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Sex-Based Differences in Gingival Inflammatory Responses to *Porphyromonas gingivalis* in Male and Female Rats

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Abstract

Periodontitis is defined as the inflammation of periodontal tissue. It is primarily caused by plaque bacteria, with *Porphyromonas gingivalis* being one of the most predominant bacteria. The inflammatory response to *P. gingivalis* varies by sex, as evidenced by higher serum titers in females than in males. This study aimed to compare the inflammatory responses in the gingiva of female and male rats following injection with *P. gingivalis*. The study was experimental laboratory using post-test control group design. Both male and female rats received injections of 2.10^9 cells/mL of *P. gingivalis* every 3 days over 19 days. The injections were into the distobuccal and distolingual gingival sulcus of the mandibular first molar. On the 19 days, tumor necrosis factor-alpha (TNF-α) levels of gingival fluid were measured using ELISA methods. Then, the rats were euthanized and removed the mandible. The number of leukocytes in the mandibular gingival tissue was assessed histologically using hematoxylin eosin staining. All of data were analyzed by Independent T-Test ($p < 0.0$ %). The findings revealed that the TNF-α levels in the gingival crevicular fluid of female rats (161.14 ng/mL) were significantly 1.5 times higher than male rats (109.57 ng/mL) after *P. gingivalis* injection ($p < 0.05$). The numbers of neutrophils and macrophages in female rats (neutrophils = 35.57 cells and macrophages = 13.71 cells) were significantly higher than those in male rats (neutrophils = 30.14 cells and macrophages = 8.43) ($p < 0.05$). However, the lymphocyte count was insignificantly comparable between sexes following injection (females = 22.24 cells and males = 22 cells) ($p > 0.05$). Sex differences might influence the immune response to *P. gingivalis* infection, with females exhibiting a stronger response characterized by increased leukocyte infiltration and TNF-α level than males. After injections of *P. gingivalis*, the inflammatory response observed in female rats was notably greater than that in male rats.

Keywords: Female rats, Lymphocytes, Macrophage, Male rats, Neutrophils, *Porphyromonas gingivalis*, Sex, Tumor necrosis factor-alpha

Introduction

Periodontitis is a chronic inflammatory disease that affects the periodontal tissue. It poses a significant health concern and has affected approximately 12.5 % of the global population by 2021 [1]. The incidence of severe periodontitis consistently increased by 8.44 % from 1990 to 2019, correlating with the population growth. However, the prevalence of periodontitis varies according to region, age, and sex [2]. The highest prevalence of periodontitis (17.57 %) is found in South and Southeast Asia, including Indonesia [1]. According to the 2018 National Basic Health Research Report of Indonesia, the prevalence of periodontitis in Indonesia is extremely high, exceeding 65 % across various age groups and both sexes [3]. Several epidemiological studies have indicated that men had a higher prevalence of periodontitis than women, particularly in the pre-

older age group (45 - 59 years). This variation in prevalence is also influenced by factors such as smoking habits. However, it was noteworthy that with increasing age, progressive damage to periodontal tissue occured more frequently in women [4-7].

Periodontal pathogens play a significant role in the development of periodontitis, and notable bacteria include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Treponema denticola*, and *Prevotella nigrescens* [8]. The composition of periodontal pathogens varies according to sex. In men, the dominant periodontal pathogens belong to the genera *Pseudomonas* and *Papillibacter*, whereas in women, they belong to the genera *Fusobacteriales* and *Tannerella*. Interestingly, the presence of *P. gingivalis* in the gingival sulcus is similar in both sexes, indicating its plays role in the severity of periodontal disease in men and women [9]. *P. gingivalis* is often referred to as the "keystone pathogen." This term is used because, although it constitutes a small proportion of the microbial community in the gingival sulcus, it has a considerable impact on the progression of periodontitis and promotion of dysbiosis [10,11]. Several studies have reported that the gingival sulcus of patients with periodontitis contained 70 - 90 % of *P. gingivalis*, whereas this value was less than 30 % in healthy individuals [12,13].

Although the proportion of *P. gingivalis* in the periodontal tissues of men and women is similar, there are notable differences in how the immune systems of each sex respond to this bacterium [14-16]. Several studies have shown that women's immune responses are generally less effective than those of men, leading to more severe inflammation in periodontal tissues for women [15]. Additionally, hormonal changes in women enhance biofilm formation and activate the virulence factors of *P. gingivalis*. This increased activity contributes to greater colonization and invasion of *P. gingivalis* into epithelial tissues, ultimately increasing its virulence in the periodontal tissues of women [17].

Studies examining sex-based differences in inflammatory responses to *P. gingivalis* infection have yielded conflicting results in human and animal studies. Human studies have indicated that men are more susceptible than women to periodontitis, resulting in a higher prevalence of the disease [7]. In contrast, animal studies exhibited that female rats experience more significant periodontal tissue damage and produce higher levels of proinflammatory cytokines than male rats [18]. Additionally, serum antibody titers against *P. gingivalis* and other inflammatory markers are higher in female than in male rats. It indicated a stronger correlation between sex to the development of periodontal disease [19]. Moreover, there is sex dimorphism in gingival apoptosis, with female rats exhibiting higher levels of caspase-3 at the sites of disease than the male rats [20]. These findings underscore the complexity of sex-based differences in periodontal disease and highlight the need for further studies to elucidate the underlying mechanisms. In this study, we compared the inflammatory responses in the gingiva of female and male rats injected with *P. gingivalis*. Using both female and male rats as research models, through this study, we aimed to compare the inflammatory responses in the gingiva of female and male rats following injection with *P. gingivalis*. Inflammation parameters assessed included the concentration of tumor necrosis factor-alpha (TNF-α) in gingival fluid and the number of leukocytes present in gingival tissue.

Materials and methods

This study was approved by the Ethics and Advocacy Commission of the Faculty of Dentistry of Gadjah Mada University, Indonesia (reference number: 0029/KKEP/FKG-UGM/EC/2022). This study used Wistar-strain white rats (*Rattus norvegicus*). Both male and female rats aged between 10 and 12 months, corresponding to a productive age range of 25 - 35 years in humans, were selected for this study [21]. In addition, the female rats needed to be in the same estrus cycle, specifically in the estrous phase, to prevent bias from hormonal fluctuations. The number of rats in each group was determined using the Lemenshow formula with a correction factor of 20 %, resulting in seven rats per group. The male rat group comprised 14 rats, divided into 2 subgroups: 1 without *P. gingivalis* injection and one with *P. gingivalis* injection, by the same to the female rat groups. Thus, the total number of rats used was 28.

The strain of *P. gingivalis* used in this study was sourced from a commercial stock of *P. gingivalis* (*Porphyromonas gingivalis* ATCC 33277, Thermo

Fisher Scientific, USA). The stock was inoculated onto solid Brain Heart Infusion Agar (BHI-A) (Oxoid). The inoculated plates were placed in a desiccator for 48 h and supplied with CO2 gas (Oxoid) under anaerobic conditions. Subsequently, one dose of P. gingivalis was taken from BHI-A; and it transferred to 2 mL of Brain Heart Infusion Broth (BHI-B) (Oxoid), which was enriched with vitamin K and hemin. The suspension was homogenized using a vortex mixer (Thermo Fisher Scientific) for 30 s. The suspension was then incubated in the desiccator with CO_2 gas at 37 °C for 24 h. The resulting growth exhibited turbidity in the BHI medium, which was then diluted with sterile distilled water and shaken until homogeneous. The concentration was measured manually and determined to be 2.10^9 CFU [22,23].

All rats were acclimated for 2 weeks prior to treatment. In the group of rats that did not receive *P. gingivalis* injection, both male and female rats were left undisturbed without induction. In the group of rats that received P. gingivalis injection, both male and female, *P. gingivalis* suspension (0.05 mL) was injected into the distobuccal gingival sulcus and distolingual area of the first mandibular molar on both sides. This injection was administered using a tuberculin syringe with a 30-gauge needle every 3 days for a total of 19 days. Consequently, the total number of injections per mouse was seven [22,23]. Periodontitis in rats injected with *P. gingivalis* was characterized by gingival redness in the areas of the right and left first and second molars, swelling of the gingival margin, and tooth mobility rated at 2 - 3 for the first and second molars of the lower jaw.

Gingival fluid was collected from all rats on day 19; however, in the groups injected with *P. gingivalis*, the gingival fluid was obtained 6 h after the last induction. The gingival fluid was taken using a paper point with size 20, which was inserted into the gingival sulcus for 2 min on both the buccal and lingual sides of the first molars on the right and left sides. The total number of paper points collected from each rat was then placed in a modified microcentrifuge tube (0.5 mL) with a hole at its end. This tube, containing the paper point, was placed inside a larger Eppendorf tube (1.5 mL). Subsequently, 50 µL of phosphate-buffered saline was added to moisten the paper point and incubated for 5 min. After this incubation, the samples were centrifuged at 2,200 rpm for 20 min. The liquid collected at the bottom of the second Eppendorf tube constituted the diluted gingival fluid. The procedure followed was as previously described [24]. Finally, the gingival fluid was analyzed for TNF-α levels using an enzyme-linked immunosorbent assay, following a standard procedure provided by fabricated (Elabscience).

After collecting gingival fluid, the rats were euthanized using a lethal dose of ketamine/xylazine. The mandibular tissue was removed, and the mandibular bone specimens were cleaned and fixed in 10 % buffered formalin. Subsequently, the specimens were decalcified in a 10 % ethylenediaminetetraacetic acid solution for 30 days to extract inorganic materials while preserving the existing proteins [25]. The tissues were processed and stained with hematoxylin and eosin in accordance with previously published procedures. Observations were conducted using a light microscope with a magnification ranging from 10× to identify the observation area. Subsequently, the number of leukocytes was counted at 1000× magnification. Measurements were taken across 10 fields of view [22].

All data, including TNF-α levels and leukocyte counts, were statistically analyzed using SPSS 18.0 software (SPSS for Windows; SPSS, Chicago, IL, USA). Normality tests, including the Shapiro–Wilk and Levene tests ($p > 0.05$), were performed to determine whether the data were normally distributed and homogeneous. All od data were analyzed using Independent T-Test to assess the significance of differences between groups ($p < 0.05$).

Results and discussion

TNF-α is a pro-inflammatory cytokine, and its presence indicates an immune response in the gingiva to periodontal pathogens, specifically *P. gingivalis*. The results of the study revealed that the concentration of TNF-α in the gingival fluid increased in both male and female rats on the 19^{th} day after being injected with *P. gingivalis*. In male rats, the TNF-α concentration after injection was 109.57 ng/mL, which was 1.5 times higher than that in the non-injected group (72.86 ng/mL) ($p < 0.05$). In female rats, the TNF-α concentration reached 161.14 ng/mL following the injection, nearly 3 times higher than that in the non-injected group (68.86 ng/mL) ($p < 0.05$). Additionally, we found that the TNF-α concentration in female rats injected with *P. gingivalis*

was significantly higher than that in male rats injected with *P. gingivalis* ($p < 0.05$) (**Figure 1**).

The results indicated that injecting *P. gingivalis* into the gingival sulcus of both male and female rats led to an increase in TNF-α concentration in the gingival fluid. This injection probably stimulated immune cells in the periodontal tissue to produce TNF-α. Several studies revealed that the presence of TNF-α in gingival fluid was a marker of periodontal tissue inflammation or the development of periodontal disease [26-28]. TNF-α levels increased significantly during the inflammatory response triggered by infections from periodontal pathogens, particularly *P. gingivalis*. The virulence factors of *P. gingivalis* activated both immunocompetent cells (such as macrophages and neutrophils) and non-immunocompetent cells (such as epithelial cells) in the gingiva, resulting to the production of pro-inflammatory cytokines, including TNF-α [28-30].

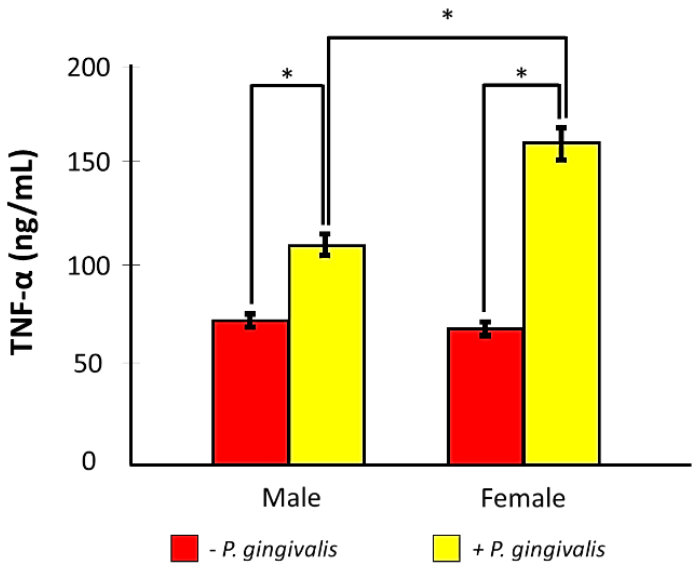

Figure 1 Tumor necrosis factor-alpha (TNF-α) concentration in the gingival sulcus fluid of male and female rats on day 19. The figure presented the mean and standard error values; *, indicated a significant difference analyzed Independent T-Test ($p < 0.05$).

The results also indicated that the concentration of TNF-α in female rats injected with *P. gingivalis* was significantly higher than in male rats injected with the same pathogen. This result suggests that sex differences might influence the immune response to *P. gingivalis* infection. Female rats exhibited a stronger response characterized by increased TNF-α production than male rats. Several studies revealed that female rats with periodontitis showed higher serum concentrations of TNF-α, interleukin-6, and C-reactive protein compared to male rats. Female rats experienced a greater acute phase response to periodontal inflammation than male rats. Estrogen may modulate the inflammatory response [31,32]. According to Grover *et al.* [33], women generally presented a more robust immune response to *P. gingivalis* compared to men, including the production TNF-α. The observed variations in TNF-α levels probably help explain the differences in prevalence and severity of periodontal disease between the sexes. Female animal models with IL-17RA receptor deficiency showed increased inflammation after P. gingivalis infection. In contrast, male models with IL-17RA receptor deficiency did not demonstrate a heightened inflammatory response. The IL-17RA receptor is crucial for regulating TNF-α. This finding indicates that TNF-α regulation in response to *P. gingivalis* may vary according to the sex [32]. However, Johnson and John highlighted that TNF-α concentrations were often higher in men with periodontal disease than in women with periodontal disease. The presence of estrogen in women help to regulate TNF-α production [20]. Although women

demonstrated effective TNF-α regulation, inflammation and damage to periodontal tissue presented more severe in women than in men. This paradox might be attributed to fluctuations in estrogen levels, which lead to a more aggressive immune response during inflammation compared to that in men [34].

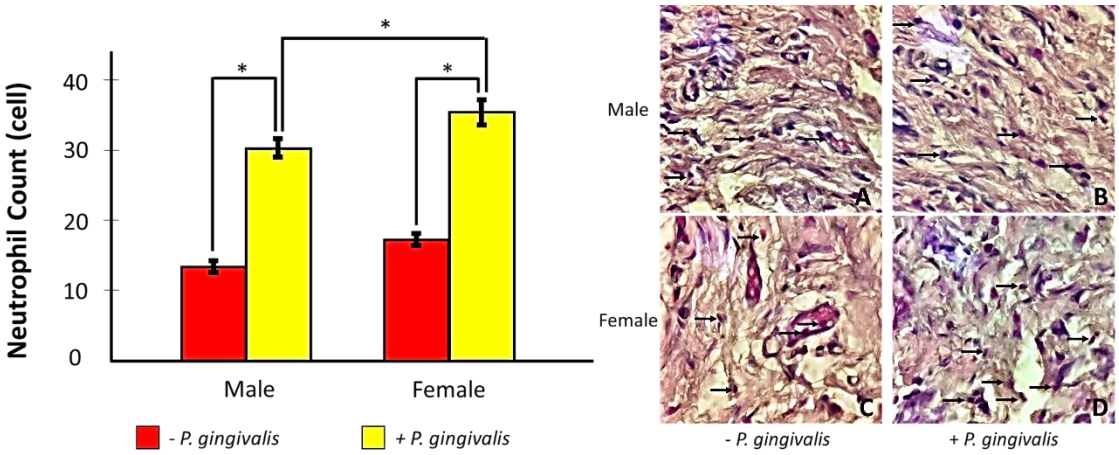

Figure 2 Number of neutrophils in the gingival tissue of male and female rats at day 19. The figure presented the mean and standard error values; * indicated a significant difference analyzed Independent T-Test ($p < 0.05$); A, male rats without *P. gingivalis* injection; B, male rats with *P. gingivalis* injection; C, female rats without *P. gingivalis* injection; D, female rats with *P. gingivalis* injection; black arrows, neutrophils in the gingival tissue.

Neutrophils are white blood cells that play a crucial role in the immune response, particularly in combating infections. The study results indicated that the number of neutrophils was higher in both male and female rats that were injected with *P. gingivalis*, compared with those not injected with the bacteria ($p < 0.05$). Additionally, female rats injected with *P. gingivalis* (35.57 cells) had a significantly higher number of neutrophils than male (30.14 cells) ($p < 0.05$) (**Figure 2**).

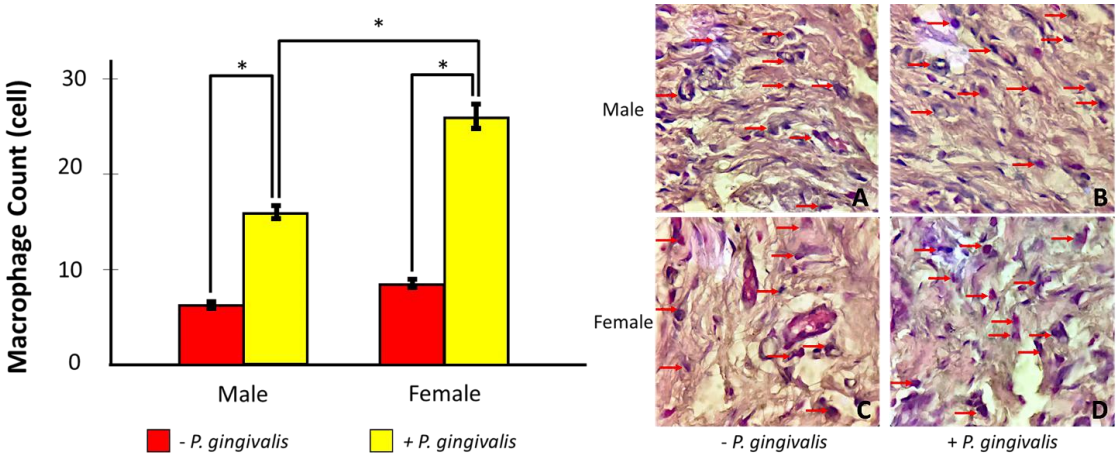

Figure 3 Number of macrophages in the gingival tissue of male and female rats at day 19. The figure presented the mean and standard error values; * indicated a significant difference analyzed Independent T-Test ($p < 0.05$); A, male rats without *P. gingivalis* injection; B, male rats with *P. gingivalis* injection; C, female rats without *P. gingivalis* injection; D, female rats with *P. gingivalis* injection; red arrows, macrophages in the gingival tissue.

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The number of macrophages present in the gingival tissue is a key indicator of inflammation caused by infection. In this study, similar to the assessment of neutrophil counts, the results demonstrated that the number of macrophages in male and female rats injected with *P. gingivalis* was significantly higher than in rats not injected with the bacteria ($p < 0.05$). Furthermore, female rats injected with *P. gingivalis* had a significantly higher number of macrophages than their male counterparts ($p < 0.05$) (**Figure 3**).

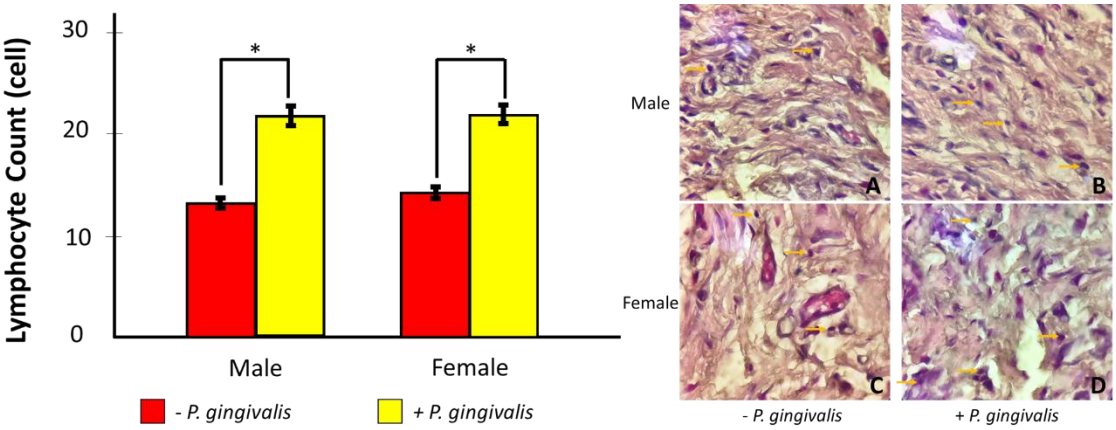

Figure 4 Number of lymphocytes in the gingival tissue of male and female rats at day 19. The figure presented the mean and standard error values; * indicated a significant difference analyzed Independent T-Test ($p < 0.05$); A, male rats without *P. gingivalis* injection; B, male rats with *P. gingivalis* injection; C, female rats without *P. gingivalis* injection; D, female rats with *P. gingivalis* injection; yellow arrows, lymphocytes in the gingival tissue.

Unlike neutrophils and macrophages, which are involved with phagocytosis, lymphocytes play a key role in recognizing pathogens. In this study, the number of lymphocytes in male and female rats injected with *P. gingivalis* was higher than that in rats not injected with *P. gingivalis* ($p < 0.05$). However, the study found that the number of lymphocytes in female rats injected with *P. gingivalis* was similar to that in male rats injected with the same *P. gingivalis* ($p > 0.05$) (**Figure 4**).

This study revealed that the number of leukocytes was significantly higher in rats injected with *P. gingivalis* than in those without *P. gingivalis* injection. *P. gingivalis* might stimulate and activate immune cells in the gingiva, leading to an inflammatory response. Previous studies revealed that periodontitis caused by *P. gingivalis* infection leads to heightened recruitment of neutrophils and macrophages. In addition to the influx of immune cells, there was a release of inflammatory mediators in the periodontal tissue, gingival sulcus, and peripheral blood [35-38]. Inflammatory mediators released by immune cells facilitate the migration and infiltration of leukocytes into the gingival tissue [39]. Beside it, TNF-α triggered vasodilation of vascular. The vasodilation allows leukocytes to exit blood vessels more easily and migrate to the site of inflammation. Neutrophils initiate the infiltration of leukocytes into periodontal tissues, followed by macrophages, lymphocytes, plasma cells, and mast cells [40,41]. However, the present study focused on neutrophils, macrophages, and lymphocytes.

In addition, the number of leukocytes in the gingival tissue of female rats injected with *P. gingivalis* was significantly higher than that of male rats injected with *P. gingivalis*. This difference in leukocyte counts between the sexes might be related to hormonal differences that affect the immune response to *P. gingivalis* infection. Sex hormones such as estrogen and testosterone influence the ability of the immune system to eliminate *P. gingivalis* through various mechanisms, including the recruitment, infiltration, and migration of leukocytes. These hormones affect immune responses differently in males and females. In women, estrogen and progesterone modulate the inflammatory response by enhancing the production of cytokines to eliminate periodontal pathogens. In contrast, testosterone in men is immunosuppressive, which reduces certain

components of the immune response [42-44]. Although estradiol enhances the virulence of *P. gingivalis* and increases immune cells such as neutrophils and macrophages [17,45,46], this study did not specifically investigate the influence of hormones on the inflammatory response in male and female rats injected with *P. gingivalis*. In contrast, male hormones promote a vascular response by increasing capillary blood vessel vasodilation in the gingiva, which facilitates leukocyte migration [47,48].

In this study, the number of lymphocytes in male rats injected with *P. gingivalis* was not significantly different from that in female rats injected with *P. gingivalis*. The role of lymphocytes in the immune system is consistent across both sexes, with men and women sharing the same functions and capabilities, particularly those of T lymphocytes. However, this study did not differentiate T and B lymphocytes. In response to *P. gingivalis*, both male and female lymphocytes are involved in pathogen recognition, production of proinflammatory cytokines, stimulation of macrophage activity, and elimination of pathogens (excluding phagocytosis). Sex hormones such as estrogen and testosterone influence lymphocyte function. For instance, estrogen enhances B-lymphocyte activation and antibody production, whereas testosterone inhibits immunoglobulin production [49,50]. Estrogen in women regulate immune responses. Estrogen deficient reduce Toll-like receptor expression and downregulate inflammatory responses in human monocytes exposed to *P. gingivalis* lipopolysaccharide [51]. Although there were no significant differences in the total number of lymphocytes between men and women, the proportion of lymphocyte subpopulations, particularly T lymphocytes, may vary according to the sex [42,52-54].

Several studies have indicated that women have a higher risk of developing periodontitis in the absence of smoking and systemic disorders than men [4-6]. Furuta *et al.* [19] demonstrated that women are more susceptible to the virulence factors of *P. gingivalis*, leading to more severe inflammation than that observed in men. Correspondingly, several studies using animal models revealed that female rats exhibited periodontal inflammation, bone loss, and increased complexity of periodontal pathogen in the gingival sulcus than male rats [18,31,32,55]. This increased susceptibility in women is attributed to hormonal changes that they experience throughout their lives. The virulence of *P. gingivalis* is influenced by female sex hormones [17,56,57]. LPS decreased the synthesis and production of estradiol. LPS initiates an inflammatory response that leads to an elevation in pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). These elevated cytokines can disrupt the normal processes necessary for estradiol biosynthesis. [58-60]. Consequently, the deficiency of sexual hormones increased the virulence of *P. gingivalis*. A decrease in these hormones increases the susceptibility to infection and inflammation in periodontal tissues [51]. Additionally, the administration of LPS decreases the production of estrogen and progesterone, both of which play critical roles in the remodeling and regeneration of periodontal tissue [61-63].

This study demonstrated differences in inflammatory responses between male and female rats after injection with *P. gingivalis*; however, this study has a few limitations, the role of hormones from both sexes in the inflammatory process associated with this infection was not investigated in this study. Furthermore, the study did not examine the specific types of lymphocytes involved in the inflammatory response to *P. gingivalis* in either sex, despite the fact that both sexes exhibit different lymphocyte activation.

Conclusions

In conclusion, female rats demonstrated a stronger inflammatory response than male rats after being injected with *P. gingivalis* 7 times over 19 days. While this study could not identify the underlying causes of the differences in inflammatory responses between male and female rats, it may serve as a reference for future research on factors influencing host responses to *P. gingivalis* in both sexes. The clinical implications of this study suggest that further investigation is necessary regarding factors that impact health and host responses in both men and women, enabling more tailored prevention, diagnosis, and treatment strategies. Therefore, it is essential for everyone to prioritize their oral health by visiting a dentist regularly to manage plaque effectively, as treatment and plaque control may differ between the sexes.

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