



The Open Dentistry Journal

Content list available at: <https://opendentistryjournal.com>



RESEARCH ARTICLE

The Affinity of Human Fetal Osteoblast to Laser-Modified Titanium Implant Fixtures

Lee Kian Khoo¹, Sirichai Kiattavorncharoen¹, Verasak Pairuchvej¹, Nisanat Lakkhanachatpan², Natthamet Wongsirichat¹ and Dutmanee Seriwatanachai^{2,*}

¹Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Mahidol University, 6 Yothi Street, Rachathewe District, Bangkok, 10400, Thailand

²Department of Oral Biology, Faculty of Dentistry, Mahidol University, 6 Yothi Street, Rachathewe District, Bangkok, 10400, Thailand

Abstract:

Introduction:

Implant surface modification methods have recently involved laser treatment to achieve the desired implant surface characteristics. Meanwhile, surface modification could potentially introduce foreign elements to the implant surface during the manufacturing process.

Objectives:

The study aimed to investigate the surface chemistry and topography of commercially available laser-modified titanium implants, together with evaluating the cell morphology and cell adhesion of human fetal osteoblast (hFOB) seeded onto the same implants.

Method:

Six (6) samples of commercially available laser-modified titanium implants were investigated. These implants were manufactured by two different companies. Three (3) implants were made from commercially pure grade 4 Titanium (Brand X); and three were made from grade 5 Ti6Al4V (Brand Y). The surface topography of these implants was analyzed by scanning electron microscope (SEM) and the surface chemistry was evaluated with electron dispersive x-ray spectroscopy (EDS). Human fetal osteoblasts were seeded onto the implant fixtures to investigate the biocompatibility and adhesion.

Results & Discussion:

Brand X displayed dark areas under SEM while it was rarely found on brand Y. These dark areas were consistent with their organic matter. The hFOB cell experiments revealed cell adhesion with filopodia on Brand X samples which is consistent with cell maturation. The cells on Brand Y were morphologically round and lacked projections, one sample was devoid of any noticeable cells under SEM. Cell adhesion was observed early at 48 hrs in laser-irradiated titanium fixtures from both the brands.

Conclusion:

The presence of organic impurities in Brand X should not be overlooked because disruption of the osseointegration process may occur due to the rejection of the biomaterial in an *in-vivo* model. Nevertheless, there was insufficient evidence to link implant failure directly with carbon contaminated implant surfaces. Further studies to determine the toxicity of Vanadium from Ti6Al4V in an *in-vivo* environment should indicate the reason for different cell maturation.

Keywords: Bone formation, Biomaterial, Dental prosthesis, Osseointegration, Titanium, Affinity.

Article History

Received: August 05, 2019

Revised: December 20, 2019

Accepted: January 02, 2020

1. INTRODUCTION

The success of dental implants hinges on an integral phenomenon where direct contact between bone and an implanted biomaterial occurs, this event is termed as osseointe-

gration. The current consensus supports the use of titanium implants with rough surfaces for more predictable peri-implant bone adhesion [1]. With this knowledge, dental implant manufacturers either use subtractive and/or additive methods to optimize the surface characteristics of titanium implants. Subtractive methods include machining, anodization, Sandblasted Large grit acid etching (SLA) and laser irradiation. Additive methods involve coating the surface of the implant

* Address correspondence to this author at the Department of Oral Biology, Faculty of Dentistry, Mahidol University, 6 Yothi Street, Rachathewe District, Bangkok, 10400, Thailand; Tel: +662007849; E-mail: dutmanee.ser@mahidol.ac.th

with materials such as titanium plasma, hydroxyapatite, calcium phosphate or bone morphogenic protein. Apart from laser irradiation, all of the above-mentioned surface modification methods could potentially introduce foreign elements to the implant surface during the manufacturing process. Laser irradiation is a clean and economical option because the lack of direct contact with the titanium fixture during the surface modifying process could ensure a contaminant-free product [2].

Multiple animal studies have documented the superiority of laser modified (LM) titanium surfaces over other surface modifying techniques especially in terms of the removal torque post-implant insertion [3 - 5]. Therefore, there is a need for more information regarding the cellular events when cells come in contact with LM surfaces. LM titanium discs have displayed mature osteoblasts with filopodia in in-vitro experiments [6, 7]. However, these experiments employed osteosarcoma derived cell lines, which tend to exhibit abnormal cell growth patterns that are less representative of completely differentiated osteoblasts [8]. In contrast, primary cell lines like Human Fetal Osteoblast (hFOB) were reported to have minimal chromosome abnormalities alongside matrix producing properties similar to differentiated osteoblasts [8]. These properties make hFOB the ideal model for osteoblast biology experiments [8, 9].

Although the current literature related to laser-modified titanium implants exhibits rather promising results in terms of the surface purity of LM implants [2, 10], the actual product the clinician purchases could be tainted or contaminated during packaging. Duddeck *et al.*, observed contamination in many commercially available implants. They found both organic (carbon, polyethylene) and inorganic (aluminum, nickel, steel, chromium) impurities *via* Scanning Electron Microscope (SEM) and Electron Dispersive X-ray Spectroscopy (EDS) analysis of supposedly sterile-packaged implants [11]. This alarming report reinforces the requirement for more scrutiny of supposedly pristine implants, even in LM Titanium implants.

Due to the lack of information regarding commercially available LM titanium implants and the reaction of hFOB to LM titanium surfaces, the aims of this study were to:

1. Analyze the surface chemistry and morphology of commercially available laser- modified titanium implants.
2. Visualize the cell morphology of hFOB seeded onto commercially available laser- modified titanium implants.

2. MATERIALS AND METHODS

2.1. SEM and EDS of Implants

Six (6) implants were purchased, 3 from biomate (Brand X) and 3 from biohorizon (Brand Y). It is important to note here that Brand X implants were manufactured from commercially pure grade 4 titanium, whereas brand Y implants were manufactured from grade 5 titanium Alloy (Ti6Al4V).

All the samples were in sterilized packages and opened only during sample analysis. To examine the surface morphology and chemistry of the implants, a scanning electron microscope with EDS (JSM 5410LV, JEOL, Tokyo, Japan)

was used. Three (3) samples from each brand were analyzed under SEM and EDS. The visualization of the surface was performed at three sections, coronal, middle and apical sections in each one of the implants. The samples were placed on carbon discs for fixation and then inside the microscope chamber under vacuum conditions. EDS was used for qualitative and quantitative elemental analysis, and random spots were selected and analyzed.

2.2. Cell Culture

Human fetal osteoblast cell line hFOB 1.19 (ATCC® CRL11372™), American Type Culture Collection (Manassas, VA, USA), following the guidelines mentioned in the study by Harris *et al.* [12] was cultured in a 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium with 2.5 mM L-glutamine (without phenol red) (Gibco, Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.3 mg/mL geneticin (G418, Serva Electrophoresis GmbH, Heidelberg, Germany). hFOB were maintained in proliferative stage at 34 °C under 5% CO₂ in a humidified incubator. Culture media were refreshed every other day.

2.3. Cell Adhesion Experiments

All implants were cleaned and degreased with ethanol at different concentrations and sterilized in an autoclave at 134°C for 15 min. Each implant was then placed horizontally in a 6-well culture plate. The cells were seeded at a density of 100,000 cells per implant using a micropipette. After 48 hrs, the implants were removed from incubation and fixed with cold 4% glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 2 days. Then, implants were dehydrated in different concentrations of ethanol and were coated with gold for SEM analysis.

3. RESULTS

Two (2) samples from Brand X displayed dark areas around the collar and upper threads (Fig. 1) under SEM, which appeared to be indicative of organic elements. Carbon appears darker than titanium in the material viewed by the contrast image because carbon atoms have fewer electrons.

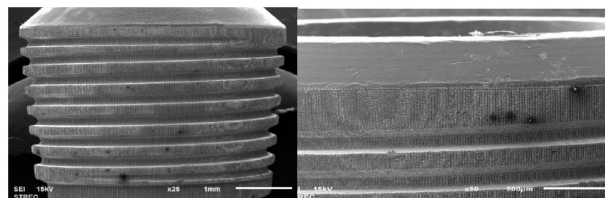


Fig. (1). SEM analysis of the collar and upper threads of laser implant from brand X at magnification at x25(a) and x50 (b).

At x5000, the typical morphology of lasered surfaces is clear; the spherical structures with microcracks were the result of rapid melting (Fig. 2) and cooling of the metal immediately after laser. EDS results displayed oxygen, titanium and small amounts of carbon in all 3 samples (Fig. 3). The presence of carbon is consistent with the dark areas.

All brand Y samples were relatively clean and displayed no distinct dark areas under SEM (Fig. 4). This could be indicative of a cleaner packaging process post-laser-irradiation. At x2000, symmetrical rods and spheres could be visualized (Fig. 5). EDS results showed oxygen, titanium, aluminum and

vanadium with a small amount of carbon in all 3 samples of brand Y (Fig. 6). The EDS results clearly showed the presence of vanadium ions which have been postulated to be non-biocompatible. The chemical composition (atomic %) of 2 brand samples is demonstrated in Table 1 (Mean \pm SD).

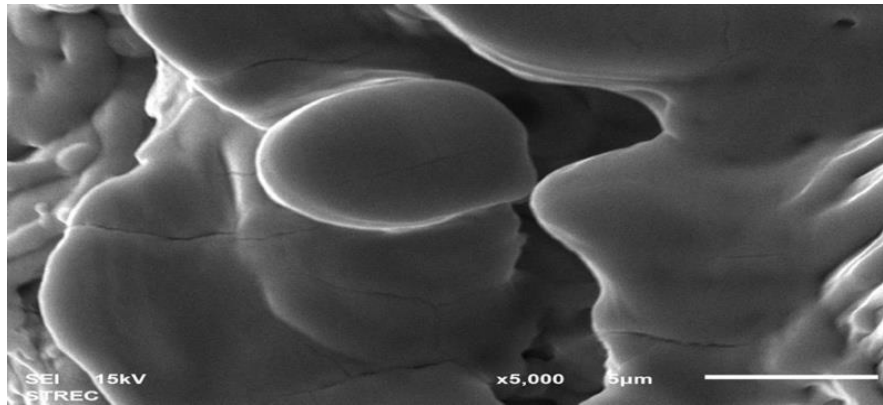


Fig. (2). Spherical structures could be visualized under SEM from a randomized area on Brand X ; at magnification x5000.

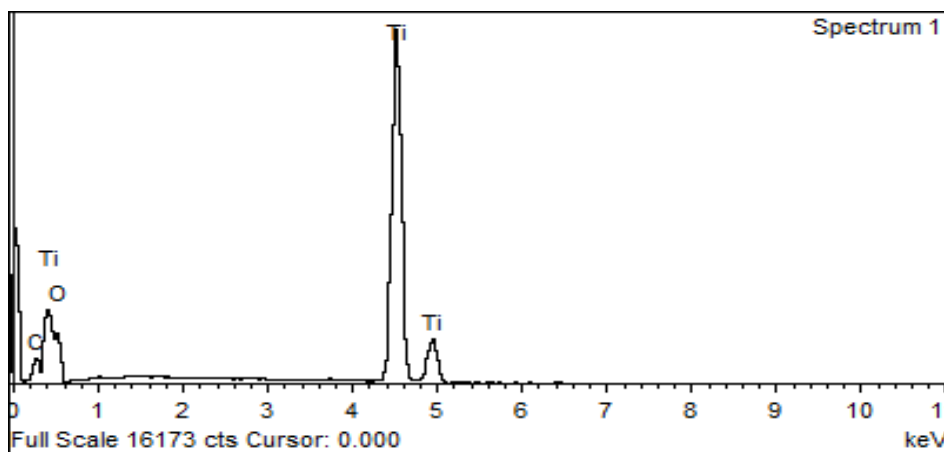


Fig. (3). EDS result from a randomized spot on one of the Brand X samples.

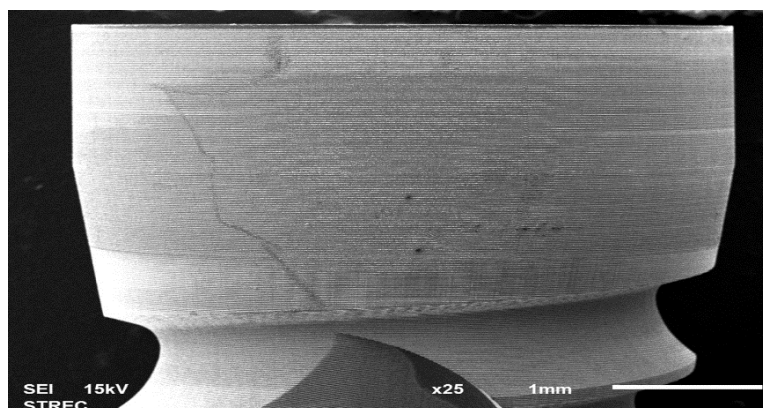


Fig. (4). SEM analysis of Brand Y sample under magnification of x25.

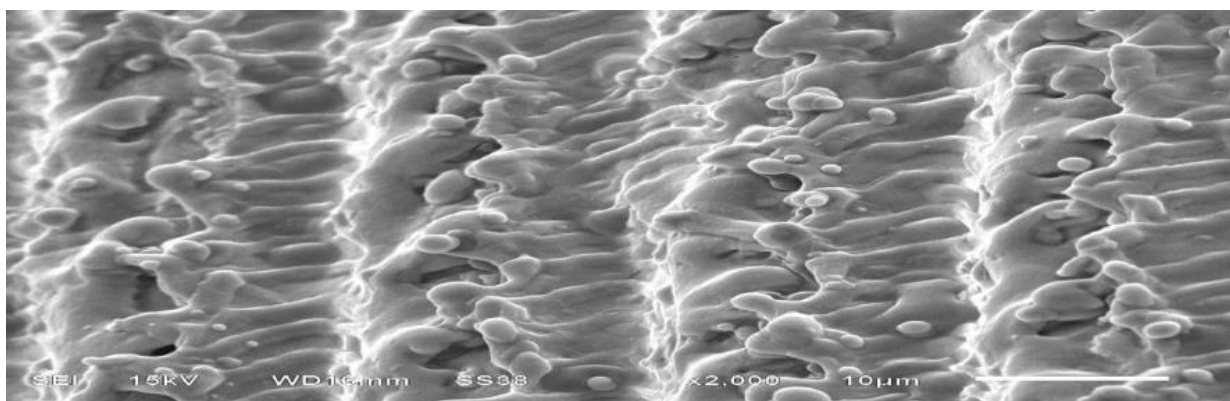


Fig. (5). SEM analysis of random surface from brand Y at magnification of x2000.

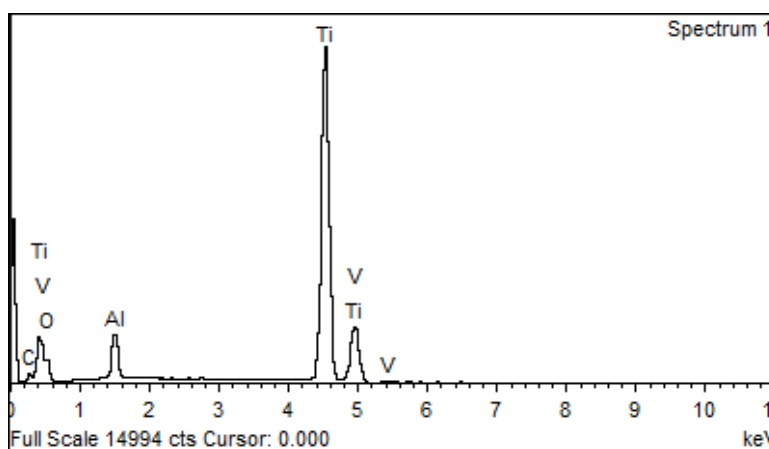


Fig. (6). EDS result from a randomized spot on one of the Brand Y samples.

Table 1. Chemical composition (atomic %) of samples (Mean ± SD); n=3, duplicated.

Elements	Brand X	Brand Y
Carbon	8.32 ± 2.92	3.94 ± 4.43
Oxygen	42.59 ± 9.37	20.71 ± 19.30
Titanium	48.94 ± 8.92	66.325 ± 14.94
Aluminum	0	6.03 ± 1.35
Vanadium	0	2.99 ± 0.79

4. CELL ADHESION EXPERIMENTS

This experiment was a descriptive analysis with the help of images. After 48 hrs, all 6 samples were visualized under SEM; a startling finding was the lack of mature cells on brand Y surfaces. One sample of brand Y displayed zero cell adherence, while the other 2 brand Y samples displayed cells that were rounded and had short processes (Figs. 7a and 7b). The short processes of osteoblast cells displayed lack of maturation at 48 hrs. Although brand Y samples were free of contaminants, the overall lack of mature cell adhesion after 48hrs could indicate the need for a longer duration for cells to mature on titanium alloys. The presence of impurities in the form of organic debris on brand X surfaces showed no impact on cell adhesion

experiments. Brand X implant samples displayed good cell maturation with filopodia (Fig. 8). The filopodia show a single osteoblast cell maturing and attachment of its arms to further sites on the implants and cell attachment with filopodia in different directions, even in the presence of darker areas, which are probably organic debris (Fig. 9).

5. DISCUSSION

Osseointegration is regarded as an essential indicator of success in oral rehabilitation with dental implants. Osseointegration is a dynamic process that involves multivariate factors that determine the direct union of bone with a biomaterial without concomitant unfavorable events such as marginal bone loss or peri-implantitis. Therefore, titanium should not be considered inert when used as an implant material, instead in an ideal scenario, after insertion into bone, it triggers a foreign body reaction which causes mild chronic inflammation, that is resolved with time by the formation of a dense bone, engulfing the implant (osseointegration) [13]. With this in mind, we can hypothesize that the introduction of additional foreign elements, whether organic or inorganic could disrupt the foreign body equilibrium, thereby, inducing more osteoclastic activity with minimal osteoblasts during bone remodeling.

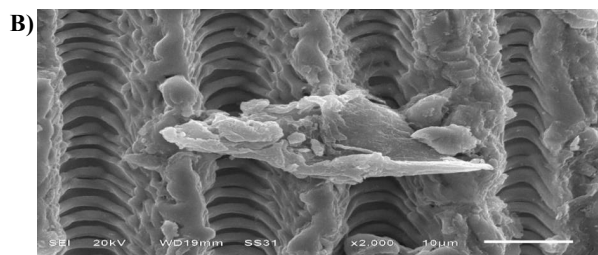
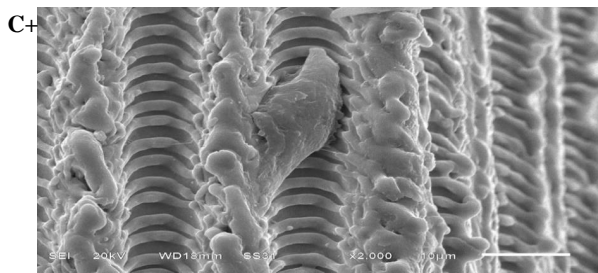


Fig. (7). (a,b) Cells that adhered to Brand Y were round and lacked projections, the cells showed signs of immature maturation and differentiation, without filopodia (magnification at x2000 on two different sites where osteoblast adhered).

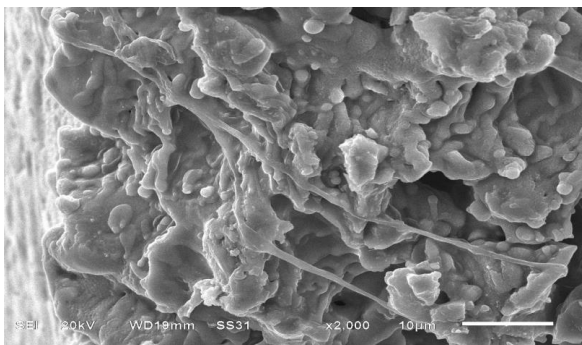


Fig. (8). Cells on Brand X, with Filopodia with long projections extending to different directions, magnification x2000.

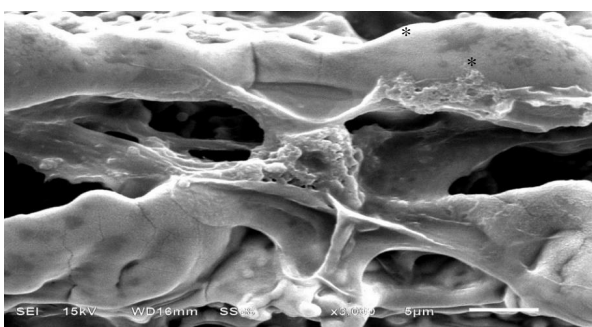


Fig. (9). Cell on Brand X, Filopodia from cell extending to different directions, note the presence of organic matter in the form of dark areas (asterisk) on the titanium surface at a magnification of x3000.

The impurities on the surface of titanium implants could occur due to residual elements generated by the surface alteration process [10] or organic contamination from the packaging of dental implants [11]. These contaminants could disrupt the healing of the implant to bone interface, thus

delaying osseointegration or causing early marginal bone loss. Therefore, quality control during the manufacturing and packaging of commercially available dental implants should be monitored carefully. Brand X samples in our study displayed dark areas under SEM which is indicative of organic impurities. A likely source of the organic debris found on brand X implants could be from the contact of the implant surface with plastic elements during the packaging process. Although the organic matter on the implant surface did not hinder cell adhesion in this study, the *in-vitro* state of the cells here does not represent the actual process of healing following implantation of a biomaterial. The *in-vitro* environment discounts the presence of inflammatory and immunomodulatory events.

The organic debris on the surface of the brand X implants is not an uncommon finding. Carbon is the most widely found contaminant on commercial implants. Morra *et al.*, reported carbon contamination in commercially available titanium implants regardless of the surface alteration process used by the manufacturers [14]. Although the exact minimum dose of carbon to elicit an unfavorable response is still nebulous, Hayashi R *et al.*, reported concentration-dependent phenomena where a higher amount of carbon caused more unfavorable osteoblast attachment and differentiation on titanium discs [15]. It is evident in our research that the concentration of carbon in the brand X implants was not enough to deter the attachment and differentiation of hFOB to the laser-modified titanium surface.

In this experiment, the 48 hrs incubation period post-seeding of hFOB onto the implants produced good adhesion and cell maturation on brand X implant samples. A longer incubation period could have produced better cell adhesion or cell maturation on brand Y samples. Longer hFOB incubation periods (7days and 15days) with lasered zirconia implants were reported to produce cells with filopodia after 7days, while intercellular contacts, which converged into a single layer that adhered onto the surface of the implants, were observed after 15 days [16].

The vast majority of dental implants on the market are made from either titanium alloys (Ti6Al4V) or commercially pure titanium (Cp-Ti). Despite having different strength and elemental composition, Shah F *et al.*, concluded that Ti6Al4V and Cp-Ti had no significant difference in their ability as an implant material to achieve osseointegration [17]. However, Steinemann documented the possibility of foreign body reactions characterized by an increase in inflammatory cells in tissues surrounding implanted Ti6Al4V as opposed to Cp-Ti implants [18]. He further attributed these findings to the leaching of vanadium ions from the Ti6Al4V implants [18]. Okazaki *et al.*, discovered small amounts of vanadium in the tissues around Ti6Al4V implants that were inserted in rat tibia [19]. Because vanadium itself is toxic, the presence of vanadium particles even in trace amounts should be a matter of concern. In this study, brand Y implants were made from Ti6Al4V, while brand X implants were made from Cp-Ti grade 4. The lack of mature cells after 48 hours of hFOB cell culture in brand Y samples could be ascribed to the presence of vanadium ions which are highly toxic.

Another potential reason for the lack of mature attachment could be simply due to the perceived shallower depth between each groove on the brand Y implants. This highlights a limitation of this research because a quantitative value of the roughness of each implant was not assessed. The use of a confocal microscope or digital profilometer could give a quantitative value of the micro-roughness average of each implant.

CONCLUSION

Laser-modified titanium implants have the potential for increasing the chances of successful osseointegration. The brand X implant samples that displayed human fetal osteoblast with filopodia infer the possibility of successful osseointegration. Although, the results in our study showed that cell adhesion is possible after 48 hrs in laser-modified titanium, there are some limitations such as the low sample size and lack of statistical analysis to make a definitive conclusion. Additionally, the main reason for the lack of cell maturity and cell adhesion in brand Y samples remains unanswerable, further studies to determine the toxicity of vanadium from Ti6Al4V in an *in-vivo* environment and titanium alloys lasered to a similar roughness of brand X should indicate the reason for the disparity between the two implant brands. The presence of organic impurities in brand X samples should not be overlooked despite good cell adhesion results. Impurities on the surface of titanium implants could potentially be hazardous in an *in-vivo* model resulting in the disruption of the osseointegration process by the rejection of the biomaterial. Nonetheless, there is currently insufficient evidence to link implant failure directly to carbon contaminated implant surfaces.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [DS], upon reasonable request.

FUNDING

The research project was financially supported by Mahidol University and Master of Science program in Oral and Maxillofacial Surgery, Faculty of Dentistry, Mahidol University.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or

otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank all the staff in the Department of Oral and Maxillofacial Surgery, Department of Oral Biology and Research Center of Faculty of Dentistry, Mahidol University.

REFERENCES

- [1] Wennerberg A, Albrektsson T. On implant surfaces: a review of current knowledge and opinions. *Int J Oral Maxillofac Implants* 2010; 25(1): 63-74. [PMID: 20209188]
- [2] Tavares HS, Faeda R, Carlos G, Guastaldi FPS, Oliveira N. *J Osseointegration* 2009; 1: 25-31.
- [3] Cho SA, Jung SK. A removal torque of the laser-treated titanium implants in rabbit tibia. *Biomaterials* 2003; 24(26): 4859-63. [http://dx.doi.org/10.1016/S0142-9612(03)00377-6] [PMID: 14530083]
- [4] Kang SH, Cho SA. Comparison of removal torques for laser-treated titanium implants with anodized implants. *J Craniofac Surg* 2011; 22(4): 1491-5. [http://dx.doi.org/10.1097/SCS.0b013e31821d4d98] [PMID: 21778844]
- [5] Kang NS, Li LJ, Cho SA. Comparison of removal torques between laser-treated and SLA-treated implant surfaces in rabbit tibiae. *J Adv Prosthodont* 2014; 6(4): 302-8. [http://dx.doi.org/10.4047/jap.2014.6.4.302] [PMID: 25177474]
- [6] Romanos G, Crespi R, Barone A, Covani U. Osteoblast attachment on titanium disks after laser irradiation. *Int J Oral Maxillofac Implants* 2006; 21(2): 232-6. [PMID: 16634493]
- [7] Chu S-F, Huang M-T, Ou K-L, *et al.* Enhanced biocompatible and hemocompatible nano/micro porous surface as a biological scaffold for functionalizational and biointegrated implants. *J Alloys Compd* 684: 726-32. [http://dx.doi.org/10.1016/j.jallcom.2016.05.134]
- [8] Subramaniam M, Jalal SM, Rickard DJ, Harris SA, Bolander ME, Spelsberg TC. Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: Bone formation *in vivo* and karyotype analysis using multicolor fluorescent *in situ* hybridization. *J Cell Biochem* 2002; 87(1): 9-15. [http://dx.doi.org/10.1002/jcb.10259] [PMID: 12210717]
- [9] Seriwatanachai D, Krishnamra N, van Leeuwen JPTM. Evidence for direct effects of prolactin on human osteoblasts: Inhibition of cell growth and mineralization. *J Cell Biochem* 2009; 107(4): 677-85. [http://dx.doi.org/10.1002/jcb.22161] [PMID: 19365811]
- [10] Wang M, Chen T, Lu S, *et al.* Osteogenic Comparison on Selective Laser Melting Printed and Sandblasting-Acid-Etching Ti Substrates for Customized Implant Applications. *Sci AdvMate* 2017; 9: 1947-2935. [http://dx.doi.org/10.1166/sam.2017.3012]
- [11] Duddeck D, Maghaireh H, Faber FJ, Neugebauer J. SEM surface analyses of 120 sterile-packed implants. *Clin Implant Dent Relat Res* 2014; 16(6): 817-26. [PMID: 23527899]
- [12] Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *J Bone Miner Res* 1995; 10(2): 178-86. [http://dx.doi.org/10.1002/jbmr.5650100203] [PMID: 7754797]
- [13] Albrektsson T, Dahlin C, Jemt T, Senerby L, Turri A, Wennerberg A. Is marginal bone loss around oral implants the result of a provoked foreign body reaction? *Clin Implant Dent Relat Res* 2014; 16(2): 155-65. [http://dx.doi.org/10.1111/cid.12142] [PMID: 24004092]
- [14] Morra M, Cassinelli C, Bruzzone G, *et al.* Surface chemistry effects of topographic modification of titanium dental implant surfaces: 1. Surface analysis. *Int J Oral Maxillofac Implants* 2003; 18(1): 40-5. [PMID: 12608667]
- [15] Hayashi R, Ueno T, Migita S, *et al.* Hydrocarbon deposition attenuates osteoblast activity on titanium. *J Dent Res* 2014; 93(7): 698-703. [http://dx.doi.org/10.1177/0022034514536578] [PMID: 24868012]
- [16] Delgado-Ruiz RA, Gomez Moreno G, Aguilar-Salvatierra A, Markovic A, Mate-Sánchez JE, Calvo-Guirado JL. Human fetal

- osteoblast behavior on zirconia dental implants and zirconia disks with microstructured surfaces. An experimental *in vitro* study Clin Oral Implants Res 2016; 27(11): e144-53. [http://dx.doi.org/10.1111/clr.12585] [PMID: 25809053]
- [17] Shah FA, Trobos M, Thomsen P, Palmquist A. Commercially pure titanium (cp-Ti) *versus* titanium alloy (Ti6Al4V) materials as bone anchored implants - Is one truly better than the other? Mater Sci Eng C 2016; 62: 960-6. [http://dx.doi.org/10.1016/j.msec.2016.01.032] [PMID: 26952502]
- [18] Steinemann SG. Titanium--the material of choice? Periodontol 2000 1998; 17: 7-21. [http://dx.doi.org/10.1111/j.1600-0757.1998.tb00119.x] [PMID: 10337309]
- [19] Okazaki Y, Gotoh E, Manabe T, Kobayashi K. Comparison of metal concentrations in rat tibia tissues with various metallic implants. Biomaterials 2004; 25(28): 5913-20. [http://dx.doi.org/10.1016/j.biomaterials.2004.01.064] [PMID: 15183605]

© 2020 Khoo *et al.*

This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International Public License (CC-BY 4.0), a copy of which is available at: (<https://creativecommons.org/licenses/by/4.0/legalcode>). This license permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.