

[Original]

Effect of Nickel and Titanium on DNA Methylation in Human Gingival fibroblast cells In Vitro

Dedy ARIWANSA^{1,2}, Koki YOSHIDA¹, Tetsuro MORIKAWA¹, Jun SATO¹, Maiko OTOMO³, Masato SAITOH³, Takashi NEZU⁴, and Yoshihiro ABIKO^{1*}

¹Division of Oral Medicine and Pathology, Department of Human Biology and Pathophysiology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan

²Faculty of Dentistry, Hasanuddin University, Makassar, South Sulawesi, Indonesia

³Division of Pediatric Dentistry, Department of Oral Growth and Development, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan

⁴Division of Biomaterials and Bioengineering, Department of Oral Rehabilitation, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan

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Abstract

Metallic dental materials are frequently used in clinics. Cytotoxicity and allergy tests in vitro and in vivo have primarily been used to evaluate their safety. However, whether these dental materials cause genetic modifications leading to pathological conditions in oral tissues has not been shown. Gene mutations and epigenetic alterations are the initial steps of genetic modification. Environmental factors, including chemical components and mechanical stimulations, can cause epigenetic modifications that are often involved in pathogenic changes in humans. Therefore, we hypothesize that dental materials induce specific alterations in DNA methylation. This study aims to demonstrate specific alterations in DNA methylations in human gingival fibro-

blast cells (HGnF) induced by metal components (Ni and Ti) of dental materials. The mRNA expression level of IL-6 for 2 weeks was significantly higher in the HGnF cells treated with 50 μ M of Ni and 10 μ M of Ti compared to the controls. Quantitative methylation-specific PCR (qMSP) revealed that the DNA methylation percentage levels of IL-6 and IFN- γ in HGnF cells treated with 10 μ M of Ti for 2 weeks were significantly lower compared to the controls. In conclusion, this study found hypomethylation of IL-6 and IFN- γ , followed by their upregulated expressions in HGnF cells stimulated with Ti. Methylation levels may serve as a new cytotoxic assessment tool, even for biocompatible materials.

Introduction

Metallic materials have been used in dentistry for a long time. They are commonly used in dental implants, orthodontic appliances, crown prostheses, and plates and screws for reconstructing jaw defects and guiding tissue regeneration. The oral cavity is a complex environment with inorganic ions, proteins, bacteria, and other organic components that can significantly impact the performance of dental metals. Dental metals can undergo such as corrosion, discoloration, bacterial adhesion, and adverse reactions, affecting their biological safety and efficacy in clinical applications (He L et

al., 2021). To assess the biological safety of metals, various in vitro assays have been utilized, including cell viability tests, cytostatic tests, cell cycle analysis, apoptosis detection, and oxidative stress assessment (Avior O et al., 2002; Issa Y et al., 2008).

Metals such as lead, silver, copper, and mercury, known for their biological toxicity, have been reported to induce epigenetic modification in certain types of cells (Issa Y et al., 2008; Manic L et al., 2022). Epigenetics refers to the changes in gene expression without changing the DNA sequence. This includes changes in DNA methylation, histone modification, and microRNA expression. DNA methylation

is a major epigenetic mechanism, and alterations are often observed in cells exposed to several biologically toxic metals. Hypermethylation and hypomethylation in CpG islands of genes usually lead to down- and up-regulated gene expression (Manic L et al., 2022). Limited alteration of the CpG island methylation may not affect their transcription, and such alterations may serve as early markers before changes in transcriptional levels occur. Therefore, even metals believed to lack biological toxicity may cause aberrant DNA methylation in cells, potentially leading to future cytotoxicity (Manic L et al., 2022). Thus, alterations in DNA methylation may serve as markers for assessing the biological safety of metals.

Inflammatory reactions are early and common responses to cytotoxic substances in the oral cavity (Issa Y et al., 2008). Alterations in DNA methylation levels in several cytokine genes have been observed during inflammatory reactions (Zhang S et al., 2022). Interleukin-6 (IL-6) and Interferon-gamma (IFN- γ) are major cytokines for inducing and modulating immune responses in the oral mucosa (Haque MF et al., 2001 ; Mazurek-Mochol et al., 2024), and alterations in DNA methylation in CpG islands of these genes have been demonstrated during the inflammatory process (Zhang S et al., 2022).

Nickel (Ni) and Titanium (Ti) are generally considered hazardous materials in ionic form but are now recognized as essential elements. Both are widely used as metallic biomaterials in dentistry and orthopedics due to their biocompatibility and mechanical properties. Ni and Ti are particularly prevalent in dental alloys (Trombetta et al., 2005 ; Olmedo et al., 2012). Ni-Ti alloy is primarily used for orthodontic wires due to its shape memory. Ti is also the most commonly used dental implant material, including implanted plates and screws, and implants placed for orthodontic anchorage and internal fixation. The oxide layer of Ti and its alloys provides an inert interface that interacts minimally with the surrounding tissue and lacks bioactivity to induce bone regeneration (He L et al., 2021). However, the poor wear resistance, large friction coefficient, and corrosion susceptibility of these metals under certain conditions cause the release of metal ions and the adhesion of plaque, inducing demineralization of the tooth surface and gingival inflammation (He L et al., 2021). Therefore, we examined whether there are alterations in DNA methylation in IL-6 and IFN- γ genes, followed by changes in their transcription, in HGnF

cells stimulated with Ni and Ti solutions.

Materials and Methods

Cell Culture

HGnF were purchased from ScienCell Research Laboratories (Carlsbad, USA) and cultured in α -MEM without Ribonucleosides and Deoxyribonucleosides medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Biowest, Nuaille, France) and 2% Penicillin-Streptomycin-Amphotericin B Suspension (FUJIFILM Wako Pure Chemical, Osaka, Japan) at 37°C in a 5% CO₂ incubator.

Cell Viability Assays

Sodium hexafluorotitanate (IV) (Ti) was purchased from Apollo Scientific (Stockport, UK) and diluted in double-distilled water (DDW) to a concentration of 500 μ M as a stock solution. Potassium hexafluoronickelate (IV) (Ni) was purchased from Strem Chemicals (Newburyport, USA) and diluted in DDW to a concentration of 1 mM as a stock solution. Cell viability was determined using the cell proliferation reagent water-soluble tetrazolium salt (WST-1 ; Thermo Fisher Scientific, Massachusetts, USA). The HGnF cells were seeded in 96-well plates (AGC TECHNO GLASS, Shizuoka, Japan) in the culture medium and cultured overnight. The cells were treated with different concentrations of Ni (5, 10, 20, 30, 40, 50, and 100 μ M) ; Ti (10, 20, 30, 40, 50, 100, and 200 μ M) ; and DDW as controls. Subsequently, the plates were incubated for 48 h, following which 10 μ L of WST-1 was added to each well, and the cells were cultured for 1 h. Absorbance was measured at 450 nm using the Multiskan FC system (Thermo Fisher Scientific). The experiment was repeated three times. Statistical analysis was performed using SPSS version 26 (SPSS, Chicago, USA). Results were compared using the Dunnet test, with a p-value of <0.001 considered statistically significant.

Sample Preparation

The HGnF cells were cultured at a density of 3.0×10^5 cells/ml in α -MEM containing 10% FBS and 2% Penicillin-Streptomycin-Amphotericin B Suspension using 60 mm dishes under the following conditions : 50 μ M of Ni ions (Ni), 10 μ M of Ti ions (Ti), and DDW (Control) for 2 weeks. The culture medium containing metal materials and DDW as control was replaced every 3 days. Total RNA samples were isolated using TRIzol (Invitrogen ; Massachu-

setts, USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Quantitative Reverse Transcription PCR

The RNA samples were reverse-transcribed into complementary DNA (cDNA) at 1 µg using the SuperScript reverse transcriptase (Invitrogen) and oligo (dT)₁₂₋₁₈ primers (Invitrogen). The reaction mixture for the quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) included the KAPA SYBR Fast qPCR Kit (Kapa Biosystems; Roche, Basel, Switzerland), primer pairs (Table 1), and cDNA. The LightCycler Nano (Roche) was used for the qRT-PCR, and the conditions included an initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 15 s. The relative expression level of each mRNA was calculated using the $\Delta\Delta Cq$ method (Livak KJ et al., 2001). Data are shown as a ratio of the target mRNA to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Results were compared using the Mann-Whitney U test, with a p-value of <0.05 being considered statistically significant.

Quantitative Methylation-Specific PCR

We confirmed the DNA methylation levels in HGnF cells treated with 50 µM of Ni, 10 µM of Ti, and DDW (Control). A DNA sample of 500 ng was treated with sodium bisulfite using the EpiTect Bisulfite Kits (Qiagen). The DNA

methylation levels of IL-6 and IFN- γ were analyzed using SYBR green-based quantitative methylation-specific PCR (qMSP) after determining the presence of CpG islands in the promoter region using the UCSC Genome Browser (<http://genome.ucsc.edu/index.html>). The primers were designed using MethPrimer (Li LC & Dahiya R, 2002) (Table 1). The bisulfite-treated DNA was mixed with KAPA SYBR Fast qPCR Kit and the primers. The qMSP conditions using LightCycler Nano were as follows: an initial incubation at 50°C for 2 min, denaturation at 95°C for 10 min, 50 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 15 s. The DNA methylation percentage in the samples was calculated using the following formula:

$$\begin{aligned} \text{Methylated DNA (\%)} &= \frac{M}{M+U} \times 100(\%) = \frac{1}{1+\frac{U}{M}} \times 100(\%) \\ &= \frac{1}{1+2^{(-\Delta Cq)}} \times 100(\%) \end{aligned}$$

Where M is the copy number of methylated DNA, U is the copy number of unmethylated DNA, and $\Delta Cq = Cq_u - Cq_m$ (Lu L et al., 2007). Results were compared using the Mann-Whitney U test, with a p-value of <0.05 being considered statistically significant.

Results

Cell Viability

To determine appropriate experimental conditions for analyzing cell viability maintenance in metal (Ni and Ti) materials-stimulated cells, we analyzed cell viability using different Ni and Ti concentrations. Using the WST-1 assay, cell viability was estimated at 48 h with different concentrations. The numbers of viable HGnF cells significantly decreased in the groups treated with 100 µM of Ni when compared to the controls (DDW) at 48 h (Fig. 1(a); $p < 0.001$; Dunnet test, $n=3$). Similarly, the numbers of viable HGnF cells significantly decreased in the groups treated with 200 µM of Ti when compared to the controls (DDW) at 48 h (Fig. 1(b); $p < 0.001$; Dunnet test, $n=3$). Consequently, we decided to use 50 µM of Ni and 10 µM of Ti respectively for further experiments (Setyawati M et al., 2013; Jakob A et al., 2017).

mRNA Expression Levels of IL-6 and IFN- γ

The mRNA expression levels of IL-6 for 2 weeks were significantly higher in the HGnF cells treated with 50 µM of

Table 1. Primer sequences used for quantitative reverse transcriptase PCR and quantitative methylation-specific PCR.

Gene	Sequence (5'-3')
GAPDH-F	GAGTCAACGGATTGGTCGT
GAPDH-R	GACAAGCTTCCGTTCTCAG
IL-6-F	CCACCGGAACGAAAGAGAA
IL-6-R	GAGAAGCAACTGGACCGAA
IFN- γ -F	ACTGTCGCCAGCAGCTAAAA
IFN- γ -R	TATTGCAGGCAGGACAACCA
IL-6-MF	GACGGATTATAGTGTACGGTTGC
IL-6-MR	ATAAAAATCATCCATTCTCACCG
IL-6-UF	GATGGATTATAGTGTATGGTTGTGG
IL-6-UR	AATAAAAATCATCCATTCTCACCAA
IFN- γ -MF	TTTTGATTAATATAGTGAAATTCGT
IFN- γ -MR	TCACCCAAACTAAAATACAATAACG
IFN- γ -UF	TTGATTAATATAGTGAAATTTTGT
IFN- γ -UR	CACCCAAACTAAAATACAATAACACA

Abbreviations : F, forward ; R, reverse ; M, methylated ; U, unmethylated

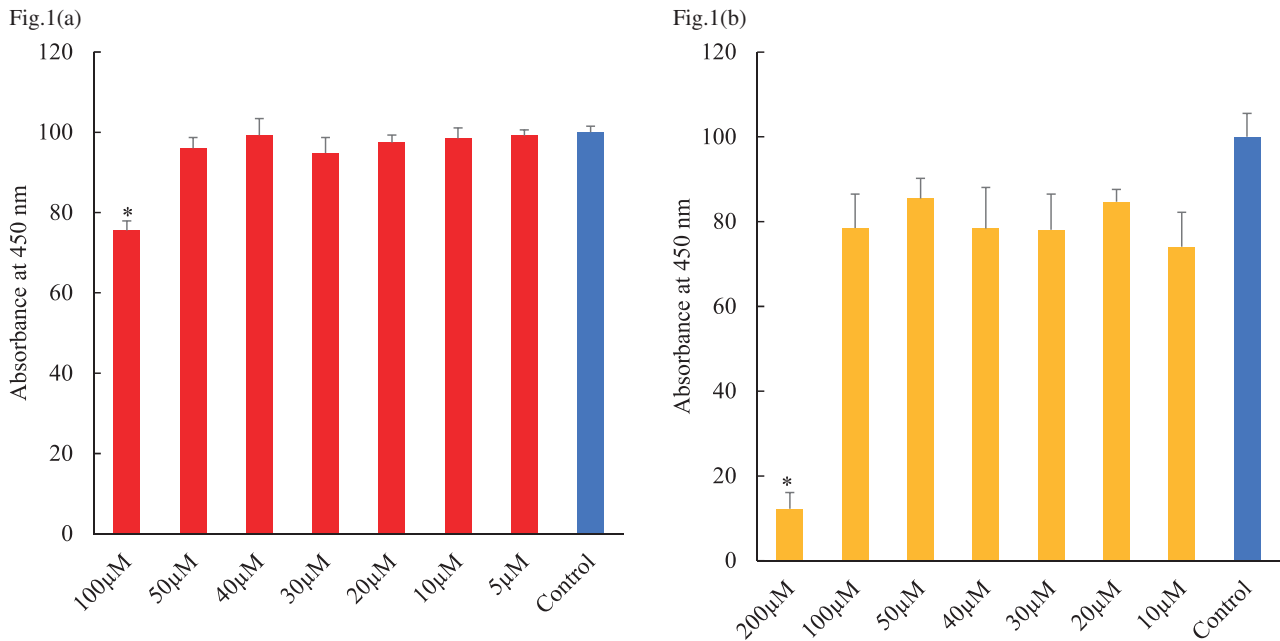


Figure 1. Cell viability.

(a) Cell viability of HGnF cells treated with different concentrations of Ni (5, 10, 20, 30, 40, 50, and 100 μM) for 48 h. The numbers of viable HGnF cells were significantly decreased in the groups treated with 100 μM of Ni compared to the controls (DDW) at 48 h (* $p < 0.001$; Dunnet test; $n = 3$).

(b) Cell viability of HGnF cells treated with different concentrations of Ti (10, 20, 30, 40, 50, 100 and 200 μM) for 48 h. The numbers of viable HGnF cells were significantly decreased in the groups treated with 200 μM of Ti compared to the controls (DDW) at 48 h (* $p < 0.001$; Dunnet test; $n = 3$).

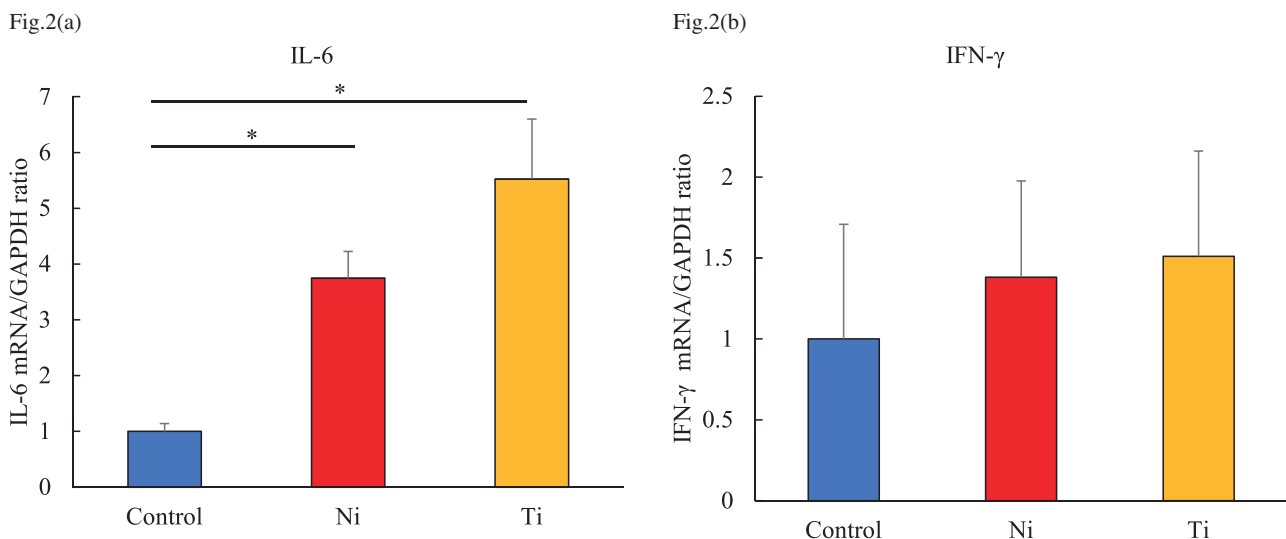


Figure 2. qRT-PCR in vitro.

(a) IL-6 expression levels in 2 weeks. The mRNA expression levels of IL-6 for 2 weeks were significantly higher in the HGnF cells treated with 50 μM of Ni and 10 μM of Ti compared to the controls (Fig. 2a; * $p < 0.05$; Mann-Whitney U test; $n = 4$).

(b) IFN-γ expression levels in 2 weeks. The mRNA expression levels of IFN-γ for 2 weeks were not significant in the HGnF cells treated with treated with 50 μM of Ni and 10 μM of Ti compared to the controls (Fig.2b).

Ni and 10 μM of Ti compared to the controls (Fig. 2(a); $p < 0.05$; Mann-Whitney U test; $n = 4$). Meanwhile, the mRNA expression levels of IFN-γ for 2 weeks were not significantly different in the HGnF cells treated with 50 μM of Ni and 10 μM of Ti compared to the controls (Fig. 2(b)).

DNA Methylation Percentage Levels of IL-6 and IFN-γ

qMSP revealed that the DNA methylation percentage levels of IL-6 in HGnF cells treated with 10 μM of Ti ($p < 0.05$) for 2 weeks were significantly lower compared to the controls (Fig. 3(a); Chi-square test; $n = 4$). Similarly, the

Fig.3(a)

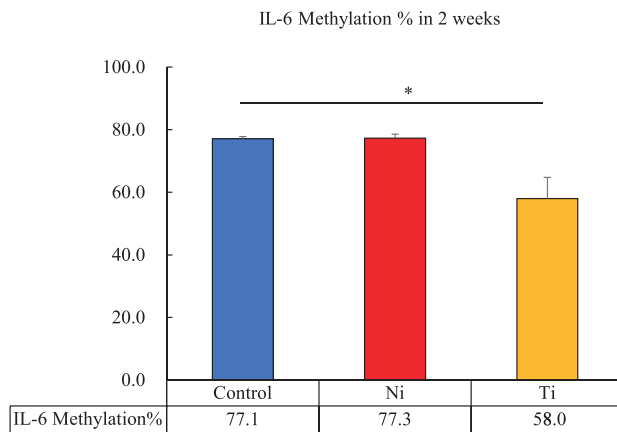
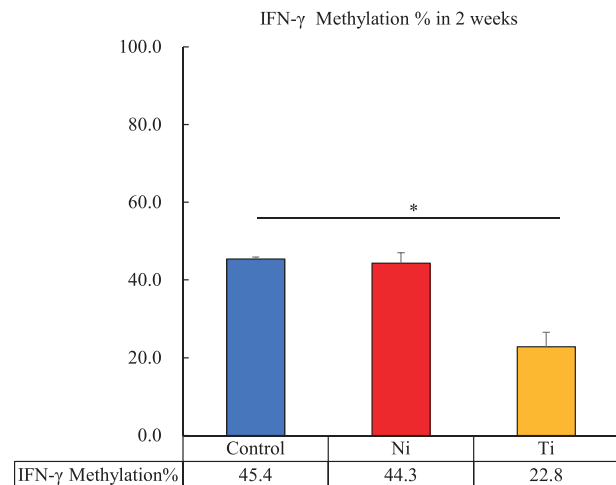


Fig.3(b)

**Figure 3.** qMSP in vitro.

(a) DNA methylation percentage levels of IL-6 in 2 weeks. The DNA methylation percentage levels of IL-6 in HGnF cells treated with 10 μ M of Ti (* $p < 0.05$) for 2 weeks were significantly lower compared to the control (Chi-square test ; $n = 4$) and the DNA methylation percentage levels of IL-6 in HGnF cells treated with 50 μ M of Ni for 2 weeks were no significant.

(b) DNA methylation percentage levels of IFN- γ in 2 weeks. The DNA methylation percentage levels of IFN- γ in HGnF cells treated with 10 μ M of Ti (* $p < 0.05$) for 2 weeks were significantly lower compared to the controls (Chi-square test ; $n = 4$) and the DNA methylation percentage levels of IFN- γ in HGnF cells treated with 50 μ M of Ni for 2 weeks were no significant.

DNA methylation percentage levels of IFN- γ in HGnF cells treated with 10 μ M of Ti for 2 weeks were significantly lower compared to the controls (Fig. 3(b) ; $p < 0.05$, Chi-square test ; $n=4$). Meanwhile, there was no significant difference in the DNA methylation percentage levels of IL-6 and IFN- γ in HGnF cells treated with 50 μ M of Ni for 2 weeks compared to the controls.

Discussion

In this study, we investigated the effects of Ni and Ti on mRNA expression and methylation levels of IL-6 and IFN- γ in the HGnF cell line in vitro. The mRNA expression of IL-6 in HGnF significantly increased when treated with 50 μ M of Ni and 10 μ M of Ti over 2 weeks. We observed hypomethylation of IL-6 in HGnF cells treated with 10 μ M of Ti for 2 weeks. Although the mRNA expression of IFN- γ in HGnF was not altered when treated with Ni and Ti, hypomethylation of IFN- γ was observed when treated with Ti. Based on these results, the hypomethylation of IL-6 may be partially responsible for the increased expression of IL-6 in HGnF cells treated with Ti. Ti may cause epigenetic modifications in the oral mucosa.

The physiological environment of the oral cavity stimulates the release of ionic components from dental alloys that come into prolonged contact with the gingival and oral mucosa (Schmalz G & Garhammer P, 2002). The released com-

ponents may have toxic effects on the oral mucosa (Trombetta et al., 2005). Various in vitro assays have been utilized to assess these toxic effects, including cell viability tests, cytostatic tests, cell cycle analysis, apoptosis detection, and oxidative stress assessment (Avior O et al., 2002 ; Issa Y et al., 2008). Previous in vitro studies have shown that both Ti and Ni induce the expression of inflammatory cytokines such as IL-6 and IFN- γ (Obando-Pereda GA et al., 2014 ; Ju HM et al., 2021 ; Huang P et al., 2023). However, alterations in methylation associated with increased expression of inflammatory cytokines have not been previously reported. Our study demonstrated hypomethylation of IL-6 and IFN- γ in HGnF when treated with Ti compared to the control. Assessing methylation levels may be a new target for cytotoxicity.

Anatase-type Ti dioxide nanoparticles (TiO₂NPs) resulted in decreased global DNA methylation and altered expression levels of methylation-related genes and proteins in human bronchial epithelial cells, suggesting that these nanoparticles induce cellular epigenomic toxicity and damage human respiratory cells (Ma Y et al., 2017). TiO₂NPs caused DNA hypomethylation in peripheral blood mononuclear cells (PBMCs) in vitro in a dose-response manner (Malakootian M et al., 2021). These DNA hypomethylations are associated with uncontrolled cell proliferation, abnormal cell cycle arrest, and apoptosis, all of which are considered risk factors

for cancer, malignant tumors, and metastases (Malakootian M et al., 2021). Further studies are needed to understand the effect of epigenetic changes due to Ti on the oral mucosa.

In conclusion, this study found hypomethylation of IL-6 and IFN- γ , followed by their upregulated expressions in HGnF cells stimulated with Ti. Methylation levels may serve as a new cytotoxic assessment tool, even for biocompatible materials.

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Conflict of Interests

The authors declare no conflict of interest.

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Dedy ARIWANSIA

Division of Oral Medicine and Pathology, Department of Oral Biology, School of Dentistry, Health Sciences University of Hokkaido.

August 2011 – March 2015 : Undergraduate Student, Dentistry Faculty of Hasanuddin University, Makassar, Indonesia

March 2015 – July 2018 : Clinical Student, Dentistry Faculty of Hasanuddin University, Makassar, Indonesia

July 2018 – May 2019 : Oral & Maxillofacial Surgeon Assistant, Celebes Cleft Center, Makassar, Indonesia

July 2018 – Present : Dentist (General Practitioner), Dentamedica Care Center, Makassar, Indonesia

July 2018 – May 2019 : Dentist (General Practitioner), Hikmah Hospital, Makassar, Indonesia

July 2018 – May 2019 : Dentist (General Practitioner), Mega Buana Hospital, Palopo, Indonesia

Juni, 2019 – March 2021 : Visiting Researcher, School of Medicine & School of Pharmacy, Kitasato University, Japan

January 2024 – Present : Research Assistant, Dentistry Faculty of Hasanuddin University, Makassar, Indonesia

April 2021 – Present : Postgraduate student, Graduated School of Dentistry, Health Sciences University of Hokkaido.