อความสวยงามและทันตกรรมรากเทียม	ลายมือชื่อนิสิต .....
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KEYWORDS: ABUTMENT, CYTOKINES, DENTAL IMPANT, GINGIVAL CREVICULAR FLUID, PERI-IMPLANT CREVICULAR FLUID

RAVIPORN MADARASMI: HOST RESPONSES IN PERI IMPLANT TISSUE IN COMPARISON TO PERIODONTAL TISSUE. ADVISOR: ASST. PROF. DR. ANJALEE VACHARAKSA, Ph.D., CO-ADVISOR: ASSOC. PROF. PRAVEJ SERICHETAPHONGSA, 70 pp.

The host response in peri-implant tissue may differ from that in periodontal tissue in a healthy individual. The purpose of this study is to investigate the expression of inflammatory cytokines in peri-implant crevicular fluid (PICF)from single implant with two different abutment types in comparison to healthy periodontal tissue. Moreover, the effect of these inflammatory cytokines on the expression of FAK and RANKL were also studied.

Nineteen participants with healthy implants and teeth were recruited according to the inclusion and exclusion criteria. PICF and gingival crevicular fluid (GCF) was collected using sterile paper points. The expression level of inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8, TNF-α, IFN-y was assessed using enzyme-linked immunosorbent assay (ELISA). varying concentrations of PICF obtained around two abutment materials were treated with human gingival fibroblast cell cultures to study the expression of FAK and RANKL using qPCR.

Expression of IL-6, TNF-α and IFN-y was not statistically different from GCF among titanium and UCLA abutment group. However, the level of IL-1α in the PICF from the implants restored with UCLA abutment was significantly higher than GCF (p- value= 0.030). Inaddition, the level of IL-1β in PICF from implants restored with titanium abutment was significantly higher than GCF (p-value=0.032). IL-8 expression in PICF from implant restored with UCLA abutment was significantly higher than titanium abutment (p-value 0.003). Moreover an up- regulation of RANKL and down regulation of FAK gene was found to be in a concentration dependent manner for both the abutment groups.

Field of Study:	Esthetic Restorative and Implant Dentistry	Student's Signature	.....
		Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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	Titanium 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01	-	26.32	-	26.01	7.78	10.22	-2.44	5.426417
FAK	34.10	36.23	34.10	-	36.23	-				

	UCLA 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	25.77	26.01	-	25.77	-	26.01	9.43	10.22	-0.79	1.729074
FAK	35.20	36.23	35.20	-	36.23	-				

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	Titanium 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
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CHAPTER I

INTRODUCTION

Background and significance

Implant-supported prostheses have become the treatment of choice in oral rehabilitation to restore function and esthetic in partial or complete edentulous arch. The use of dental implant has been increased steadily due to its high predictability and survival rates (1). Although dental implants share some similarities to the natural teeth, dental implant and natural tooth are different in some aspects, and that makes the peri-implant tissue respond differently to the microbial challenge. Upon exposure to the oral environment, the implant-supported prostheses are colonized by the early colonizers, such as *Streptococcus oralis*, *Streptococcus gordonii*, and *Actinomyces naeslundii*, through interactions with the salivary pellicle (2). These early colonizers facilitate the adherence of secondary colonizers by co-adhesion, and biofilm formation proceeds through growth and division of surface associated microorganisms. Like other biofilms, multiple species live in close physical contact and this increases the probability of

microbial interactions, both synergistically and antagonistically, between microbial cells within implant-associated biofilms. Mucosal epithelium represents the first barrier and first line of defense against external stimuli (3). The adherence of the oral microbiota, as well as the characteristics of implant materials has an influence on the host response and recruitment of inflammatory mediators in preventing the microbial penetration (4). In this study, we investigated the host response in the peri-implant tissue in comparison to the host response in the periodontal tissue. Moreover, the differences in the host response between two abutment materials were also recorded. This finding may provide baseline understanding of host responses in peri-implant tissue and also the responses to differential implant abutments.

Research Questions

Whether the host response in peri-implant tissue differs from the healthy periodontal tissue?

Hypothesis

Dental implant materials induce host responses in peri-implant tissue differently from healthy periodontal tissue.

Research Objectives

1. To investigate the host response in peri-implant tissue and to compare to the expression of inflammatory mediators in healthy periodontal tissue by using crevicular fluid as an assessment tool.

The peri-implant crevicular fluid (PICF) and gingival crevicular fluid (GCF) obtained from healthy implant-supported prostheses were assessed for the expression of cytokines, including IL-1α, IL-1β, IL-6, IL-8, IFN-*y* and TNF-α, using single-analyte ELISArray. The expression was compared between tooth and implant of the same individual, and also between different implant abutment materials.

2. To investigate the gene expression in fibroblasts responding to peri-implant crevicular fluid by using quantitative RT-PCR. The human gingival fibroblasts (HGF) were cultured in monolayer before incubated with PICF obtained from implants with different abutment materials. The expression of focal adhesion kinase (FAK) and Receptor activator of nuclear factor kappa-B ligand (RANKL)-specific mRNA in fibroblasts were assessed using quantitative RT-PCR.

Keywords

Abutment, Cytokines, Dental implant, Gingival Crevicular fluid and Peri-implant crevicular fluid

Research design

Cross sectional analytical study

Population: Patients who received anterior implant-supported fixed partial prostheses at Faculty of Dentistry, Chulalongkorn University Bangkok, Thailand from year 1998 to 2011.

Intervention: None

Control: None

Outcome measurement: The expression of IL-1α, IL-1β, IL-6, IL-8, IFN-*y* and TNF-α.

CHAPTER II

Literature Review

The immune system is believed to play a role in the biological mechanisms that determine the fate of any implant placed within the living tissues (5). This is because when the implant is placed in the living tissue protein absorption takes place. This protein absorption is the first key to tissue integration and hence influencing a group reaction, modulating the host response in its entirety (5). After the protein absorption, the complement system is activated and macrophage guides the inflammatory response to the biomaterial because macrophages are only recruited if a biomaterial is present. Macrophages are found frequently on the surface of titanium oral implants and justify the concept of osseointegration being a foreign body reaction (5). However, the concept of foreign body equilibrium was introduced by Albrektsson and colleagues which stated that osseointegration is the result of a foreign body reaction that with the right intensity in the inflammatory response, will balance itself out by hormones and cytokines and allow for bone to grow on the implant surface. On the other hand, the disturbance of the

foreign body equilibrium will result in peri-implant bone loss (6).

There are many etiologic factors that can compromise implant- tissue integration and cause tissue inflammation or bone loss such as surgical trauma, microgap, occlusal overload, and peri-implantitis. Peri-Implantitis is one of the leading problems during maintenance phase in implant dentistry. The definitions of peri-implant diseases, revised in the 6th European Workshop on Periodontology in 2008, were that “peri-implant mucositis is the presence of inflammation in the mucosa at an implant with no signs of loss of supporting bone; and peri-implantitis, in addition to inflammation in the mucosa, is characterized by the loss of supporting bone” (7).

Histological analysis in dogs has revealed that peri-implant tissue presents an inflammatory cell infiltrate at the level of implant abutment junction, even at sites that had been exposed to plaque control (8). It is suggested that, peri-implant infiltrate is produced by host cells to limit bacterial invasion. An early and reliable detection of any adverse peri-implant tissue reaction is peri-implant crevicular fluid (PICF) (9). PICF is an

osmotically mediated inflammatory exudate originating from the vessels of the gingival plexus. Its composition is similar to that of the gingival crevicular fluid (GCF), containing host-derived enzymes and their inhibitors, inflammatory mediators and host response modifiers and tissue breakdown products. Thus, analysis of cytokine levels in the PICF may help in detecting inflammatory lesions at an early stage, which might be clinically latent. Simultaneously, PICF analysis may also help in monitoring the osseointegration process and the bone response to occlusal loading and infection, thereby improving the long-term success of implants(10).

According to many studies, many inflammatory and immune mediators, including inflammatory cytokines, can be detected in PICF. These inflammatory mediators have an essential role in pathogenesis of diseases such as peri-implantitis. The soluble mediators present in PICF may be products of the physiological interaction between host immune cells to the oral microorganisms for generating tissue homeostasis. In periodontology and implantology they are involved in inflammation-related alteration and repair of periodontal or peri-implant tissues. Certain cytokines

have been proposed as diagnostic markers of periodontal or peri-implant tissue destruction. The inflammatory mediators secreted by various cells of the oral mucosa can mediate many cellular functions as summarized in Table 1 (11-15).

Table 1 List of inflammatory mediators, their sources and action

Cytokine/Chemokine	Cellular source	Function	Inflammation	Reference
IL-1α/β	Phagocytes, Epithelial and Fibroblasts	Induces inflammatory cell migration and induces bone resorption	Increased in chronic inflammation	Bloemenet al, 2010
TNF-α	Phagocytes, Epithelial, osteoblasts and Fibroblasts	Upregulates adhesion molecules. Regulates production of IL-1 and IL-6, Increases of RANKL expression	Increased in chronic inflammation	Dinarello, 2000
IL-8	Lymphocytes, Mast cells Epithelial cells Fibroblasts	Inflammatory chemokine Induces osteoclast differentiation	Increased	Yoshimura et al., 1995

	Endothelial cells, Osteoclasts.	and activity		
IL-6	Phagocytes T and B cells Epithelial Fibroblasts Osteoblasts	Promotes bone resorption Pro-inflammatory properties	Increased in chronic inflammation	Pestka et al., 2004
IL-10	Th2 cells Treg cells	Protective role and Stimulates OPG production	Increased	Pestka et al., 2004
IFN-y	CD4 and CD8	Activation, growth and differentiation of T and B cells, macrophages, Upregulates MHC expression	Increased	Pestka et al., 2004

Nowzari et al; have reported many biological molecules and cytokines in PICF similar to GCF of natural teeth. Possible biological markers include: pro-inflammatory cytokines, enzymes, and catabolic metabolites. However, they noted a general increase level in pro-inflammatory cytokine concentration in PICF. This observation suggested the possibility of the peri-implant tissue reaction to small metal ions releasing from dental implant or abutment materials. These molecules represent a biological marker to better monitor implant health and disease progression. Pro-inflammatory molecules found and studied in the dental literature include: interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin- 8 (IL-8), and prostaglandin E-2 (PGE_2). Some of the PICF enzymes present in the dental literature include: matrix metalloproteinases (MMPs), neutral protease (NP), neutrophil elastase (NE), myeloperoxidase, β-glucuronidase, cathepsin, and elastase (16).

A study by A.B. Petkovic et al; showed that concentrations of cytokines and chemokines (IL-1β, TNF-α, MIP-1α and IL-8) were calculated in relation to the PICF

volume. Patients with tissue inflammation had significantly higher concentrations of IL-1β, TNF-α, MIP-1α and IL-8 in PICF around the dental implant (17).

The study conducted by Salcetti et al; provided new data regarding levels of inflammatory and growth factor mediators and bacterial pathogens associated with failing implants compared with healthy implants. This study revealed significant elevations of IL-1β in PICF according to the progression of inflammation (18).

Ataoglu et al. study determined IL-1β and TNF-α levels in PICF. The peri-implant inflamed gingival tissue had higher levels of IL-1β than peri-implant gingival tissue with non-inflamed or slightly inflamed gingiva. The findings of that study indicated that IL-1β levels in PICF might be used to evaluate implant health status (19).

Regarding PICF components, several studies have been carried out on inflammatory markers and growth factors such as IL-1β, platelet-derived-growth factor, PGE_2 and aspartate aminotransferase. Lamster & Grbic noticed that the volume of gingival fluid of natural teeth and implants and the signs of inflammation seemed to be the same, but the amount of gingival fluid from 'healthy sites' is less than that from

gingiva with inflammation(20). Several previous studies have shown IL-1 and TNF-α level in higher concentrations in the GCF of patients with periodontitis in relation to the control group. Those cytokines were Th1 monocyte related and were synergistic in their capacity to enhance bone resorption (11, 14, 16).

The local balance of cytokines reflects local cell activity. According to the profile of the cytokines produced and the type of cell infiltrate, three contradictory theories of the pathogenesis of peri-implant disease arising from mucositis have been established.

First, Seymour et al proposed that susceptibility to disease progression depends on a shift in the lymphocyte population in the inflammatory infiltrate from predominantly T-cells in mucositis to an increased proportion of B-cells in peri-implantitis, which is the result of local hyperproduction of the Th2 type of cytokines (IL-4, IL-5, IL-10, and IL-13). They cause local proliferation and differentiation of B-lymphocytes, local secretion of non-protective antibody (specific for antigen determinant of bacteria) and hyperproduction of IL-1 from activate B-lymphocytes, which leads to progression of the lesion (21).

Second, Ebersole & Taubman; suggest that local production of Th2 cytokines stimulates local production of specific antibody and local production of anti-inflammatory cytokines (IL-4, IL-10, and IL-1ra) from epithelial cells, which stop or delay progression of the periodontal lesion. The formation, maintenance and progression of the lesion are caused by activity specific CD8+ T lymphocytes, which produce proinflammatory cytokines (IFN, IL-1) leading to the activity of local macrophages and osteoclasts, which results in local destruction(22).

Third, Dennison & Van dyke; suggest that macrophages play a central role in the immune response to bacteria, producing proinflammatory cytokines and other mediators that induce activity in osteoclasts and osteoblasts. Alteration in the monocyte response may lead to abnormal disease patterns. Adequate production of IL-4 (Th2 cytokines) inhibits macrophage activity, antigen presenting, production of essential proinflammatory cytokines necessary for starting the immune response and increases macrophage apoptosis, which delay the formation of a periodontal lesion (23).

CHAPTER III

METHODOLOGY

1. Participant selection

Patients who received implant-supported fixed partial prostheses from Esthetic restorative and implant dentistry clinics, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand from January 1998 to December 2016 were examined. Participants were randomly included in this study based on the following criteria (Table 2). All participants were thoroughly explained about the steps of PICF and GCF collection, benefits and possible risks of the study approved by ethics committee of Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2016-046). The consent forms were signed before attending the study and participants were free to withdraw from the study at any time.

Participants were categorized according to abutment materials placed into UCLA group and Titanium abutment group. Demographic data including name, date of

birth, age, sex, medical status, medication, oral health care, and detailed information on dental implant prostheses were assessed through an interview.

Table 2: Criteria for participant selection.

Inclusion Criteria	Exclusion Criteria

• Healthy patients over 21 years of age
• Having at least one implant supported fix partial dentures in the anterior sextant
• Implant being loaded for at least 12 months
• UCLA or Titanium abutment
• Healthy peri- implant soft tissue
  ○ No bleeding on probing
  ○ Probing depth ≤ 3mm
  ○ No pus exudate
	• Patient presented with systemic disease
	• Having immunosuppressant medications or antibiotics within 3 months
	• Pregnancy and lactating
	• Smoking or having a history of smoking

○ No visible tissue inflammation ○ No visible bone resorption ● Not underwent previous periodontal treatment for at least 3 months ● Obtained consent from patient	

Periodontal parameters such as bleeding on probing (BOP), gingival index (GI), plaque index (PI) and probing depth (PD) were evaluated. (Periowise, Premier Dental, Playmouth Meeting, PA,). The Silness and Loe plaque index was used for measuring the amount of plaque present on four surfaces of a tooth; mesial, distal, buccal, and lingual. Numeric values were assigned to each surface based on the plaque detected. Based on this scoring system: 0 = no plaque is present, 1 = plaque is present at the free gingival margin, 2 = a moderate amount of plaque present within the gingival sulcus, and 3 = the presence of abundant plaque within the gingival sulcus. The four numbers are then added together and divided by four. The resultant number is the relative plaque

score for that tooth. mPI is a modified version of PI for implants (24, 25).

The Loe and Silness gingival inflammation index was recorded in a similar manner as the plaque index. Numeric values were assigned to each tooth surface analyzed using the following scale: 0 = normal gingiva; 1 = mild gingival inflammation, noted by slight change in color; 2 = moderate inflammation, redness, glazing, and bleeding on probing; 3 = severe inflammation, marked redness, edema, ulceration, spontaneous bleeding. The tooth surface values are added and divided by the total surfaces evaluated (21).

Intraoral radiographs were taken on implant using periapical technique to ensure no bone loss occur till the first thread of implant.

2. Peri-implant fluid and gingival crevicular fluid collection

For PICF and GCF collection, the tooth that has the same probing depth as the peri-implant was chosen for the collection of GCF. The site for peri-implant and gingival

crevicular fluid collection was isolated using a cotton roll and air dried to ensure there was a good moisture control. Supragingival calculus or plaques were removed 2 weeks prior to sample collection. PICF and GCF were collected using four sterile absorbent paper points size M (Kerr, CA, USA), the absorbent paper point were marked at 15mm and then placed in the sulcus for 30 seconds. After 30 seconds, they were removed and cut with scissors at the marked length (15mm). PICF absorbed from each strip were stored in 1.5 mL plastic tube containing 100 μL of phosphate buffer saline (PBS), pH 7.2, supplemented with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). It was then centrifuged at 8000 rpm for three minutes and the paper points were then discarded, and the supernatant were stored at -80°C until used.

3. Single-analyte ELISArray kits

Single analyte ELISArray kit was designed to quantitatively measure amount of individual protein analyte using a standard sandwich enzyme-linked immunosorbent assay (ELISA) technique. A target specific capture antibody was coated on the plate.

The single-analyte ELISArray kit contains assay buffer, detection antibody, avidin-HRP and a 96 well plate. ELISA was performed according to the manufacturer's instruction (Qiagen Valencia, USA). Briefly, All required reagents and samples for ELISA were brought to room temperature before proceeding of ELISA. Assay buffer and sample dilution buffer were prepared according the manufacturer's instruction Assay (50 μl) was added into each well, 50 μl of serially diluted standard and 50 μl of sample were added to the respective wells. The plate was incubated at -4^{0}C for 24 hours. After incubation, the plate was washed for 3 times with 1x wash buffer. Detection antibody (100 μl) was added and incubated at room temperature for 1 hr. Plate was washed three times. Avidin-HRP (100 μl) was added into each well and incubated for 30 minutes at room temperature. Plate was washed four times. Development solution (100 μl) was added and the kinetics of plate was read at 450nm.

4. Human Gingival Fibroblast (HGF) Cell culture

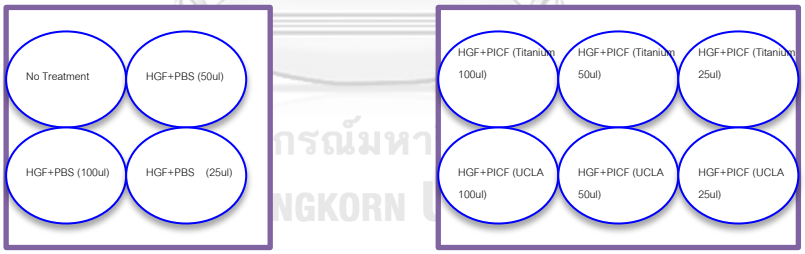
Gingival tissue for primary gingival fibroblasts (PGF) culture were obtained from healthy human subjects (age ranging from 14 to 40 years), who are patients undergoing crown lengthening surgery at the Esthetic Restorative and Implant Dentistry clinic, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.

The tissue specimens were washed with PBS containing antibiotics. Gingival tissue specimens were cut into pieces, approximately 1x1 mm in size, and placed in the culture plate containing DMEM pH 7.2 supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1% amphotericin B (Gibco BRL, New York, USA) to prevent growth of micro-organisms, as culture medium and incubated at 37^0C in a humidified atmosphere of 95% air and 5%CO_2 (Corning, New York, USA) (26). The medium was changed every two days. When the primary cell culture reaches a confluence of 70-80%, the HGF were detached with 0.025% trypsin-EDTA (Life Technology, USA), and 0.05% trypsin-EDTA respectively,

diluted with culture medium and then subcultured in a ratio of 1:3. HGF from passage three till seven were used in the following experiments.

For the experiment, $3x10^4$ cells were seeded in 6-well plates with a diameter of 34mm and cultured for 24 hours prior to the treatment. HGF seeded in 10-well plates were incubated 5%, 2.5% and 1.25% volume of peri- implant crevicular fluid (PICF) respectively as shown in Figure 1.

Figure 1: Shows HGF culture with treatments



After the treatment, all well plates were incubated at 37^0C in a humidified atmosphere of 5% CO_2 for 24 hours. Cells were then harvested for total RNA using TRIzol reagent (Qiangen Valencia,USA), and stored in -80^0C.

5. RNA isolation and quantitative RT-PCR

Total RNA were extracted with TRIzol® reagent (Molecular research Center, Cincinnati, Ohio, USA) according to manufacturer's instructions. Briefly, 1 ml. of TRIzol®reagent were added to each culture well. Then, TRIzol®reagent were transferred into 1.5 ml tube, and 200 µl of chloroform were added and shaken vigorously. The mixtures were then centrifuged at 14,000 rpm for 15 minutes, before the aqueous phase were collected. Isopropanol (500 µl) were added to precipitate RNA. After centrifugation, RNA pellet were dissolved in nuclease-free water and the amount of RNA were determined by the absorption at 260/280 nm using a micro-Volume UV-Vis Spectrophotometer for Nucleic Acid and Protein Quantitation (NanoDrop2000, Thermo Scientific).

One µg of each RNA sample were converted to cDNA by Improm-II™ reverse transcriptase system (Promega, Madison, WI, USA) as recommended by the manufacturer. Subsequently, quantitative PCR (qPCR) was performed by using QuantiTect SYBR® Green PCR Kits (Qiagen Valencia, USA). Primers sequences used in

this study are shown in Table 3. PCR amplification of the cDNA template were performed using Light Cycler® 480 SYBR Green I Master kit (Roche Diagnostic) on MiniOpticon™ Real–Time PCR Detection System (Bio-Rad, California,USA). PCR conditions were 95°C for 1min followed by 40 cycles of amplification consisting of 95°C for 10s, 60°C for 10s, and 72°C for 20min. Reactions were run in triplicate, and results were averaged. Each value was normalized to GAPDH as the housekeeping gene to control for variations in the amount of cDNA input.

Table 3: Primer sequences using the RT-PCR

Target	Sequence	Accession no.*
FAK	5GACCTGTTATCCTAAGCCCGAGA3 5ATGCCTGACCTTGGTAAATGCTG3	BC081646
GAPDH	5-TGTGTCCGTCGTGGATCTGA-3 5-TTGCTGTTGAAGTCGCAGGAG-3	TC198136

RANKL	5-CACTATTAATGCCACCGAC-3 5-GGGTATGAGAACTTGGGATT-3	AF019047

*NIH Genbank Accession number

6. Statistical analyses

The data were analyzed by using SPSS program (SPSS version 16.0, SPSS Inc., Chicago, IL). A normal distribution of all data was tested. Paired sample t-test was used for the comparison of cytokines concentration between peri-implant and natural teeth. Independent t test was used to compare the concentration of cytokines between UCLA and titanium abutment. One-way ANOVA was used for FAK and RANKL gene expression between fibroblast cells treated with PICF obtained around titanium and UCLA abutment.

CHAPTER IV

RESULTS

All participants had healthy dental implant restored with single unit prosthesis. Ten had ten implants restored with UCLA abutment while nine participants had a titanium abutment installed. A pristine tooth without any restoration located in the same sextant of the implant was selected for the periodontal sample collection. The demographic data and clinical parameters of implants and teeth are shown in Table 4 and 5, respectively. The mean age (± standard deviation) of all participants was 47.5±11.12. The implants and neighboring teeth appeared in healthy state with no bleeding on probing. Progressive bone resorption is not observed in any participant. The average month of implant in function was 49.7±19.1 months. Among these, the average month of implant restored with titanium abutment in function (n = 9) was 60.2±9.8, which is statistically significant (P value= 0.023) higher than the average month of implant restored with UCLA abutment in function (n = 10) was 40.2±22.1 months.

No significant differences were found in gingival index, plaque index and probing depths between the natural teeth and dental implant. Moreover, independent t test revealed no significant differences in modified gingival index, modified plaque index and probing depths between two abutment materials.

The expression of inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8, IFN-*y* and TNF-α, are investigated and compared between natural tooth and dental implant in the same individuals. Means (pg/mL) and standard error of the inflammatory cytokines detected by ELISA from periodontal or peri-implant crevicular fluid or titanium (Figure 2) and UCLA abutments are shown (Figure 3). In healthy state, most of the cytokines such as IL-6, IL-8, IFN-*y* and TNF-α are expressed in peri-implant crevicular fluid similar to the natural tooth of the same individual. Nonetheless, paired sample t-test demonstrated a significant difference in the level of IL-1β (p value = 0.032) between implants restored with titanium abutment and natural tooth (Figure 2c), and the level of IL-1α was also significantly different (p value = 0.030) between implants restored with UCLA abutment and natural tooth (Figure 3d).

To compare the host responses between the implants restored with titanium or UCLA abutments, the expression of the inflammatory cytokines from PICF from these two abutments was demonstrated (Figure 4). Independent t test shows a significant difference in the level of IL-8 expression (P-value= 0.003).

Table 4: The Demographic data of participants and characteristics of implant-supported prostheses including in this study

Participants (n)	19
Age (years)	
• Titanium	44.7±(10.68), 46[#]
• UCLA	41.2±(15.3), 45[#]
Gender	
Male (n)	
• Titanium	3
• UCLA	6
Female (n)	
• Titanium	6
• UCLA	4
Implant-supported crown (n)	19
Implant system	
• Astra Tech-Titanium (n)	8
• Straumann- Titanium (n)	1
• Straumann-UCLA (n)	10
Loading period (months)	
• Titanium abutment	60.2(±9.8), 64[#]
• UCLA abutment	40.2±(22.1), 34[#]
Distance between implant shoulder to bone contact (mm)	
• Titanium abutment	2.62±(0.82), 2.60[#]
• UCLA abutment	2.92±(0.76), 2.87[#]

[#] Mean(±SD), Median

Table 5: The clinical parameters

	Teeth	Titanium	Teeth	UCLA
Gingival Index	0.69±0.27	0.64±0.25	0.67±0.33	0.65±0.29
Plaque Index	0.67±0.31	0.42±0.25	0.65±0.31	0.5±0.31
Probing depth	1.94±0.27	2.61±0.44	1.57±0.42	2.42±0.33

Mean±SD

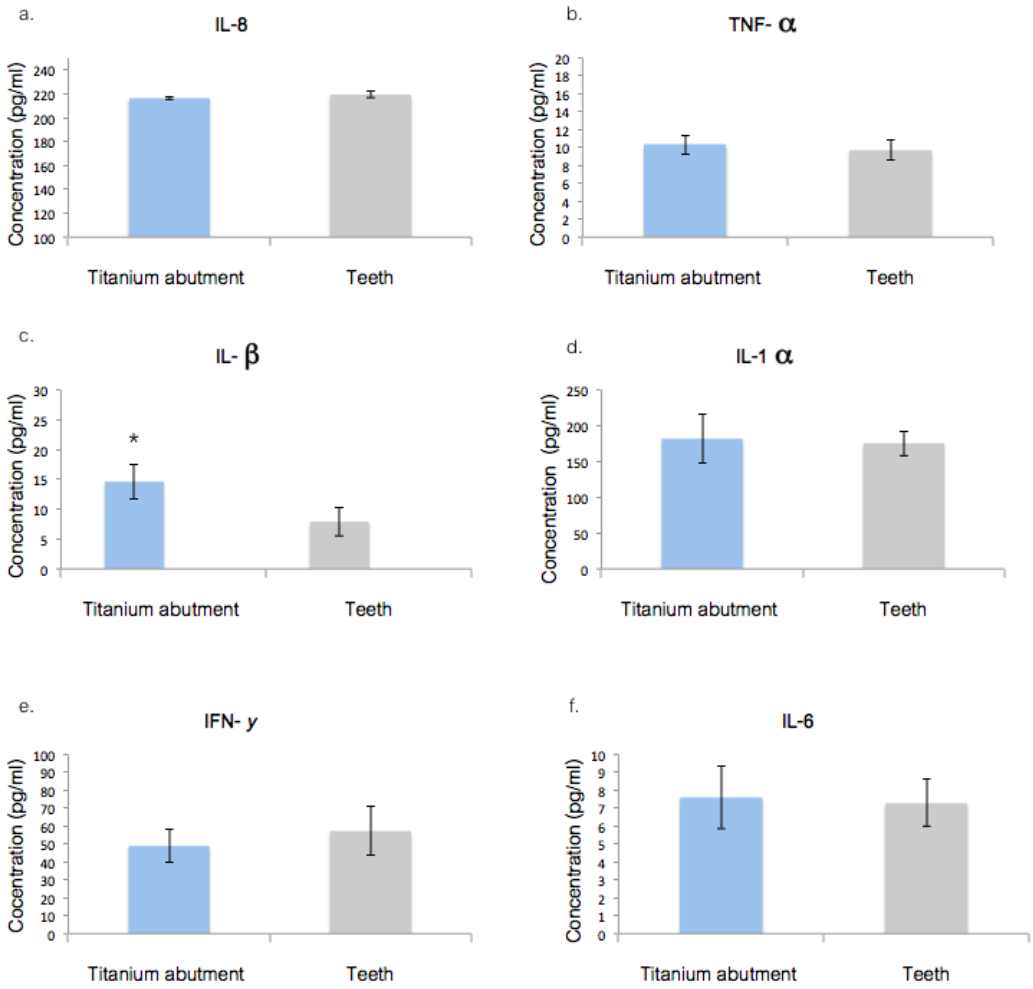


Figure 2: Cytokines expression (pg/ml) in periodontal and peri-implant crevicular fluid from implant-supported titanium abutment. Nine participants had implants restored with titanium abutment. Expression of IL-8 (a), TNF-α (b), IL-1β (c), IL-1α (d), IFN-y (e), and IL-6 (f) is demonstrated. The levels of IL-1β expression in peri-implant crevicular fluid is significantly higher than natural tooth (p value = 0.032) as indicated with *. These

data are collected from three independent experiments and means (SE) are

demonstrated.

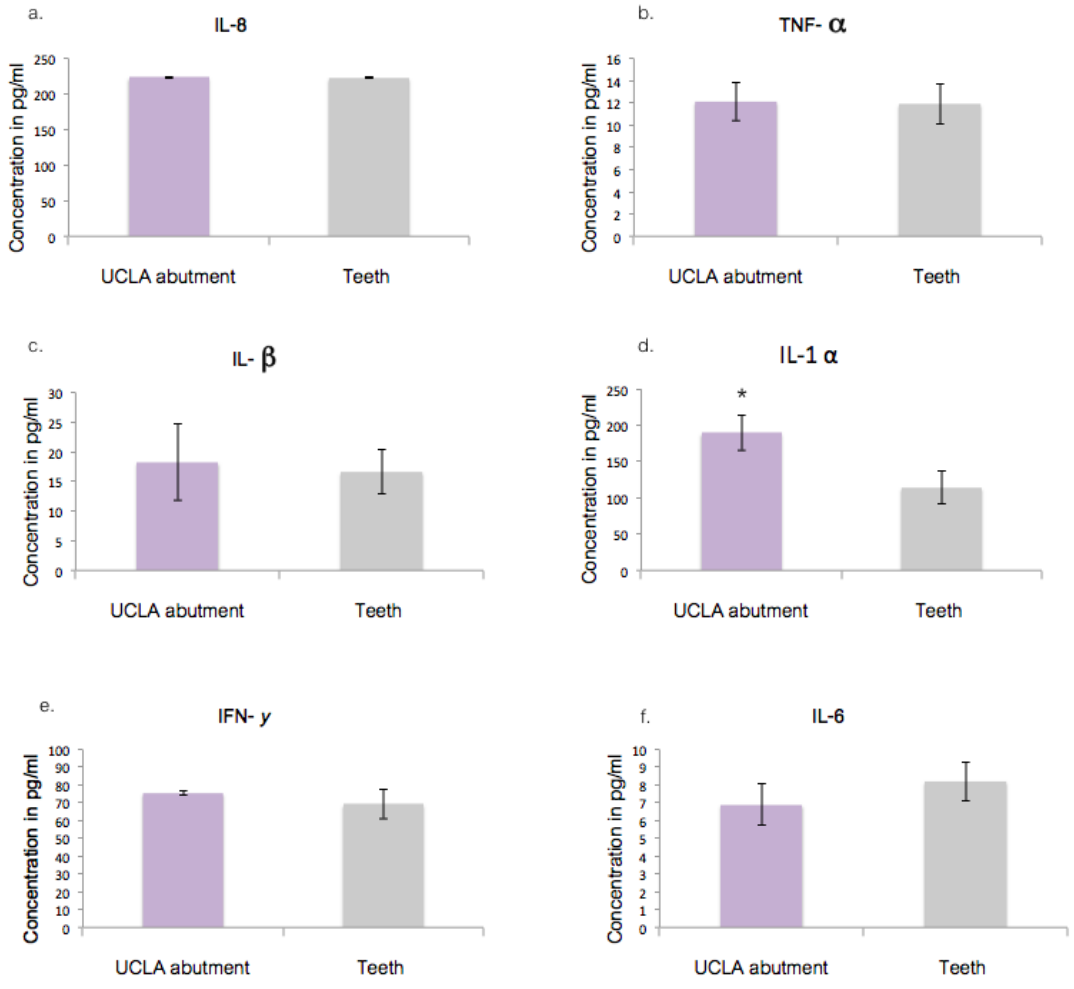


Figure 3: Cytokines expression (pg/ml) in periodontal and peri-implant crevicular fluid

from implant-supported UCLA abutment. Ten participants had implants restored with

UCLA abutment. Expression of IL-8 (a), TNF-α (b), IL-1β (c), IL-1α (d), IFN-y (e), and IL-6 (f) is demonstrated. The levels of IL-1α expression in peri-implant crevicular fluid is significantly higher than natural tooth (p value =0.030) as indicated with *. These data are collected from three independent experiments and means (SE) are demonstrated.

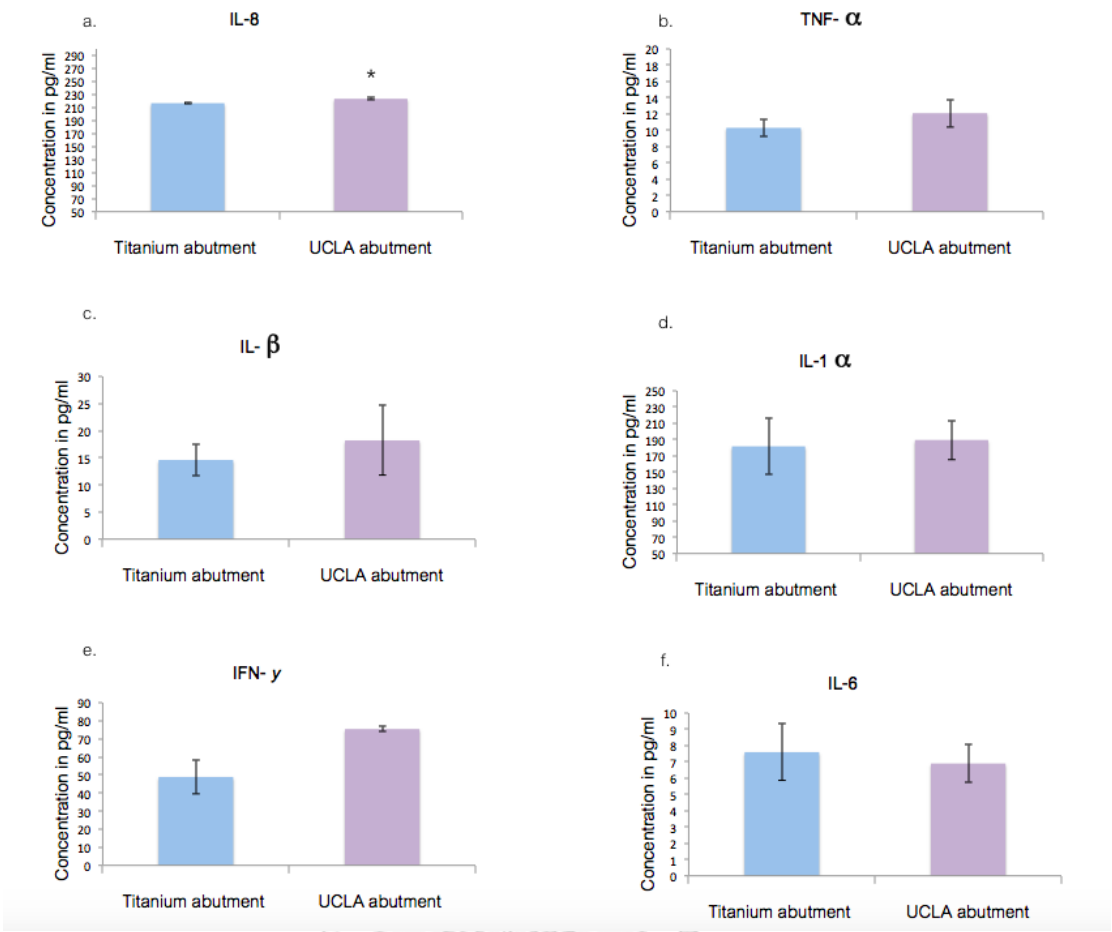


Figure 4: Cytokines expression (pg/ml) in periodontal and peri-implant crevicular fluid from implant-supported Titanium or UCLA abutment. Expression of IL-8 (a), TNF-α (b), IL-1β (c), IL-1α (d), IFN-y (e), and IL-6 (f) is demonstrated. The levels of IL-8 expression in peri-implant crevicular fluid obtained around UCLA abutment is significantly higher than peri-implant crevicular fluid obtained around titanium abutment (p value =0.003) as indicated with *. These data are collected from three

independent experiments and means (SE) are demonstrated.

From the first part of the study, the differential expression of some inflammatory cytokines was demonstrated in periodontal and peri-implant crevicular fluid. Then, it was questioned whether these inflammatory cytokines affected on Focal Adhesion Kinase (FAK) and receptor activator of nuclear factor-ΚB ligand (RANKL) expression of human gingival fibroblasts. Human gingival fibroblasts (HGFs) were cultured with PICF obtained from dental implants restored with titanium or UCLA abutment for 24 hours as described in materials and methods. The expression of FAK and RANKL mRNA was normalized with a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Normal HGF culture in growth medium was used as a negative control. Quantitative SYBR PCR revealed that PICF from both titanium and UCLA abutments (Figure 5) down-regulated FAK gene expression in HGF culture in a concentration-dependent manner.

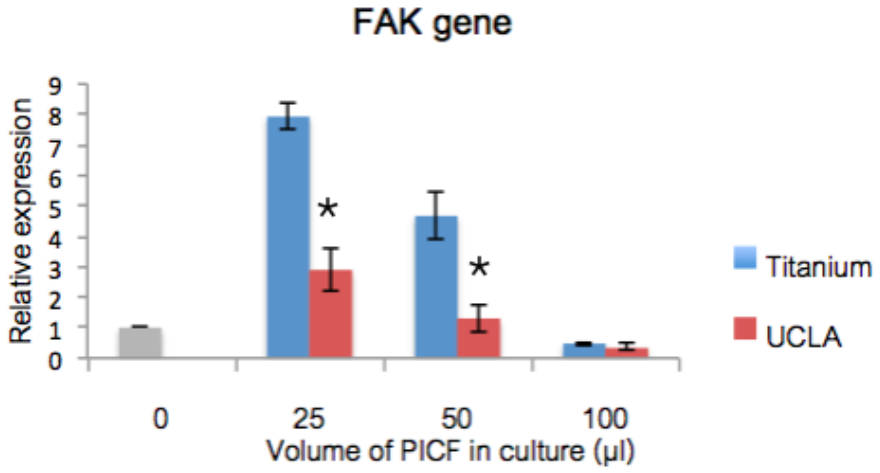


Figure 5: Peri-implant crevicular fluid down-regulates focal kinase mRNA in HGF cultures. HGF cultures are incubated with 25 µl, 50 µl and 100 µl of PICF from dental implants restored with titanium or UCLA abutment respectively. Means of relative expression detected by quantitative SYBR PCR assay from 2 independent experiments are shown. Error bars indicate standard deviation. One-way ANOVA showed statistically significance difference in expression of FAK gene between HGF treated with 3 different volumes of PICF obtained from both UCLA and titanium abutment. While HGF treated with 25 µl and 50 µl of PICF from titanium showed a statistically significant higher expression than HGF treated with 25 µl or 50 µl of PICF obtained around UCLA

abutment with a p-value of 0.007 and 0.001 respectively.

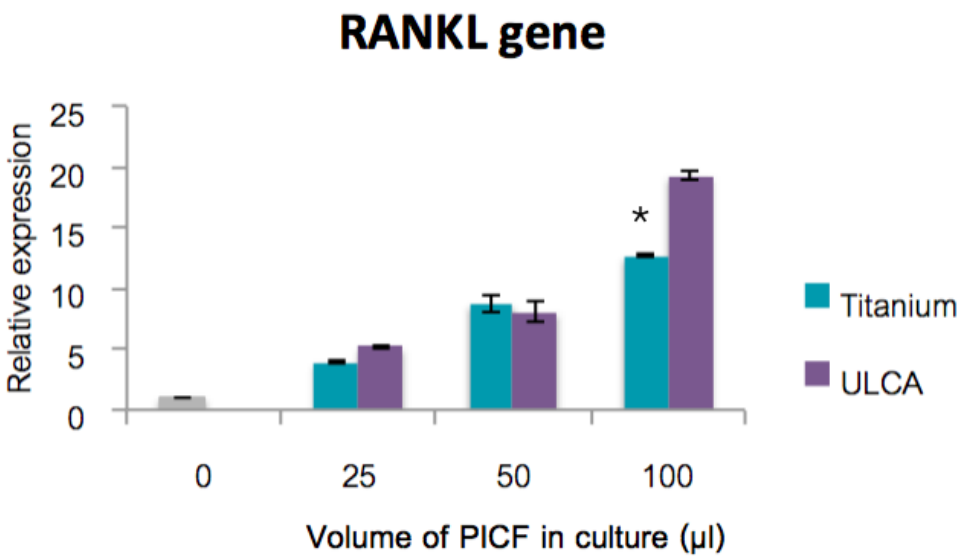


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Figure 6: Peri-implant crevicular fluid up-regulates receptor activator of nuclear factor-

K B ligand (RANKL) mRNA in HGF cultures. HGF cultures are incubated with 25 µl,

50 µl and 100 µl of PICF from dental implants restored with titanium or UCLA abutment

respectively. Means of relative expression detected by quantitative SYBR PCR assay

from 2 independent experiments are shown. Error bars indicate standard deviation. The

highest expression of RANKL was found to be in HGF treated with 100 µl of PICF

obtained around UCLA abutment. One-way ANOVA showed statistically significance difference in expression of RANKL gene between HGF treated with 3 different volumes of PICF obtained from both UCLA and titanium abutment. While HGF treated with 100 µl of PICF from titanium showed a statistically significant lower expression than HGF treated with 100 µl of PICF obtained around UCLA abutment with a p-value of 0.000.

Table 6: Shows the concentration of each cytokines present in 5% (100 µl), 2.50% (50 µl) and 1.25% (25 µl) of Supernatant added around (a) Titanium abutment or (b) UCLA abutment respectively

a. Volume of PICF from titanium abutment

Cytokines (pg)	100 µl	50 µl	25 µl
IFN- y	2.4	1.2	0.6
IL-1α	9.1	4.5	2.3
IL-1β	0.7	0.4	0.2
IL-6	0.4	0.2	0.1
TNF-α	0.5	0.3	0.1

IL-8	10.8	5.4	2.7

b. Volume of PICF from UCLA abutment

Cytokines (pg)	100 µl	50 µl	25 µl
IFN- y	3.8	1.9	0.9
IL-1α	9.5	4.7	2.4
IL-1β	0.9	0.5	0.2
IL-6	0.3	0.2	0.1
TNF-α	0.6	0.3	0.2
IL-8	11.2	5.6	2.8

CHAPTER V

DISCUSSION AND CONCLUSIONS

Host responses can be monitored through molecules expressed in PICF. Changes in osseointegration surrounding dental implants as a response to occlusal loading and oral microbiota thereby affected the long-term success of the implants (1, 10). Early detection of peri-implant inflammation along with other clinical signs and symptoms is beneficial for prevention of bone loss.

To study the host response to microenvironment surrounding natural teeth or dental implants, the inflammatory cytokines in GCF or PICF was assessed in this study. Similar level of cytokine expression in natural teeth and dental implant is reported except for the level of IL-1α and IL-1β. The results of this study are in consistent with previous

studies that showed an elevated level of IL-1β in PICF associating with the use of titanium abutment (25). It has been proposed that Ti particles may act as a secondary stimulus, to activate the inflammasome in the macrophages, resulting in the release of active IL-1β from surrounding mucosal cells, and potentially stimulates bone resorption by up-regulating RANKL (27). Titanium ions, as low as 9 p.p.m, could increase RANKL/osteoprotegerin ratio and resulted in osteoclastogenesis in stromal or osteoblastic cells (28).

Another study using multiplex proteomic immunoassays assessed the differences between the levels of interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17A, tumor necrosis factor (TNF)-α, C-reactive protein, osteoprotegerin, leptin, and adiponectin in GCF and PICF of 73 healthy individuals. All cytokines were detectable in PICF and GCF; however, only IL-17A and TNF-α concentration in PICF were significantly higher than GCF (29). Differences in study design, number of participants, selected biomarkers panel and the inclusion criteria between our study and theirs could have influenced the cytokine detection. This study uses a single ELISA array to assess cytokine levels.

Unlike the multiplex assay, a single array for each cytokine resulted in a precise standard curve and reliable data. While, multiplex assay includes a broad range of different proteins being assayed in one detection, therefore the efficacy of multiplex assay may be questioned.

In addition, a higher concentration of inflammatory cytokines were found in PICF obtained around yellow gold abutment than PICF obtained around titanium abutment however only IL-8 levels reached a statistical significance. This finding is in accordance with histological analysis by Sampatanukul et al, which showed that gold alloy exhibited highest percentage of inflammatory cellularity grades in comparison to titanium and zirconia. Moreover, a lower amount of collagen and fibroblast and higher fractions of leukocytes were found in the connective tissue interface with gold alloy abutments (30).

Plaque accumulation on the abutment surface is one of the cause of inflammatory reactions and recruitments of inflammatory cytokines. There are various factors that affect the bacterial adhesion on the abutment surface such as surface free energy, hydrophilicity, surface chemistry, surface charge, roughness, and the presence of

proteins. Many studies were carried out on bacterial adhesion on biomaterials with various degrees of roughness and physicochemical properties (31, 32). A study by *Ha-Young Kim* et al, showed that Gold alloy had the highest polar surface energy and the lowest nonpolar surface energy which displayed the strongest bacterial adhesion in comparison to titanium and zirconia with the same surface roughness (33). Moreover, weaker fibroblast adhesion strength was observed in Au and Cr–Co alloys compared to polished ZrO and Ti specimens (30),(34). These findings support the idea that material type has a strong influence on cellular adhesion strength. The latter fact shows that the chemical composition of Au and Cr–Co alloys may be less compatible with gingival fibroblasts despite similar or even higher roughnesses compared to ZrO and Ti specimens. Welander et al. studied mucosal barrier with various implant abutments in vivo and concluded that abutments made of ZrO and Ti favored better mucosal barrier than Au alloy because Au led to an apical shift of the barrier epithelium as well as marginal bone occurring between 2 and 5 months of healing (34).

Only implants placed at crestal or subcrestel level in the anterior region were evaluated in this study. All implants have been in function from 1 to 5 years and no visible mucosal inflammation is observed in all patients consistently with the previous report by Mohammad et al (35) that subgingival level of implant placement appears to have no effect on clinical status and supporting tissue of single implant restorations. Regardless of the position of the implant being placed, the differential expression of inflammatory cytokines in PICF is likely due to abutment materials (35).

Peri-implant mucosal tissues, including the epithelium, connective tissue and alveolar bone, play an important role in success of treatment. After implantation, two distinct responses may occur on the implant surface. The bone tissue grows to contact the implant surface establishing complete osseointegration and a successful initial stability. Fibrous encapsulation involves the soft tissue covering the neck of implant surface. Gingival fibroblast cell around dental implant is the first line body defense against the oral microorganisms and plays a crucial role in recruiting many defense mechanisms needed to combat infection (6). Inflammatory responses may lead to destructive bone

resorption where RANKL upregulation plays a crucial role in osteoclastogenesis. Mice with a knock-down level of RANKL protein show severe osteopetrosis and defective tooth eruption resulting from completely lacking of osteoclast function. These genetic experiments for the first time proved that RANK and RANKL are essential for osteoclastogenesis (36).

Cytokines including M-CSF, IL-1, IL-6, IL-11, IL-17, and TNF-α are reported to up-regulate RANKL expression and promotes osteoclastogenesis (37). In contrast, IL-10, GM-CSF, IL-12, IL-10, IL-18, IFN-y are reported to inhibit osteoclast differentiation (38). This study was limited only to the assessment of pro-inflammatory cytokines in GCF and PICF around the differential abutment materials. Further studies may be needed to assess the expression of anti-inflammatory cytokines on the expression of RANKL. Nonetheless, the inflammatory cytokines present in PICF in this study stimulate HGF culture to express much higher RANKL mRNA, while decreasing FAK mRNA, in a concentration-dependent manner. IL-8 concentration in PICF obtained from UCLA abutments was significantly higher than titanium abutments.

Simultaneously, the effect of PICF from UCLA abutments on RANKL and FAK expression appears to be higher than that of PICF from titanium abutments.

This result is consistent with the previous study that showed IL-8, IL-1α and TNFα decreases the expression of FAK gene in HGFs (39) (40). IL-8 (0.005-5 ng/ml) is chemotactic for fibroblasts and resulted in focal adhesion disassembly and cell migration (40). Thus, IL-8 stimulates a loss of focal adhesions in both rat and human primary fibroblasts. The down-regulation of FAK mRNA compromises the ability of fibroblast adhesion around the dental implants (40). Interestingly, previous studies demonstrated that HGF responded to titanium disc better than gold alloys of the same roughness in terms of FAK expression and cell adhesion. Despite the surface roughness, chemical composition of gold alloys may be less biocompatible as compared to inert material like titanium (34). However, both titanium and gold alloy UCLA abutments are clinically successful in providing soft tissue seal around the dental implants. None of them showed gingival recession nor bone loss after 5 years of function. This is in accordance with previous prospective study which shows no

different response amongst titanium or gold alloy in peri-implant marginal bone and soft tissue (38). If only the soft tissue response is considered, the choice between using gold or titanium abutment is merely up to the clinician's preference. The thickness of the peri-implant soft tissue is a crucial factor in the abutment options. Abutment materials have an impact on the color and esthetics when the thickness of soft tissues is 2 mm or less. In the cases of thin peri-implant soft tissue, gold or zirconia abutments may be preferred rather than titanium due to its low colorimetric performances (39).

Conclusions

The expression of some inflammatory cytokines in healthy peri-implant tissues, including IL-1α and IL-1β, may be different from those in healthy periodontal tissue. The implant abutment materials have an influence on the immune response. Therefore, this study demonstrated that the concentration of inflammatory cytokines in PICF, especially from UCLA abutments, potentially increase osteoclast function while decrease fibroblast adhesion.

Further studies

Inflammatory response of zirconia abutment compared to titanium abutment.

Clinical implications

The use of yellow gold abutment should still be considered due to its esthetic appearance compared to titanium abutment especially in thin periodontal biotype. However, when yellow gold abutment material is used special attention should be given to patient's oral hygiene as the material itself has the capability to recruit inflammatory cytokines.

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APPENDIX

Appendix A. The demographics

Sex	Age	Implant	Tooth	Implant type	Abutment	Loading period in months
F	46	11	22	Straumann	Titanium	70
M	56	21	11	Straumann	UCLA	26
F	59	22	12	Straumann	UCLA	50
M	23	23	13	Straumann	UCLA	26
M	27	31 32	42	Straumann	UCLA	15
F	54	31	41	Straumann	UCLA	26
F	23	21	11	Straumann	UCLA	15
M	45	12	22	Straumann	UCLA	76
M	56	22	13	Straumann	UCLA	56
M	24	31	41	Straumann	UCLA	42
F	32	13	23	Astratech	Titanium	70
F	45	11	21	Straumann	UCLA	26
M	53	21	11	Astratech	Titanium	50
M	31	21	11	Astratech	Titanium	26
F	58	21	12	Astratech	Titanium	15
F	46	12	22	Astratech	Titanium	26

M		22	12	Astratech	Titanium	15
F	58	12	22	Astratech	Titanium	76
F	33	31	41	Astratech	Titanium	56

Appendix B. The expression of IL-8

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	223.403	10	4.6074	1.4570
	TeethUCLA	222.340	10	2.9594	.9359
Pair 2	Titanium	216.656	9	3.7608	1.2536
	TeethTitanium	219.344	9	7.8162	2.6054

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.096	.792
Pair 2	Titanium & TeethTitanium	9	-.092	.814

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	1.0630	5.2311	1.6542	-2.6791	4.8051	.643	9	.537
Pair 2	Titanium - TeethTitanium	-2.6889	8.9798	2.9933	-9.5914	4.2136	-.898	8	.395

Group Statistics

	Abutment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	1	10	223.4030	4.60740	1.45699
	2	9	216.6556	3.76077	1.25359

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
Concentration	Equal variances assumed	.309	.586	3.472	17	.003	6.74744	1.94362	2.64677	10.84812
	Equal variances not assumed			3.511	16.862	.003	6.74744	1.92205	2.68973	10.80516

Appendix C. The expression of IL-1 alpha

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	189.520	10	76.2310	24.1064
	TeethUCLA	114.029	10	71.4299	22.5881
Pair 2	Titanium	181.667	9	103.1836	34.3945
	TeethTitanium	175.196	9	51.3865	17.1288

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.212	.557
Pair 2	Titanium & TeethTitanium	9	.526	.146

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	75.4914	92.7612	29.3337	9.1340	141.8488	2.574	9	.030
Pair 2	Titanium - TeethTitanium	6.4714	87.8119	29.2706	-61.0267	73.9696	.221	8	.831

Group Statistics

	Abutment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	1	10	189.5204	76.23098	24.10635
	2	9	181.6670	103.18357	34.39452

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
Concentration	Equal variances assumed	.455	.509	.190	17	.852	7.85340	41.31839	-79.32078	95.02758
	Equal variances not assumed			.187	14.648	.854	7.85340	42.00119	-81.85765	97.56445

Appendix D. The expression of IL-1 Beta

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	18.161	10	20.2135	6.3921
	TeethUCLA	16.617	10	11.7925	3.7291
Pair 2	Titanium	14.628	9	8.6781	2.8927
	TeethTitanium	7.873	9	7.0790	2.3597

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.659	.038
Pair 2	Titanium & TeethTitanium	9	.521	.150

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	1.5440	15.2798	4.8319	-9.3865	12.4745	.320	9	.757
Pair 2	Titanium - TeethTitanium	6.7552	7.8349	2.6116	.7328	12.7777	2.587	8	.032

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	18.161	10	20.2135	6.3921
	TeethUCLA	16.617	10	11.7925	3.7291
Pair 2	Titanium	14.628	9	8.6781	2.8927
	TeethTitanium	7.873	9	7.0790	2.3597

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.659	.038
Pair 2	Titanium & TeethTitanium	9	.521	.150

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	1.5440	15.2798	4.8319	-9.3865	12.4745	.320	9	.757
Pair 2	Titanium - TeethTitanium	6.7552	7.8349	2.6116	.7328	12.7777	2.587	8	.032

Appendix E. The expression of IFN-gamma

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	75.354	10	41.8989	13.2496
	TeethUCLA	69.270	10	25.7713	8.1496
Pair 2	Titanium	49.860	9	28.3094	9.4365
	TeethTitanium	57.337	9	40.9285	13.6428

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.293	.411
Pair 2	Titanium & TeethTitanium	9	.560	.117

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	6.0840	42.2605	13.3640	-24.1474	36.3154	.455	9	.660
Pair 2	Titanium - TeethTitanium	-7.4763	34.3310	11.4437	-33.8655	18.9128	-.653	8	.532

Group Statistics

	Abutment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	1	10	75.3540	41.89886	13.24958
	2	9	49.8603	28.30938	9.43646

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	Upper
Concentration	Equal variances assumed	1.767	.201	1.535	17	.143	25.49367	16.60792	-9.54597	60.53331
	Equal variances not assumed			1.567	15.856	.137	25.49367	16.26648	-9.01514	60.00247

Appendix F. The expression of IL-6

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	6.857	10	3.7132	1.1742
	TeethUCLA	8.233	10	3.5293	1.1161
Pair 2	Titanium	7.586	9	5.1875	1.7292
	TeethTitanium	7.266	9	3.9027	1.3009

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	-.174	.631
Pair 2	Titanium & TeethTitanium	9	.356	.347

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	UCLA - TeethUCLA	-1.3761	5.5501	1.7551	-5.3464	2.5942	-.784	9	.453
Pair 2	Titanium - TeethTitanium	.3200	5.2646	1.7549	-3.7268	4.3668	.182	8	.860

Group Statistics

	Abutment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	1	10	6.8569	3.71317	1.17421
	2	9	7.5856	5.18752	1.72917

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration	Equal variances assumed	.938	.346	-.355	17	.727	-.72866	2.05291	-5.05991	3.60260
	Equal variances not assumed			-.349	14.364	.732	-.72866	2.09017	-5.20098	3.74367

Appendix G. The expression of TNF-alpha

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	UCLA	12.140	10	5.4491	1.7232
	TeethUCLA	11.940	10	5.7338	1.8132
Pair 2	Titanium	10.322	9	3.2687	1.0896
	TeethTitanium	9.719	9	3.4674	1.1558

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	UCLA & TeethUCLA	10	.785	.007
Pair 2	Titanium & TeethTitanium	9	.650	.058

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	UCLA - TeethUCLA	.2000	3.6761	1.1625	-2.4297	2.8297	.172	9	.867
Pair 2	Titanium - TeethTitanium	.6037	2.8254	.9418	-1.5681	2.7754	.641	8	.539

Group Statistics

	Abutment	N	Mean	Std. Deviation	Std. Error Mean
Concentration	1	10	7.2170	5.02779	1.58993
	2	9	7.5856	5.18752	1.72917

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration	Equal variances assumed	.002	.968	-.157	17	.877	-.36856	2.34493	-5.31593	4.57882
	Equal variances not assumed			-.157	16.660	.877	-.36856	2.34902	-5.33227	4.59516

Appendix H. The expression of FAK gene

Titanium 100	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
28.23	26.10	-	28.23	-	26.10	5.86	4.97	0.89	0.539614
34.09	31.07	34.09	-	31.07	-				
UCLA 100	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
29.16	26.10	-	29.16	-	26.10	6.04	4.97	1.07	0.476319
35.20	31.07	35.20	-	31.07	-				

	Titanium 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01	-	26.32	-	26.01	7.78	10.22	-2.44	5.426417
FAK	34.10	36.23	34.10	-	36.23	-				

	UCLA 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	25.77	26.01	-	25.77	-	26.01	9.43	10.22	-0.79	1.729074
FAK	35.20	36.23	35.20	-	36.23	-				

	Titanium 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01	-	26.32	-	26.01	7.78	10.22	-2.44	5.426417
FAK	34.10	36.23	34.10	-	36.23	-				

	UCLA 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	25.77	26.01	-	25.77	-	26.01	9.43	10.22	-0.79	1.729074
FAK	35.20	36.23	35.20	-	36.23	-				

Appendix I. The expression of RANKL gene

	Positive control	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	30.90	29.02	-	30.90	-	29.02	1.10	3.10	-2.00	4
RANKL gene	32.00	32.12	32.00	-	32.12	-				

	Titanium (25)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	31.13	27.00	-	31.13	-	27.00	3.73	5.43	-1.70	3.24901
RANKL gene	34.86	32.43	34.86	-	32.43	-				

	UCLA (25)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	27.53	27.00	-	27.53	-	27.00	3.10	5.43	-2.33	5.028053
RANKL gene	30.63	32.43	30.63	-	32.43	-				

	Titanium (50)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	29.88	26.01	-	29.88	-	26.01	4.23	7.47	-3.24	9.447941
RANKL gene	34.11	33.48	34.11	-	33.48	-				

	UCLA (50)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01		26.32	-	26.01	4.31	7.47	-3.16	8.938297
RANKL gene	30.63	33.48	30.63	-	33.48	-				

	Titanium (100)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	28.45	31.76	-	28.45	-	31.76	3.96	7.64	-3.68	12.81712
RANKL gene	32.41	39.40	32.41	-	39.40	-				

	UCLA (100)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	30.21	31.76	-	30.21	-	31.76	2.30	7.64	-5.34	40.50421
RANKL gene	32.51	39.40	32.51	-	39.40	-				

	Positive control	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	30.13	29.02	-	30.13	-	29.02	1.14	3.10	-1.96	3.89062
RANKL gene	31.27	32.12	31.27	-	32.12	-				

	Titanium (25)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	31.13	27.00	-	31.13	-	27.00	3.60	5.43	-1.83	3.555371
RANKL gene	34.73	32.43	34.73	-	32.43	-				

	UCLA (25)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	27.53	27.00	-	27.53	-	27.00	3.01	5.43	-2.42	5.35171
RANKL gene	30.54	32.43	30.54	-	32.43	-				

	UCLA 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01	-	26.32	-	26.01	4.61	7.47	-2.86	7.260153
RANKL gene	30.93	33.48	30.93	-	33.48	-				

	Titanium(50)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	29.88	26.01		29.88	-	26.01	4.46	7.47	-3.01	8.055644
RANKL gene	34.34	33.48	34.34	-	33.48	-				

	Titanium (100)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	28.23	31.76	-	28.23	-	31.76	3.99	7.64	-3.65	12.55335
RANKL gene	32.22	39.40	32.22	-	39.40	-				

	UCLA (100)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	29.16	31.76	-	29.16	-	31.76	1.57	7.64	-6.07	67.18187
RANKL gene	30.73	39.40	30.73	-	39.40	-				

	UCLA (25)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	27.53	27.00	-	27.53	-	27.00	3.01	5.43	-2.42	5.35171
RANKL gene	30.54	32.43	30.54	-	32.43	-				

	UCLA 50	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	26.32	26.01	-	26.32	-	26.01	4.61	7.47	-2.86	7.260153
RANKL gene	30.93	33.48	30.93	-	33.48	-				

	Titanium(50)	Control Well 1	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	29.88	26.01		29.88	-	26.01	4.46	7.47	-3.01	8.055644
RANKL gene	34.34	33.48	34.34	-	33.48	-				

REFERENCES

(1)

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