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Quantitative Evaluation of NFE2L2/NRF2 and Cytokine Response Induced by Porphyromonas Gingivalis Lipopolysaccharide and Nicotine in a Titanium-Oral Mucosa Model

Porphyromonas Gingivalis Lipopolisakkarit ve Nikotin Tarafından Uyarılan Titanyum-Ağız Mukoza Modelinde NFE2L2/NRF2 ve Sitokin Yanıtının Kantitatif Olarak Değerlendirilmesi

Gökhan KASNAK[a], Mervi GÜRSOY[b,c], Eija KÖNÖNEN[a], Erhan FIRATLI[d], Stina SYRJÄNEN[b,e], Ulvi Kahraman GÜRSOY[b]

[a]İstanbul Kent University Faculty of Dentistry, Department of Periodontology, İstanbul, Türkiye
[b]Turku University Institute of Dentistry, Turku, Firland
[c]City of Turku Social Welfare Division, Department of Oral Health, Turku, Finland
[d]İstanbul University Faculty of Dentistry, Department of Periodontology, İstanbul, Türkiye
[e]Turku University Hospital, Clinic of Pathology, Turku, Finland

ABSTRACT Objective: Titanium can alter peri-implant mucosa cytokine response to microbiological and chemical stresses. This work used organotypic titanium-oral mucosa model to evaluate oxidant resistance and cytokine response to *Porphyromonas gingivalis* lipopolysaccharide (Pg LPS) and nicotine. **Material and Methods:** Seeding gingival keratinocytes on collagen gels with fibroblasts and adding titanium grade (Ti-Gr) 4, Ti-Gr 5, or hydroxyapatite discs developed an organotypic titanium-oral mucosa model. Pg LPS (1 l/mL), nicotine (1.54 mM), or both were given to the model. The Luminex® xMAPTM technology measured interleukin (IL)-1β, IL-1Ra, IL-8, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor levels in culture media. Cultured tissues were paraffin-blocked and immunohistochemistry was used to measure nuclear factor erythroid 2 like 2 (NFE2L2/NRF2), 8-hydroxyguanosine (8-OHdG), and Parkinsonism-associated deglycase (PARK7/DJ-1). Statistical analysis included one-way analysis of varianceand Tukey's HSD. **Results:** Nicotine and Pg LPS+nicotine increased IL-1β and IL-1Ra secretions in Ti-Gr 4, Ti-Gr 5, and hydroxyapatite groups ($p<0.01$). Pg LPS alone ($p<0.01$) and in conjunction with nicotine ($p=0.021$) enhanced MCP-1 production, but nicotine alone lowered it ($p=0.024$). IL-8 concentrations rose in all test groups ($p<0.01$). All groups treated with nicotine, LPS, or both showed increased 8-Hydroxydeoxyguanosine and NFE2L2/NRF2 immunostainings ($p<0.01$), whereas PARK7/DJ-1 expression remained unchanged. When assessed without inducers, Ti-Gr 4, Ti-Gr 5, or hydroxyapatite groups showed no significant variations in cytokine secretion patterns, NFEL2/NRF2, 8-OHdG, or PARK7/DJ-1 immunostainings. **Conclusion:** Titanium did not affect cytokines or oxidant resistance molecules in our organotypic model without inducers. However, Pg LPS, nicotine, and their mixtures enhance cytokines and oxidative resistance molecules.

Keywords: Cell culture, three dimensional; keratinocyte; fibroblast; porphyromonas gingivalis lipopolysaccharide; nicotine

ÖZET Amaç: Peri-implant mukozası, mikrobiyal ve kimyasal uyaranlara karşı çeşitli sitokinleri salgılamaktadır. Titanyum varlığı ise dokunun bu yanıtını değiştirebilmektedir. Bu çalışmada, organotipik titanyum-ağız mukozası modeli kullanarak *Porphyromonas gingivalis* lipopolisakkaridi (Pg LPS) ve nikotine karşı oksidatif direnç ve sitokin yanıtının incelenmesi amaçlanmıştır. **Gereç ve Yöntemler:** Organotipik model, fibroblast içeren kolajen jelin üzerine gingival keratinositlerin ekilmesini takiben üzerlerine titanyum grade (Ti-Gr) 4, Ti-Gr 5 ve hidroksiapatit disklerin yerleştirilmesi ile oluşturuldu. Uyaran olarak Pg LPS (1 l/mL), nikotin (1,54 mM) veya bileşimleri (Pg LPS+nikotine) modellere uygulandı. Kültür ortamındaki interlökin (IL)-1β, IL-1 reseptör antagonisti (IL-1Ra), IL-8, monosit kemoatraktan protein [Monocyte chemoattractant protein-1 (MCP-1)] ve vasküler endotelyal büyüme faktörünü seviyeleri Luminex® xMAPTM yöntemi ile ölçüldü. Kültüre edilen örnekler parafin bloklara alınarak, nükleer faktör, eritroid 2 benzeri 2 (NFE2L2/NRF2), 8-hidroksiguanozin (8-OHdG) ve Parkinsonizm ile ilişkilendirilen deglikaz [Parkinsonism-associated deglycase (PARK7/DJ-1)] protein ekspresyonları immünohistokimya ile analiz edildi. İstatistiksel analiz için tek yönlü varyans analizi ve Tukey's HSD testi kullanıldı. **Bulgular:** Ti-Gr 4, Ti-Gr 5 ve hidroksiapatit gruplarında, nikotin ve Pg LPS+nikotin IL-1β ve IL-1Ra ekspresyonunu arttırdığı görüldü ($p<0,01$). Pg LPS tek başına ($p<0,01$) ve nikotin ile kombinasyon halinde ($p=0,021$) uygulandığında MCP-1 seviyesinde artış gözlemlenirken, tek başına nikotin varlığında MCP-1 seviyesinde azalma görüldü ($p=0,024$). Tüm test gruplarında IL-8 konsantrasyonlarının arttığı tespit edildi ($p<0,01$). Nikotin, Pg LPS veya bunların kombinasyonun uygulandığı tüm gruplarda 8-OHdG ve NFE2L2/NRF2 ait immün boylamalar daha yoğun iken ($p<0,01$), PARK7/DJ-1eşit düzeyde gözlemlendi ($p>0,05$). Her hangi bir uyaran olmadığında Ti-Gr 4, Ti-Gr 5 veya hidroksiapatit grupları arasında sitokin seviyeleri ve NFEL2/NRF2, 8-Hidroksideoksiguanozin veya PARK7/DJ-1 immün boyamalarında herhangi bir fark bulunamadı ($p>0,05$). **Sonuç:** Organotipik titanyum-ağız mukozası modelinde, titanyumun tek başına gingival keratinosit ve fibroblastları etkileyerek sitokin yanıtı uyaramadığı ve oksidatif direnç moleküllerinin artışına neden olmadığı, ancak Pg LPS, nikotin ve kombinasyonlarının varlığında sitokin yanıtını uyardığı ve oksidatif direnç moleküllerinin ortaya çıkmasına neden olduğu çalışmamızca gösterilmiştir.

Anahtar Kelimeler: Hücre kültürü, üç boyutlu; keratinosit; fibroblast; porphyromonas gingivalis lipopolisakkaridler; nikotin

Correspondence: Gökhan KASNAK
İstanbul Kent University Faculty of Dentistry, Department of Periodontology, İstanbul, Türkiye
E-mail: gkasnak@gmail.com

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Peri-implant mucositis is a reversible inflammatory condition that affects only the soft tissue around dental implants and, if not treated, might lead to the formation of peri-implantitis.[1] Several risk factors, such as bacterial and chemical insults, take part in the formation of peri-implant mucositis.[2] Bacteria are capable of initiating multiple signaling pathways, including the toll, protein kinase, and complement pathways, which subsequently lead to the synthesis of ROS.[3] Reduced levels of ROS play a crucial role in the intracellular signaling cascade.[4] Conversely, an excessive buildup of ROS produced by pathogenic bacteria leads to oxidative stress, which in turn damages the tissue of the oral mucosa by breaking down its structural components.[3] The biomarker known as 8-Hydroxydeoxyguanosine (8-OHdG) exhibits a high degree of sensitivity in detecting oxidative stress, since it has the ability to accurately indicate even minimal levels of oxidative DNA damage. One potential indication of periodontal tissue breakdown is the observation of an elevated amount of 8-OHdG in both saliva and gingival crevice fluid.[4] In addition to this, the incidence of peri-implant tissue problems is heightened in individuals who smoke, which might also be related to nicotine as an accepted source of exogenous ROS.[1,2,5]

Peri-implant mucosa forms the first barrier against physical, chemical, and microbiological insults, and its integrity is vital for long-term success in implant treatment.[6] Keratinocytes and fibroblasts, which are the cells found in this barrier, regulate the inflammatory response by secreting interleukin-1 (IL-1) and IL-1-receptor antagonist (IL-1Ra). interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) are also involved in the regulation of neutrophil and macrophage migration.[7] Additionally, they regulate the growth of endothelial cells by secreting vascular endothelial growth factor.[8] Moreover, previous studies have shown evidence that titanium had the ability to alter the immune response in human gingival fibroblasts through its chemical composition and surface properties.[9] There is currently a lack of empirical data about the impact of titanium on the inflammation of the peri-implant mucosa, which is caused by ROS.

The interactions between titanium and oral mucosa cells have been generally studied on monolayer cell culture models in which cells have been seeded on titanium discs.[10] Although it is possible to obtain consistent and significant results from these in-vitro experiments, they are constrained by the lack of a polarized cell phenotype, insufficient cell differentiation, and limited cell-to-cell interaction.[11] Thus, an organotypic titanium oral mucosa model, which contains both oral keratinocytes and fibroblast cells, can be useful for representing the cellular activities of the peri-implant mucosa.[11] Furthermore, it has been established that three-dimensional cell culture models are preferable to monolayer cell culture models in in-vitro oral research.[12] The main objective of this investigation is to examine the cytokine response, namely IL-1b, IL-1Ra, IL-8, MCP-1, and VEGF, in response to oxidative stress caused by bacterial *Porphyromonas gingivalis* lipopolysaccharide (Pg LPS) and chemical (nicotine) assaults in an organotypic titanium oral mucosa model. Plus, an evaluation was conducted to examine the impact of titanium on the modulation of cytokine activity.

MATERIAL AND METHODS

CONSTRUCTION OF THE ORGANOTYPIC TITANIUM ORAL MUCOSA MODEL

The 3D organotypic model resembled the structure of native oral mucosal tissue. It was created by using commercially obtained gingival keratinocytes and fibrobroblasts, which consisted of three compartments: a collagen matrix at the base, seeded keratinocytes above the base, and the disc placed on top.[12-14] The organotypic oral mucosa-titanium model was constructed in four steps. In the first step, gingival keratinocytes were grown in a medium that had keratinocyte-serum-free medium and antibiotics added (penicillin-streptomycin, 10,000 U/mL, Gibco, Thermo Fisher Sci. Co., Waltham, MA, USA) at 37 °C with a 5% CO2 concentration. Similarly, gingival fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C and 5% CO2 concentration. Both cell types were passaged on a weekly basis until they reached a confluence of 80-90%. Secondly, to create the collagen matrix, the gingival fibroblast cells (passages 15 to 20) were suspended in a collagen solution (PureCol, Advanced BioMatrix, Tucson, AZ, USA)

mixed with DMEM at a density of $3x10^5$/mL. This suspension was then plated into culture inserts (10 mm diameter, 3 mm pore size, ThinCert, Grenier Bio-One, Monroe, NC, USA). Subsequently, the inserts were meticulously put in 12-well tissue cultivation plates (Corning Inc., Corning, NY, USA) and allowed to harden for a duration of 24 hours. Thirdly, the gingival keratinocytes were cultured on collagen-fibroblast gels at a density of $8x10^5$/mL. Finally, when the cells had reached confluence after a seven-day period, inserts were positioned on metal grids to establish the air-liquid interface. After the two days of epithelial cells grown commercially available polished hydroxyapatite (HA, Clarkson Chromatography Inc., South Williamsport, PA, USA), machined surface titanium grade (Ti-Gr) 4 (Cp-Ti, contains 0.4% oxygen, Servo Dental GmbH & Co. KG, Hagen, Germany), and Ti-Gr 5 (Ti-6Al-4V, includes 0.2% oxygen, Implance Dental Implant System Ltd., Trabzon, Türkiye), discs with a height of 2 mm and a diameter of 5 mm were placed on top of the cultures, and the models were allowed to grow for an additional 14 days in antibiotic-free Green's medium for the formation of multi-layered epithelium around the discs. The medium was replaced on a triweekly basis (Figure 1).

In the current study, the experiment involving the cultivation of cell lines did not require any kind of ethical permission because the research was conducted solely through the utilization of cell passages rather than primary cells.

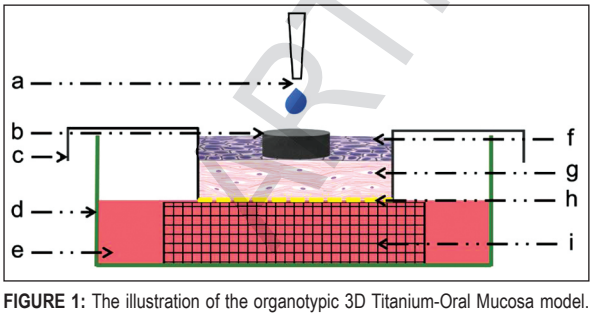

FIGURE 1: The illustration of the organotypic 3D Titanium-Oral Mucosa model. After the confluence of epithelial cells grown for two days commercially available titanium and hydroxyapatite disks were placed on the top. After a 14-day incubation period, the models were treated with 1µl/mL Porphyromonas gingivalis lipopolysaccharide (Pg LPS), 1.54 mM nicotine (NIC), or their combinations for 24 hours. **a)** Stimulant (Pg LPS or NIC or Pg LPS+NIC) **b)** Commercially available titanium/hydroxy apatite disks **c)** Insert **d)** 12-well plate **e)** Medium **f)** Gingival keratinocyte monolayer **g)** Collagen layer with gingival fibroblasts h) Air/liquid interface **i)** Metal grid

STIMULATION OF THE ORGANOTYPIC TITANIUM ORAL MUCOSA MODEL AND SAMPLE COLLECTION

At the end of incubation (14-day) of the organotypic oral mucosa Pg LPS (1µl/mL, tlrl-pglps, InvivoGen, San Diego, CA, USA), nicotine (1.54 mM, N0267, Sigma Aldrich, St. Louis, MO, USA) and their combination (Pg LPS+nicotine) were applied to the top of each inserted disc. In control cultures only phosphate-buffered saline was added. The administration quantities of nicotine and Pg LPS were calculated in accordance with the research of Kasnak et al.[15,16] The inserts were extracted from the 12-well plates after a 24-hour incubation period, and the discs were carefully removed. Cytokine analysis was conducted by collecting and storing the medium from each well in a freezer at a temperature of -80 °C. The oral mucosa samples of organotypic titanium were subjected to fixation in a 10% buffered formalin solution for a duration of 16 hours. Subsequently, the samples were meticulously washed with tap water. Each sample underwent dehydration for 2x30 minutes by gradually increasing the concentration of ethanol. Following a brief xylene cleaning process, all samples were bisected, and the exposed sides of each division were aligned for paraffin embedding.

ANALYSIS OF CYTOKINE LEVELS

The determination of IL-1β, IL-1Ra, IL-8, MCP-1, and vascular endothelial growth factor (VEGF) levels in the culture media of the organotypic titanium oral mucosa models was conducted using the Luminex® xMAP™ technique (Luminex Corporation, Austin, TX). Commercially accessible kits (pro-human cytokine group I assays; Bio-Rad, Santa Rosa, CA) were employed for this purpose. The assay detects IL-1β at a level of 0.6 pg/mL, IL-1Ra at 5.5 pg/mL, IL-8 at 1.0 pg/mL, MCP-1 at 1.1 pg/mL, and VEGF at 3.1 pg/mL. Just the elute and medium were used to determine the background absorbance. The determination of the detection limit involved identifying the analytical level at which the detected fluorescence intensity signal surpassed the background signal by a margin of two standard deviations.

IMMUNOHISTOCHEMICAL EXAMINATION OF THE ORGANOTYPIC TITANIUM ORAL MUCOSA MODEL

Immunohistochemistry was performed by cutting sections of paraffin blocks with a thickness of 5 µm. Cytokeratin (M0821, DAKO, Glostrup, Denmark) and vimentin (M7020, DAKO) antibodies were employed for the purpose of localizing epithelial and mesenchymal tissue. To detect ROS, the tissue samples were subjected to immunostaining using 8-OHdG (SC 393870, Santa Cruz Biotechnology, Dallas, TX, USA). Additionally, the anti-oxidative response was identified by immunostaining with nuclear factor erythroid 2 like 2NFE2L2/NRF2 (SC 722, Santa Cruz Biotechnology, Dallas, TX, USA) and Parkinsonism-associated deglycase (PARK7/DJ-1) (SC 32874, Santa Cruz Biotechnology, Dallas, TX, USA). The usual procedures were employed to perform immunohistochemical stainings for all markers utilizing an automated immunostainer (TechMate, DAKO). Cytokeratin, vimentin, and PARK7/DJ-1 antibodies were diluted at a ratio of 1:50, whilst NFE2L2/NRF2 and 8-OHdG antibodies were diluted at a ratio of 1:75. In summary, the antigen was extracted using a microwave for 5 minutes twice in a citrate buffer with a concentration of 1 mol/L and a pH of 6.0. This was then followed by the inhibition of endogenous peroxidase using 3% H2O2. The biotinylated secondary antibody (Dako REAL™ Detection System, K5001, DAKO) was used to detect the primary antibody. This antibody was coupled with streptavidin-horseradish peroxidase and visualized using 3,3'diaminobenzidine tetrahydrochloride in HRP buffer (Dako REAL™ Detection System, K5001, Dako, Glostrup, Denmark).

To assess the immunostainings the regions of interest (ROI) were first determined under microscopy. After this, all ROIs, either left or right contact areas to the discs with unimpaired cell integrity, were scanned using original magnifications ranging from 20X to 100X, and high-resolution pictures were taken. Immunopositivity was graded into three categories: non-stained, moderately stained, and fully stained, based on the intensity of the overall staining for 8-OHdG, PARK7/DJ-1, and NFE2L2/NRF2 to evaluate the ratio of positively stained cells. The validation of the staining intensity of keratinocytes and fibroblasts in the organotypic oral mucosal model was conducted using the ImageJ software (version 1.46c; Rasband WS, National Institutes of Health, Bethesda, MD, USA) with the immunohistochemical image analysis toolbox plugin version 2 (National Institutes of Health, Bethesda, MD, USA). In a prior investigation, our research team demonstrated the effectiveness of the stains through experimentation on human tissues.[16]

STATISTICAL ANALYSIS

Utilizing the software IBM SPSS Statistics for Windows V24.0, which was developed by IBM Corp. in Armonk, New York, USA, statistical analysis was performed. The statistical analysis utilized a one-way analysis of variance in conjunction with Tukey's HSD. P-values less than 0.05 were regarded as indicating statistical significance.

RESULTS

The characterization of the oral mucosa in organotypic titanium model by vimentin, and cytokeratin immunostainings and the surface characteristics were presented as supplementary figures (Figure 1, Figure 2). Induction of oxidative stress by Pg LPS, nicotine, and Pg LPS+nicotine is demonstrated with 8-OHdG immunostainings; the response to those oxidative stimulants is validated with NFE2L2/NRF2 and PARK7/DJ-1 immunostainings. 8-OHdG and NFE2L2/NRF2 stainings were substantially more intense in all disc types after nicotine exposure than in the nontreated controls (8-OHdG stainings p=0.028 for hydroxyapatite, p=0.040 for Ti-Gr 4, and p=0.045 for Ti-Gr 5; NFE2L2/NRF2 stainings p=0.033 for hydroxyapatite, p=0.39 for Ti-Gr 4, and p=0.045 for Ti-Gr 5). Likewise, the combined administration of nicotine and Pg LPS resulted in a significant increase in the 8-OHdG and NFE2L2/NRF2 immunopositivity in all samples derived organotypic titanium-oral mucosa models. The significances of the differences in immunopositivity between exposed and none-exposed samples were as follows: 8-OHdG immunostainings p=0.047 for hydroxyapatite, p=0.038 for Ti-Gr 4, and p=0.042 for Ti-Gr 5; NFE2L2/NRF2 stainings p=0.046 for hydroxyapatite, p=0.048 for Ti-Gr 4, and p=0.041 for Ti-Gr 5). On the other hand, the application only with Pg LPS resulted in an in-

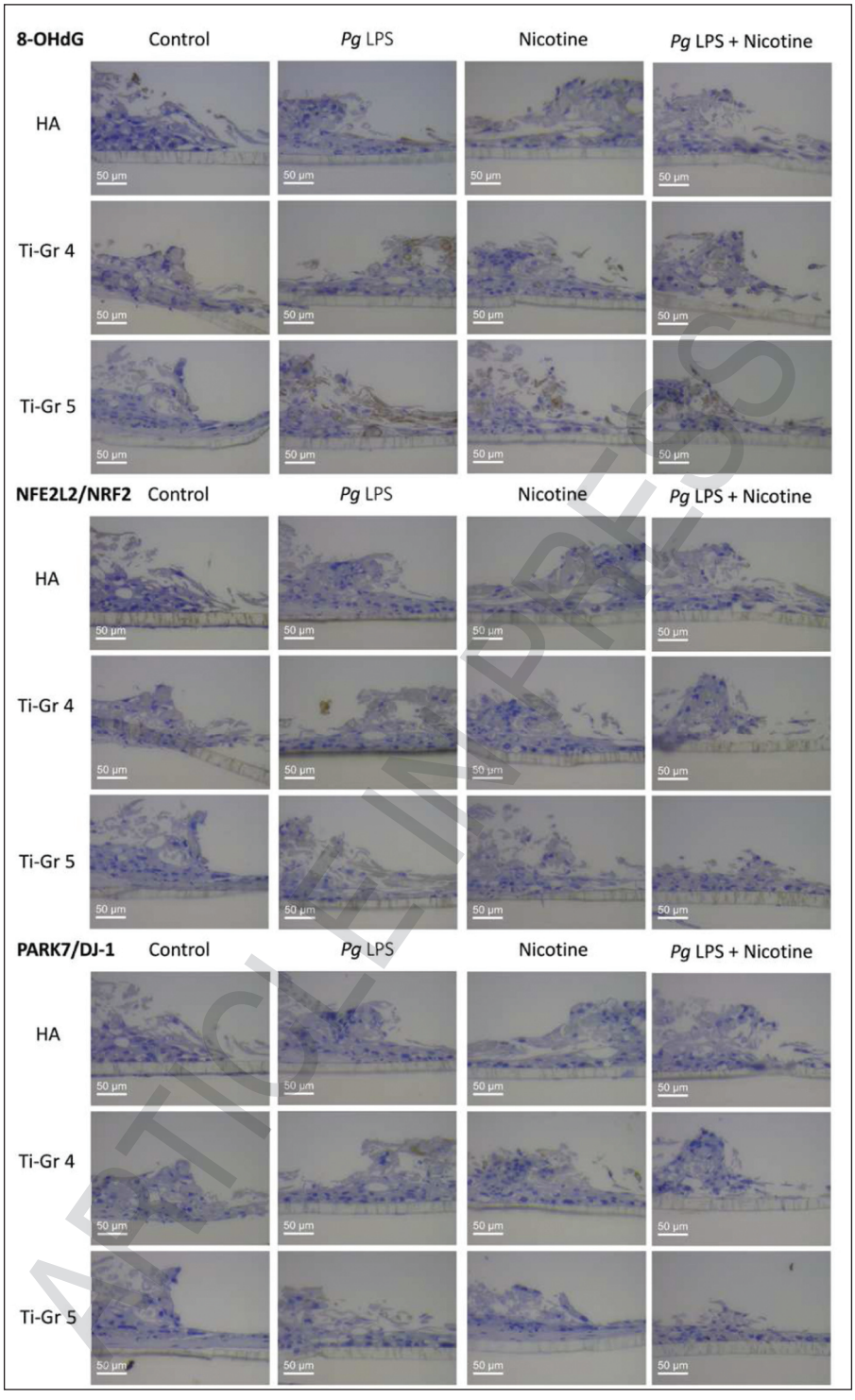

FIGURE 2: Images (50X magnification) of titanium-oral mucosal models immunostained with NFE2L2/NRF2, 8-OHdG, and PARK7/DJ-1 antibodies in the absence or presence of irritants (LPS of Porphyromonas gingivalis, nicotine, and their combination).
8-OHdG:8-Hydroxydeoxyguanosine; Ti-Gr: Titanium grade; NFE2L2/NRF2; Nuclear factor erythroid 2 like 2; Pg LPS: Porphyromonas gingivalis lipopolysaccharide; PARK7/DJ-1: Parkinsonism-associated deglycase.

crease intensity of 8-OHdG immunostaining in all of the test samples ($p<0.01$ for hydroxyapatite, $p<0.01$ for Ti-Gr 4, and $p<0.01$ for Ti-Gr 5), but no difference was observed in the staining levels of

NFE2L2/NRF2 (p>0.05 for all disc types). Regardless of the stimulant, the PARK7/DJ-1 immunopositivity did not reveal significant differences in any of the specimens (Figure 2 and Figure 3).

According to our cytokine analyses, nicotine significantly increased the levels of IL-1β (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5), IL-1Ra (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5), and IL-8 (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5) in all groups in comparison to their controls. Similarly, the combinational application of Pg LPS and nicotine elevated IL-1β (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5), IL-1Ra (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5), IL-8 (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5) and MCP-1 (p=0.021 for hydroxyapatite, p=0.018 for Ti-Gr 4, and p=0.02 for Ti-Gr 5) levels significantly. Nicotine inhibited the secretion of MCP-1 (p=0.024 for hydroxyapatite, p=0.021 for Ti-Gr 4, and p=0.01 for Ti-Gr 5). At the same time, the Pg LPS increased its secretion in all test groups (p<0.01 for hydroxyapatite, Ti-Gr 4, and Ti-Gr 5). Neither alone nor combinational application of Pg LPS and nicotine did not induce any change in VEGF levels (Figure 4).

Additionally, between-group comparisons were performed based on the disc type, and no difference was observed for any cytokine levels and 8-OHdG, NFE2L2/NRF2, and PARK7/DJ-1 immunostainings (Figure 3, Figure 4).

DISCUSSION

The role of soft tissue surrounding dental implants has been examined using several models. Furthermore, previous research has investigated the impact of titanium's surface topography and chemical structure on the peri-implant mucosa. To date, there is a lack of research investigating the effects of oxidative stress caused by bacteria and nicotine on the control of cytokines in the organotypic titanium oral mucosa model. This work presents empirical findings that demonstrate the considerable increase in levels of IL-1Ra, IL-1β, and IL-8 following treatment with Pg LPS, nicotine, or a combination of both. Conversely, nicotine alone was shown to reduce MCP-1 levels, while Pg LPS was observed to boost it in the organotypic titanium oral mucosa model. Additionally, we have successfully shown that there is no significant distinction between the machined surface Ti-Gr 4 and Ti-Gr 5 in relation to their impact on the modification of these cytokines.

Oral keratinocytes, together with underlying fibroblasts, are acknowledged as the primary defensive mechanism against bacterial and environmental threats, such as nicotine, within the peri-implant soft tissue and its response is similar to healthy gingiva.[6,17] The current investigation examined the cytokine response of gingival keratinocytes and fibroblasts in an organotypic titanium oral mucosa model, specifically in response to Pg LPS and nicotine-induced ROS.

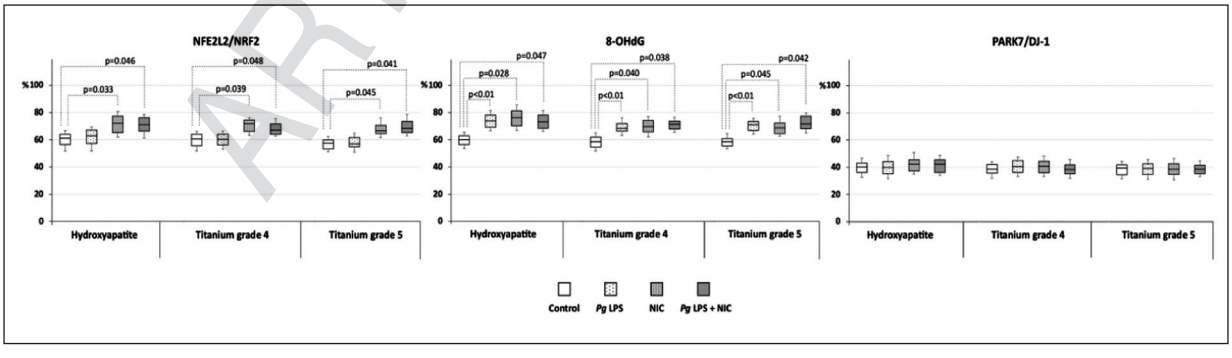

FIGURE 3: A comparison of the immunostaining intensities of NFE2L2/NRF2, 8-OHdG, and PARK7/DJ-1 between the control and the organotypic models that were exposed to Pg LPS, NIC, or a combination of the two. The results are given as a bar graph. p values above the bars represents the level of statistical significance in digitally analysed immunopositivity when compared the exposured models with controls.

NFE2L2/NRF2; Nuclear factor erythroid 2 like 2; 8-OHdG:8-Hydroxydeoxyguanosine; PARK7/DJ-1: Parkinsonism-associated deglycase; Pg LPS: Porphyromonas gingivalis lipopolysaccharide; NIC: Nicotine.

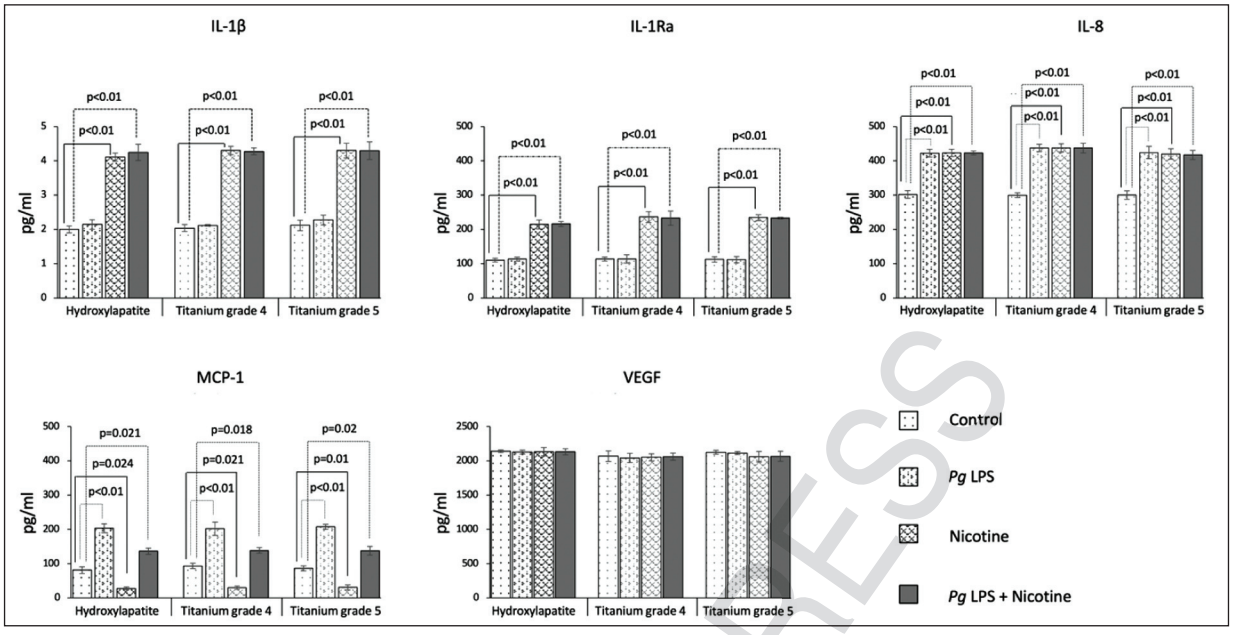

FIGURE 4: The secreted concentrations of IL-1β, IL-1Ra, IL-8, MCP-1, and VEGF (pg/mL) in the culture media of the organotypic titanium oral mucosa models were illustrated as a bar graph. The significant differences between exposured and nonexposured cultures were indicated with p values above the bars.
IL: Interleukin; IL-1Ra: Interleukin-1receptor antagonist; MCP-1: Monocyte Chemoattractant Protein-1; VEGF: Vascular endothelial growth factor; Pg LPS: Porphyromonas gingivalis lipopolysaccharide.

Human and animal studies have established the importance of the peri-implant soft tissue.[18,19] However, these study models might have some restrictions due to ethical considerations and complexities in the study protocol. Cell cultures are extensively employed in the evaluation of the biological impact of various substances, but monolayer structure with only one cell type do not allow the mimic of the *in-vivo* situation.[20,21] Hence, in order to address these constraints, researchers have employed organotypic oral mucosa models that consist of many layers of epithelial cells.[12] Moreover, the confluence of the epithelial cells, basement membrane, and air are crucial for the modulation of epithelial cell differentiation, which maintains an advantage for the organotypic oral mucosa models over the other in-vitro protocols.[22] Based on these facts, Chai WL. et al. have introduced an organotypic oral mucosa model to explore the implant-soft tissue interface.[11,12] The application of Pg LPS, nicotine, and their combination to induce oxidative stress in the organotypic titanium oral mucosa model was a novel approach in this study. On the other hand, the short-term culture protocol, 24 hours, was a study limitation. Despite the inherent limitations of cell culture models, such as the inability to replicate dento-gingival or implant-oral mucosal apparatus, our study may be limited by the fact that we solely applied Pg LPS. LPSs from various periodontopathogens, including the briding bacteria *Fusobacterium nucleatum*, may potentially have a significant impact on the development and progression of periodontal and periimplant diseases. Consequently, the building of biofilms or the implementation of LPS mixtures could be helpful for future research.

The role of ROS in periodontal disease has been extensively investigated.[22,23] It has been shown that a rise in ROS levels can be caused by metal ions, which contributes to the activation of fibroblasts and osteoclasts, causes osteolysis surrounding dental implants.[24] Up to date, only in one study, the salivary concentrations of the oxidative biomarkers evaluated in peri-implantitis patients, and it has been reported that total saliva would not be useful to quantify the oxidative damage in peri-implantitis.[25] In the present study, the elevated levels of ROS indicated with the

presence of 8-OHdG and the application of nicotine, Pg LPS, and the combination of these factors result in an elevation of IL-1β, IL-8, and IL-1Ra levels. Notably nicotine inhibited the expression of MCP-1, whereas in the presence of Pg LPS, alone or with nicotine, its levels were elevated, and this observation may suggest that the observed effect was contingent upon Pg LPS more than nicotine. Prior researches have substantiated the presence of heightened levels of IL-8 and MCP-1 subsequent to the incubation of gingival keratinocytes and fibroblasts with *P. gingivalis*.[8,26] Contradictory to that , any change in the IL-8 expression levels in nicotine exposed gingival epithelial cells was not observed by Johnson et al.[27] This might be related to the different methodology; Johnson et al., used gingival epithelial cells as monolayers our organotypic titanium oral mucosa model, which contains both gingival keratinocytes grown on collagen gel containing fibroblasts.[27] Fitzsimmons et al., showed elevated IL-8 and MCP-1 secretions from gingival fibroblasts when incubated with Pg LPS but not that of VEGF which is in line with our results.[8,9]

It is well known that not only *P. gingivalis* is capable of initiating the inflammatory-dependent oxidative response, but also nicotine causes the formation of oxidative stress in several types of oral cells, including gingival keratinocytes and fibroblasts.[28] In the current study, we found that all of the test groups had a significantly higher number of 8-OHdG-stained cells than their respective controls, which is in line with the literature.[29] On the other hand, also the evaluation of the levels of NFE2L2/NRF2, the primary controller of anti-oxidative response, in gingival keratinocytes and fibroblasts might be helpful in understanding the role of the response mechanism against the ROS.[30] To our knowledge, we are the first to study the anti-oxidative response in a titanium-oral mucosa model. Our results showed that the treatment of nicotine and its combination with Pg LPS induced protein expression of NFE2L2/NRF2 in the organotypic model. However, the exposure with Pg LPS only did not stimulate NFE2L2/NRF2 levels. The fact that the antioxidant response against Pg LPS can be controlled by mechanisms other than NFE2L2/NRF2 is one possible explanation for this phenomenon. According to Wang et al. findings, *P. gingivalis* has the ability to activate the forkhead box-O gene, which plays a role in the transcription of antioxidants in gingival epithelial cells.[31] Regarding the levels of PARK7/DJ-1, we did not find any significant differences between the test group and the control group. The in-vitro study may have certain inherent weaknesses, one of which might be responsible for this outcome.

The cruciality of the interaction between peri-implant mucosa and the dental implant abutment for a long-term successful treatment outcome has been recently reviewed in detail.[32] The adhesion, migration, proliferation, and differentiation of cells (epithelial, fibroblast, osteoblast, and macrophage) may be influenced by changes in the topography, chemistry surface energy, and hydrophilicity of titanium, either individually or in combination. These alterations may occur through modifications in cytokine regulation.[9] Another important finding of the present study was that Ti-Gr 4 (C: 0.08%, N: 0.05%, O: 0.4%, Fe: 0.5%, H: 0.015%, and Ti: balance) and Ti-Gr 5 (C: 0.08%, N: 0.05%, O: 0.2%, Fe: 0.3%, H: 0.015%, Al: 6.75%, V: 4.5%, Y: 0.005%, and Ti: balance) even have different compositions resulted in similar pattern of cytokine release after Pg LPS and nicotine exposures. Furthermore, the effects of Ti-Gr 4 and 5 on cytokine expression in both stimulated and non-stimulated organotypic oral mucosa models are shown to be insignificant when compared to the organotypic oral mucosal model with hydroxyapatite. Our findings suggest that the cytokine expression in the peri-implant mucosa is mostly influenced by *P. gingivalis* and/or nicotine-induced ROS, rather than the type of titanium used. However, due to the complexity of the immune system, even a very minor alteration in the secretion of a cytokine may have biological importance in vivo. The majority of the studies related to cytokine expression mainly focused on the surface properties of the titanium and investigated its effect on different cell types but not gingival keratinocytes. Up to our knowledge, no data compare the modifying effect of Ti-Gr 4 and Ti-Gr 5 on cytokine secretion. Previous studies have shown evidence indicating that titanium elicits the synthesis of pro-inflammatory cytokines, including IL-1b, IL-6,

and tumor necrosis factor-alpha (TNF-a), within human macrophages.[33] According to Bruni et al., Ti-Gr 5 stimulated TNF-a secretion in the cultured human umbilical endothelial cells.[34] The primary reason for the discrepancy between our results and those of other researchers may be based on the selection of different cell lines and the design of the cell culture model. In our study, we examined the impact of titanium on oral gingival keratinocytes and fibroblasts, while Bruni et al. selected umbilical cord vein endothelial cells, and Östberg et al. used neutrophils.[33,34] Changes in surface properties may have been another reason for the inconsistent results. Andrukhov et al.'s study, similar to ours, might provide an answer to this.[35] They compared gingival fibroblasts' cytokine expression between smooth and rough surfaces, as well as between titanium and zirconia surfaces. According to their findings, surface characteristics had only a minor effect on gingival fibroblasts' secretion of IL-8. Thus, newly developed titanium alloys and surface treatments should be tested in further studies

CONCLUSION

In conclusion, exposure with Pg LPS and nicotine resulted in elevated IL-1b, IL-Ra, and IL-8 secretions by inducing ROS in the organotypic titanium oral mucosa model. On the other hand, MCP-1 secretion declined by nicotine but increased by Pg LPS. Importantly, also the cytokine secretion pattern was similar for the two machined surfaces of Ti-Gr 4 and 5.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Gökhan Kasnak, Ulvi Kahraman Gürsoy;* ***Design:*** *Gökhan Kasnak, Ulvi Kahraman Gürsoy;* ***Control/Supervision:*** *Eija Könönen, Stina Syrjänen, Ulvi Kahraman Gürsoy;* ***Data Collection and/or Processing:*** *Gökhan Kasnak, Mervi Gürsoy;* ***Analysis and/or Interpretation:*** *Gökhan Kasnak, Mervi Gürsoy, Ulvi Kahraman Gürsoy;* ***Literature Review:*** *Gökhan Kasnak;* ***Writing the Article:*** *Gökhan Kasnak;* ***Critical Review:*** *Mervi Gürsoy, Eija Könönen, Erhan Fıratlı, Stina Syrjänen, Ulvi Kahraman Gürsoy;* ***References and Fundings:*** *Gökhan Kasnak, Ulvi Kahraman Gürsoy;* ***Materials:*** *Ulvi Kahraman Gürsoy.*

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