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RESEARCH ARTICLES

Vitamin D inhibits TNF-α serum level in wistar rats stimulated with *Porphyromonas gingivalis*

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ABSTRACT

Periodontitis is a chronic inflammation of the periodontal tissues initiated by increased proinflammatory cytokines such as TNF-α due to the induction of *Porphyromonas gingivalis* (*Pg*). This study aimed to analyze the effect of vitamin D (cholecalciferol) on TNF-α serum level in Wistar rats stimulated with *Pg* to induce periodontitis. Twenty-seven male Wistar rats (n = 27) were divided into three equal groups. Group I was healthy Wistar rats that received 2000 IU vitamin D once a day. Group II was Wistar rats stimulated with *Pg* and received 2000 IU vitamin D, while group III was Wistar rats stimulated with *Pg* but did not receive vitamin D. Blood was collected through the orbital sinus and centrifuged to get the serum. TNF-α serum levels were assessed using Elisa method on the 7th, 14th, and 28th days. The data were normally distributed and homogeneous. The mean TNF-α data was analyzed for differences between groups using the one-way ANOVA and LSD post hoc test. Significant differences were seen in group II (418.49 ± 161.08 ng/mL) and group III (172.16 ± 104.18 ng/mL) on the 28th day (p = 0.001). The findings suggest that vitamin D inhibits the TNF-α serum level in Wistar rats stimulated with *Pg* on the 28th day.

Keywords: periodontitis; *Porphyromonas gingivalis;* TNF-α; Vitamin D

INTRODUCTION

The prevalence of periodontitis in the United States reaches 45.9% in adults over the age of 30 years.[1] The results of basic health research in 2018 showed that the prevalence of dental and oral diseases in Indonesia was 57.6%, and the prevalence of periodontitis was 74.1%.[2] According to these findings, the prevalence of periodontitis among Indonesians remains high compared to Americans.

Periodontitis is a chronic inflammation of the periodontal tissues caused by bacteria. Periodontitis is characterized by loss of the periodontal attachment, increased periodontal pocket depth, and alveolar bone resorption. Periodontitis is preceded by an increase in induced proinflammatory cytokines as a result of bacterial invasion. The process of destruction of the periodontal tissue in periodontitis is initiated by the accumulation of plaque, which contains pathogenic bacteria and toxins. *Aggregatibacter actinomycetemcomitans, Eikenella corrodens, Fusobacterium nucleatum, Prevotella intermedia, Porphyromonas gingivalis,* and *Tannerella forsythia* are gram-negative bacteria that are often found in periodontal pockets.[3,4] Bacteria and their products such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, and proteases in chronic periodontitis can increase local cytokines, which can then modulate the host response, thereby accelerating alveolar bone resorption.[3,4]

Porphyromonas gingivalis (*Pg*) is a gram-negative anaerobe found in dental plaque. It has been closely linked to the development of periodontal disease. It contains a wide range of virulence factors, including those that allow it to adhere to and infiltrate host epithelial cells.[5] *Pg* invasion is carried out through altering host signal

transduction and cytoskeletal architecture. *Pg* products stimulate the production of the cytokine tumor necrosis factor alpha (TNF-α), which is involved in osteoclastogenesis and osteoclast activity, accelerating bone resorption. TNF-α is a proinflammatory cytokine that contributes to periodontal tissue destruction. These proinflammatory cytokines are secreted by various types of cells: monocytes, macrophages, dendritic cells, epithelial cells, keratinocytes, and fibroblasts. The amount increases during the inflammatory process and increases bacterial phagocytosis activity.[6] Several previous studies found that TNF-α levels in the blood serum of periodontitis patients were higher than in healthy people.[7]

Vitamin D is a secosteroid hormone synthesized through photochemical reactions from ultraviolet radiation on skin cells and through food consumption. Vitamin D deficiency can lead to decreased bone mineral density, osteoporosis, increased periodontal disease, and jaw bone resorption. Because vitamin D has immunomodulatory, anti-inflammatory, antiproliferative, and anti-apoptotic effects, meeting vitamin D requirements can lower the risk of gingivitis and chronic periodontitis.[8] The prevalence of vitamin D deficiency in the world's population is high; 30–50% of the population suffers from vitamin D deficiency. Vitamin D deficiency is an epidemic that affects about one billion people worldwide in both the deficiency and insufficiency categories. Vitamin D deficiency occurs when the level of 25-hydroxyvitamin D (OH-D) in the blood serum is 20 ng/ml, while the condition of insufficiency is when the level of 25-OH-D is 21-29 ng/ml. The findings of one study revealed that more than 70% of the population over 12 years had 25 OH-D levels in blood serum of 32 ng/mL.[9]

Alshoubi et al found that individuals who received more than 800 IU of vitamin D per day had a lower risk of developing chronic periodontitis than individuals who received less than 400 IU of vitamin D per day.[10] The assessment is in pocket depth, gingival attachment, and alveolar bone resorption. Hiremath et al showed that vitamin D ranging from 500 to 2000 IU per day were both safe and effective in the treatment of gingival inflammation.[11]

Systemic treatment and modulation of the host immune response in chronic periodontitis both use antibiotics and local treatment, which is also carried out using topical antibiotics. It has been widely reported that excessive and inappropriate use of antibiotics can lead to bacterial resistance. Considering this issue, it is necessary to study the effects of vitamin D, which can modulate the host immune response by reducing proinflammatory cytokines as a treatment for chronic periodontitis. This study aimed to analyze the effect of vitamin D 2000 IU in TNF-α serum level on Wistar rats stimulated with *Pg* to induce periodontitis.

MATERIALS AND METHODS

Animal ethics approval for this research was obtained from the Ethics Committee of the Faculty of Medicine, University of Udayana, on March 10, 2022 (project number 485/UN14.2.2.VII.14/LT/2022). The research design was an experimental randomized posttest only control group design. There were three experimental groups with each group consisting of 9 Wistar rats (n = 27). Group I was healthy Wistar rats that received 2000 IU vitamin D once a day. Group II was Wistar rats stimulated with *Pg* and received 2000 IU vitamin D, and group III was Wistar rats stimulated with *Pg* that were not given vitamin D. The TNF-α serum levels of each group were assessed on the 7th day (3 Wistar rats), 14th day (3 Wistar rats), and 28th day (3 Wistar rats). The number of samples was calculated using the Federer's formula (n-1) (t-1) ≥ 15 with the number of observations for each group multiplied by three, resulting in a total of nine observations (t = 9), with three samples in each observation (n = 3).

Pg ATCC 33277 bacteria were obtained from the Faculty of Dentistry, Airlangga University. The procedure for the subculture of the Pg bacterial isolate began by taking ATCC 33277 culture stock. It was then cultivated using the quadrant IV method on Brucella Agar with 5% Sheep Blood

and incubated for 48 hours under anaerobic conditions at an incubator temperature of 37 °C. The procedure for suspending Pg bacteria began by taking pure Pg colonies, which were then put into a sterile inoculum tube containing 0.45% sodium chloride, and then homogenized using a vortex. Turbidity was equalized using McFarlan nephelometer 3, and then the suspension was stored in a sterile Falcon tube and placed in an anaerobic jar.

Vitamin D used in this study was cholecalciferol TCI (Tokyo Chemical Industry Co., LTD). One IU is equivalent to 0.000025 mg, and thus 2000 IU/day equals 0.05 mg/day. The conversion dose for rats from the human dose is multiplied by 0.018; therefore, 2000 IU = 0.05 mg x 0.018 = 0.0009 mg/day. Vitamin D in powder form was weighed using a digital balance with microgram analysis, dissolved in sterile distilled water, and administered via hand feeding.

The rats used as samples were 2-3 months old healthy male Wistar rats weighing 250-300 grams. The rats were obtained from the Laboratory of Histology, Faculty of Medicine, University of Udayana. They were acclimatized for one week in cages with a size of 33 x 22 x 15 cm, and each cage consisted of three rats. The rats were placed in a ventilated room and received indirect sunlight at a room temperature of 22 °C. They were fed pellets containing 17–20% protein, 3-4% fat, and 35–40% carbohydrates, and given mineral water (15–30 ml/day). The total daily food intake of the rats was 10%-15% of their body weight.

Before being induced, the rats were anesthetized with a combination of ketamine and xylazine. Ketamine was administered intramuscularly at a dose of 40 mg/kg body weight, and xylazine was administered subcutaneously at a dose of 5 mg/kg body weight. Periodontitis was induced by injecting 0.02 ml *Pg* bacterial isolate intra sulcular in the gingival sulcus of the first right lower incisor labial part of Groups II and III using a 30 G every two days for 28 days. Following the first induction, the samples were divided into three groups of nine mice each. Group I, healthy rats which were not stimulated with *Pg*, was given regular food and Vitamin D 2000 IU/day, once a day in the afternoon. Three mice were killed by rapid decapitation on the 7^{th}, 14^{th}, and 28^{th} days. Group II was stimulated by *Pg* and was given a placebo in the form of regular food, killed by rapid decapitation on the 7^{th}, 14^{th}, and on 28^{th} days. Group III, which was stimulated with *Pg*, were given regular food and 2000 IU/day of vitamin D, and killed by rapid decapitation on the 7^{th}, 14^{th}, and 28^{th} days.

Rats were killed for blood sampling. Before they were killed, the rats were euthanized under ketamine anesthesia. Blood was collected through the orbital sinus, and the amount drawn was up to 1 ml. It was then put into a tube containing EDTA as an anticoagulant. Samples were centrifuged for 15 minutes at 2000-3000 RPM at 2-80 °C within 30 minutes after collection. TNF-α levels were determined by aspirating separated serum, and it was then stored in a freezer at -200 °C. The TNF-α serum levels (ng/mL) were assessed using Elisa Kit for Rats BT LAB (Bioassay Technology Laboratory) (Shanghai Korain Biotech Co., Ltd).

A normality test using the Shapiro-Wilk test showed that all data between groups were normally distributed ($p > 0.05$). TNF-α data were found to be homogeneous ($p > 0.05$) using Levene homogeneity testing. Because the data were normally distributed and homogeneous, the mean TNF-α data was tested for differences between groups using the one-way ANOVA test, as seen in the LSD post hoc test.

RESULTS

TNF-α data were descriptively analyzed to determine the mean, standard deviation (SD), minimum value, and maximum value obtained from the research findings (Table 1). The significance analysis with the one-way ANOVA test on the TNF-α data is shown in Table 2, with the value of $F = 54.327$ and $p = 0.005$. The values showed that there was a difference in the average of TNF-α in the three groups ($p < 0.05$). The LSD post hoc test was applied to identify which group showed a significant difference (Table 3).

Table 1. Descriptive analysis of TNF-α data between groups

Group	n	Mean (ng/mL)	SD	Max	M.n
Group I (7th day)	3	528.26	182.18	684.40	182.18
Group I (14th day)	3	300.09	129.77	449.51	215.44
Group I (28th day)	3	216.43	32.51	236.14	178.91
Group II (7th day)	3	149.46	26.88	175.43	121.75
Group II (14th day)	3	359.82	146.27	492.99	203.26
Group II (28th day)	3	418.49	161.08	587.42	266.61
Group III (7th day)	3	142.95	20.12	161.12	121.86
Group III (14th day)	3	190.88	64.15	242.65	119.10
Group III (28th day)	3	172.16	104.18	290.48	196.32

Table 2. *One Way Anova Test* to analize the differences in data between groups

Group	n	Mean (ng/mL)	SD	F	p
Group I (7th day)	3	528.26	182.18		
Group I (14th day)	3	300.09	129.77		
Group I (28th day)	3	216.43	32.51		
Group II (7th day)	3	149.46	26.88		
Group II (14th day)	3	359.82	146.27	4.327	0.005*
Group II (28th day)	3	418.49	161.08		
Group III (7th day)	3	142.95	20.12		
Group III (14th day)	3	190.88	64.15		
Group III (28th day)	3	172.16	104.18		

*Significant

DISCUSSION

Vitamin D 2000 IU was given once a day in the afternoon because the production of vitamin D3 in the skin is minimal in the early morning and late afternoon. Additionally, vitamin D is absorbed most efficiently in the presence of food.[12] In this study, TN-α serum levels were used as a marker for periodontitis. We adopted Jain et al's finding which indicates that periodontal disease is positively associated with elevated TNF-α serum levels. Subsequent analysis suggests that the quantification of TNF-α in serum may be utilized as a "marker" for periodontal disease.[7]

The results of this study showed that in the group of rats induced by periodontitis but not given vitamin D, the mean TNF-α serum level was higher on day 28 than on days 14 and 7. The findings of this study are consistent with those of previous research, which found an increase in salivary TNF-α levels in periodontitis patients compared to healthy individuals.[13,14] Jain et al conducted research on serum TNF-α levels and found that serum TNF-α levels are higher in patients with periodontitis than in healthy individuals.[7]

TNF-α plays an important role in the pathogenesis of periodontitis. TNF-α is produced

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Table 3. *Least Significant Test (LSD)* to analyze the significant difference between two groups

Groups	p
Group I (7th day) and Group I (14th day)	0.023*
Group I (7th day) and Group I (28th day)	0.003*
Group I (14th day) and Group I (28th day)	0.370
Group II (7th day) and Group II (14th day)	0.035*
Group II (7th day) and Group II (28th day)	0.009*
Group II (14th day) and Group II (28th day)	0.030*
Group III (7th day) and Group III (14th day)	0.609
Group III (7th day) and Group III (28th day)	0.755
Group III (14th day) and Group III (28th day)	0.841
Group I (7th day) and Group II (7th day)	0.084
Group I (14th day) and Group II (14th day)	0.525
Group I (28th day) and Group II (28th day)	0.042*
Group I (7th day) and Group III (7th day)	0.001*
Group I (14th day) and Group III (14th day)	0.251
Group I (28th day) and Group III (28th day)	0.637
Group II (7th day) and Group III (7th day)	0.944
Group II (14th day) and Group III (14th day)	0.830
Group II (28th day) and Group III (28th day)	0.001*

* Significant

by monocytes and macrophages in response to bacterial pathogenic components such as LPS. Elevated TNF-α levels cause gingival fibroblasts to release collagenase, resulting in cartilage collagen damage and alveolar bone resorption. TNF-α causes bone destruction by increasing osteoclastic activity while decreasing osteoblastic activity. TNF-α has been shown to be involved in the pathogenesis of periodontitis.[15] Periodontal microbiological studies have revealed that *Aggregatibacter actinomycetemcomitans* and *Pg* stimulate the production of TNF-α by monocytes and macrophages. TNF-α stimulates osteoclasts and matrix metalloproteinase (MMP), which cause periodontal breakdown.[16]

TNF-α plays an important role in inflammatory processes, including periodontitis. TNF-α is an inflammatory cytokine that promotes bone resorption by suppressing osteoblast anabolic function and inducing RANKL expression in osteoblasts and stromal cells, thereby activating osteoclastogenesis.[17] Yuce et al observed an increase in TNF-α levels in the gingival fluid of patients with periodontitis, which decreased after periodontal therapy. TNF-α inhibits osteoblast differentiation and thus bone formation.[18] TNF-α stimulates osteoclastogenesis by increasing M-CSF and RANKL production in stromal cells and osteoblasts. TNF-α is an active protein that is either biologically bound to its stem cells, monocytes, and T cells, or bound to its soluble form after enzyme cleavage. TNF-α binds to one of its two receptor cells (TNFR) to initiate a cellular response.[19]

TNF receptors (TNFR1/p55 and TNFR2/p75) are found in nearly all cell types, including macrophages, lymphocytes, neutrophils, and fibroblasts. In periodontitis, it was reported that TNFR1 and TNFR2 were expressed by sulcus epithelial cells, monocytes/ macrophages, fibroblasts, and endothelial cells. In periodontitis, there is an imbalance between TNFR1 and TNFR2. When the severity of periodontitis increases and alveolar bone resorption occurs, the ratio of TNFR2/ TNFR1 decreases. TNFR1 activation can cause cell proliferation, stimulation, and survival. It also initiates apoptotic signals and cell death. TNFR1 is a TNF receptor that causes osteoclastogenesis. Through the NF-kB signaling pathway, TNF-α and TNFR1 mediate endotoxin-induced osteoclastogenesis and bone resorption. Research has shown that reduced expression of TNFR1 significantly suppresses the RANKL signaling pathway, including NF-kB and AP1 activation. TNFR1 is needed for optimizing RANKL expression, which induces osteoclastogenesis.[20]

In our study, TNF-α serum levels tended to be stable in the Wistar rats group stimulated with *Pg* and given vitamin D. This was evident from the insignificant difference between days 7 and 14 ($p = 0.609$), days 7 and 28 ($p = 0.755$), and days 14 and 28 ($p = 0.841$). There was a significant difference in TNF-α serum levels between groups of Wistar

rats induced by periodontitis and given vitamin D and those not given vitamin D (p = 0.001) on the 28th day, with TNF-α levels being higher in the group without vitamin D. This finding is supported by a case-control study conducted by Czyz and Firkova,[21] which found that serum levels of vitamin D3 were lower in patients with periodontitis (31.34 nmol/L) than in healthy individuals (39.64 nmol/L). Patients with severe generalized periodontitis over 50 years have lower vitamin D3 serum levels than younger patients with mild periodontitis. This finding is consistent with that of Isola et al, who also found that patients with chronic periodontitis (17.4 ± 5.2 ng/mL) and chronic periodontitis with chronic heart disease had lower vitamin D serum levels (16.5 ± 5.6 ng/mL) than healthy individuals (29.9 ± 5.4 ng/mL) and individuals with only chronic heart disease (24.6 ± 3.7 ng/mL).[22]

Vitamin D supplementation can help maintain periodontal health by increasing jaw mineral density and inhibiting alveolar bone resorption. Individuals with adequate vitamin D levels outperform those with vitamin D deficiency in treating chronic periodontitis. Several studies have shown that vitamin D deficiency causes progressive damage in people with chronic periodontitis.[23] Results of various research show a positive effect of vitamin D serum levels on periodontitis healing as measured clinically by parameters of bleeding on probing, pocket depth, clinical attachment level, gingival index, and distance from cementoenamel junction to alveolar crest. The clinical parameters of periodontal disease are used to further identify the effect of vitamin D on periodontitis. The effect of vitamin D on bacteria and cytokines in periodontitis has also been widely studied. It has been reported that after six months of therapy with vitamin D, there is a decrease in the population of bacteria that cause periodontitis such as *Tannerella forsythia, Treponema denticola*, and *Pg*.[24]

The protective mechanism of vitamin D against periodontitis occurs through two biological pathways: the antimicrobial and anti-inflammatory pathways. The antimicrobial effect of vitamin D occurs as a result of the bond between $1,25(OH)_2D_3$ and receptor vitamin D (VDR), which then induces cAMP, b-def-2, and b-def-3 peptides by macrophages, monocytes, gingival epithelium, and periodontal ligament epithelium. These peptides will reduce microbes in the oral cavity and prevent the exposure of the periodontal tissues to these microbial products. The anti-inflammatory effect is achieved by inhibiting NF-kB and upregulating MKP-1 while decreasing the production of proinflammatory cytokines such as IL-6 and TNF-α. The decrease in the production of proinflammatory cytokines will inhibit the destruction of the periodontal connective tissue by weakening MMP stimulation. Reduced IL-6 and TNF-α production lowers the RANKL/OPG ratio in osteoblast stromal cells, inhibiting osteoclast progenitor differentiation as a cause of alveolar bone resorption.[25,26]

CONCLUSION

Vitamin D inhibited the TNF-α serum level in Wistar rats stimulated with *Pg* on the 28th day.

LIMITATIONS AND SUGGESTION

Although elevated levels of TNF-α in the serum may serve as an indicator of periodontitis, better quantification of TNF-α in periodontal tissue, such as gingival crevicular fluid, is recommended. This investigation was conducted on blood serum as a result of inadequate infrastructure and facilities to obtain gingival crevicular fluid from rats. Sample from gingival crevicular fluid and other periodontal tissue, such as alveolar bone, should be utilized in the development of markers in future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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