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Matrix Metalloproteinase-8 Expression and Collagen Density Alterations in Male and Female Rat Models of *Porphyromonas gingivalis*-Induced Periodontitis

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ABSTRACT

Periodontitis is periodontal tissue inflammation caused by periodontal pathogens, including *Porphyromonas gingivalis* (*P.gingivalis*). The incidence of periodontitis in men and women differs significantly, especially in the severity of periodontal tissue destruction, if it overrules smoking habits and poor oral hygiene. However, the information was inadequate and debatable. This study aimed to compare the effect of *P. gingivalis* on periodontal tissue destruction in female and male rat models, signed increased MMP-8 expression and decreased collagen density. This study was a laboratory experiment using male and female Wistar rats, aged 10–12-month-old, in estrous phase. Each sex of animal models was divided into two groups: control and periodontitis. The periodontitis group induced *P. gingivalis* every three days for 19 days in the distopalatal and distobuccal gingival sulcus of the mandibular first molar. After the final induction, they were euthanized, and their mandibles were removed for histological observation by immunohistochemistry to determine the expression of MMP-8 in periodontal tissue and Picrosirius Red to determine collagen density. Expression of MMP-8 and collagen density was observed at 400x magnification using a light microscope. However, the images of collagen density were analyzed using the ImageJ application. The data were analyzed using ANOVA and a multiple-comparison test ($p < 95\%$). The MMP-8 expression in female periodontitis models significantly (25.71 ± 3.04 cells) was higher than male periodontitis model (19.14 ± 1.77 cells) ($p < 0.05$). Therefore, the collagen density in male periodontitis model ($51.27 \pm 6.05\%$) was higher than female periodontitis models ($45.04 \pm 4.24\%$) ($p < 0.05$). Briefly, *P. gingivalis* caused periodontal tissue damage in female and male periodontitis models, whereas the female periodontitis model experienced more periodontal tissue damage than the male.

Keyword: Collagen density; Female Periodontitis Model; Male Periodontitis Model; MMP-8 expression; *Porphyromonas gingivalis*

Introduction

Periodontal disease is a chronic inflammatory disease that disrupts the integrity of tooth-supporting tissues, leading to bone resorption and

tooth loss. Periodontitis affects almost the entire global population across various age groups, approximately 10-15% [1]. The prevalence of periodontitis also differs between men and women, although most studies indicated there was no

difference by considering smoking habits, systemic diseases, and oral hygiene [2,3]. Based on the 2021 Global Burden of Disease report, the Decree of the Minister of Health of the Republic of Indonesia in 2024 described that periodontal disease is more prevalent in women than in men, about 13%, and tends to increase with age, with a peak prevalence at 60-64 years, with a 67% higher prevalence in the elderly [4].

The primary cause of periodontitis is subgingival plaque containing periodontal pathogens. The host and periodontal pathogens, especially *Porphyromonas gingivalis* (*P. gingivalis*), triggers inflammation and damage to periodontal tissues [5]. *P. gingivalis* is considered the main etiological agent in the pathogenesis and development of periodontal disease. These bacteria account for a large proportion of periodontal pathogens in the gingival sulcus of patients with periodontitis, especially women [6]. Several studies have revealed that *P. gingivalis* is more dominant in the periodontal sulci during each life phase of women [7–10]. Moreover, *P. gingivalis* can attach to the host, evade and deceive the host's immune system, and interact strongly with the host and other microbes, thereby triggering dysbiosis in the gingival sulcus [11].

The interactions activate the inflammatory signaling cascade through inflammatory pathway, which involves immunocompetent and non-immunocompetent cells of periodontal tissue. This pathway influences the leukocyte response in infiltrating periodontal tissue and producing inflammatory cytokines [12]. These inflammatory cytokines activate leukopoiesis and chemoattractants, resulting in the migration and infiltration of leukocytes into the periodontal tissue. Leukocytes produce inflammatory cytokines as an action to eliminate *P. gingivalis*. Moreover, they produce matrix metalloproteinase to degrade matrix extracellular and facilitate immune cell migrate in periodontal tissue. If the activation of MMPs is excessive, it will trigger the severity of periodontitis, especially MMP-8 [13–16]. Meanwhile, excessive activity of MMP-8 due to *P. gingivalis* infection causes excessive destruction of the collagen matrix and a decrease in gingival collagen density. Meanwhile, this causes structural weakness of periodontal tissue, which in turn can worsen periodontal conditions such as increasing periodontal pocket depth, bone resorption, and loss of tooth attachment [17].

Moreover, the excessive immune response and tissue remodeling induced by this interaction

are influenced by genetic factors, systemic disorders, smoking and oral hygiene habits, and sex hormones [18–20]. Sex hormones significantly influence periodontal bacterial activity, and the incidence of periodontitis differs significantly between men and women [21]. Several studies have revealed that gingival inflammation tends to occur in women, especially during menstruation and pregnancy [22–24]. However, these findings are still a matter of debate, not only because of differences in hormonal factors between men and women but also because of other factors that can influence them, such as smoking habits and oral hygiene practices. These habits might confound the incidence of periodontitis and the virulence of *P. gingivalis* in both sexes [25]. This study aimed to compare the effect of *P. gingivalis*, the main periodontal pathogen, on periodontal tissue damage in female and male rat models.

Methods

With the number 0029/KKEP/FKG-UGM/EC/2022, the Ethics and Advocacy Commission of the Faculty of Dentistry at Gajah Mada University approved this study. This study used Wistar rats (*Rattus norvegicus*) in both sexes, male and female. They aged 10–12 weeks (equivalent to the productive age range of 25–35 years) met the criteria for experimentation. The sample size of this study was determined using Lemeshow's formula, which indicated that each group required seven rats [26]. The total number of experimental animals was 14 male and 14 female rats each. Each sex was divided into two groups: healthy and *P. gingivalis*-induced periodontitis. All experimental animals were randomly assigned to prevent bias.

P. gingivalis was a commercial strain collection (*Porphyromonas gingivalis* ATCC 33277, Thermo Fisher Scientific, USA). The *P. gingivalis* stock was introduced into solid Brain Heart Infusion Agar (BHI-A) (Oxoid, USA). The plate was subsequently placed in a desiccator for two 24-hour periods and supplied with CO₂ gas (Oxoid, USA) to establish anaerobic conditions. Following this, a single colony of *P. gingivalis* was extracted from the BHI-A and transferred into 2 mL of Brain Heart Infusion Broth (BHI-B) (Oxoid, USA) supplemented with vitamin K and hemin. The mixture was then homogenized using a vortex (Thermo Fisher Scientific, USA) for 30 seconds and incubated in a CO₂ gas-filled desiccator at 37°C for 24 hours. This process resulted in the turbidity of

the BHI medium, which was subsequently diluted with sterile distilled water and mixed until uniform. The concentration was manually determined using the 1.5 McFarland standard, equating to 2.109 cells/ml [27].

The *P. gingivalis* suspension was administered into the distobuccal gingival sulcus and the distolingual area of the first molar in the mandible, with a dosage of 0.05 ml. This was done using a tuberculin syringe equipped with a 30-gauge needle every three days over a period of 19 days (total 7 times injections) [27,28]. Clinical and radiological examinations were performed before induction. At the time of the last injection, the gingiva in the first and second molar areas appeared reddish, and there was a loss of attachment with tooth mobility of the first and second molars to the 2nd degree. Radiography results showed a decrease in the alveolar crest and a break in the lamina dura, indicating bone resorption. Following the final injection, the animal models were euthanized with a lethal dose of ketamine/xylazine, and the mandibular tissue was extracted. The mandibular bone samples were then washed and fixed in a 10% formalin buffer. Subsequently, the specimens underwent decalcification in a 10% EDTA solution for 30 days to remove inorganic materials, excluding inorganic bone components, while preserving the proteins [29].

Paraffin-embedded tissue was cut into 5µm, then deparaffinized with xylol, and rehydrated through a graded alcohol series, finally with PBS pH 7.4 for 5 min. MMP-8 expression in periodontal tissue was examined using immunohistochemical staining (Elabscience, USA). Subsequently, endogenous peroxidase blockade was performed using 3% H₂O₂ solution for 20 min, followed by washing with PBS (pH 7.4) three times for 5 min each. Non-specific binding blockade was carried out by incubating with 5% FBS containing 0.25% Triton X 100, followed by washing with PBS pH 7.4 three times for 5 min. The preparation was then incubated with anti-rat MMP-8 primary antibody for 60 min and washed with PBS pH 7.4 three times for 5 min, followed by incubation with anti-rat HRP conjugate secondary antibody for 40 min and washing with PBS pH 7.4 three times for 5 min. The enzymatic reaction was visualized using the DAB chromogen substrate, which was incubated for 10 min. The preparation was then washed with PBS

(pH 7.4), rinsed with water, and counterstained with Mayer's hematoxylin for 10 min. After rinsing with running water and drying, the preparation was mounted with Entellan, covered with a cover glass, and observed using a 400x light microscope [27].

The other slides were immersed in Picrosirius Red solution (0.1% Sirius Red in saturated picric acid) for ±60 min at room temperature to allow the dye to selectively bind to the collagen fibers. After incubation, the slides were briefly rinsed with picric acid or distilled water and differentiated using acidic or weak alcohols to reduce background staining. They were then rehydrated using graded alcohol, cleared with xylene, and covered with a non-aqueous mounting medium and coverslips. The slides were then observed under a conventional light microscope to reveal bright-red collagen against a pale-yellow background. The collagen density percentage was calculated using ImageJ software, a Java-based digital image processing software. Image J. After the image appears, analysis is carried out using the "Plugin" in Image J; after that, "Immunization" and the results are displayed in image form, with an expression percentage. The data were analyzed using the Shapiro-Wilk and Levene tests for normality and homogeneity ($p > 0.05$), respectively. One-way analysis of variance (ANOVA) was followed by the least significant difference (LSD) test to determine the significance of the differences between groups ($P < 0.05$).

Results and Discussion

Periodontitis-induced *P. gingivalis* infection stimulates damage to periodontal tissue. Sex differences determine the severity of periodontitis. Periodontal tissue damage was assessed by MMP-8 expression and percentage of collagen density. This study showed periodontal tissue in the female-periodontitis model was more severe than the male-periodontitis model. The expression of MMP-8 in female periodontitis model (25.71 ± 3.04) was higher than male periodontitis model (19.14 ± 1.77 cells). However, the percentage of collagen density of healthy model was more than periodontitis models, in both sexes. Therefore, in periodontitis groups, collagen density in female periodontitis model ($45.04 \pm 4.24\%$) was lower than male periodontitis model ($51.27 \pm 6.05\%$).

Table 1. Periodontal Tissue Destruction of Male and Female Periodontitis Models (n=28)

Variable	Baseline		Periodontitis Models	
	Male	Female	Male	Female
MMP-8 expression (cells)	8.43±1.98	8.57±2.63	19.14±1.77	25.71±3.04*
Collagen density (%)	68.22±6.26	64.6±5.72	51.27±6.05	45.04±4.24*

The data presented the mean and standard deviation analysed by one-way analysis of variance ($p<0.05$); *, there was a significant difference between the genders; n, the total number of samples in this study

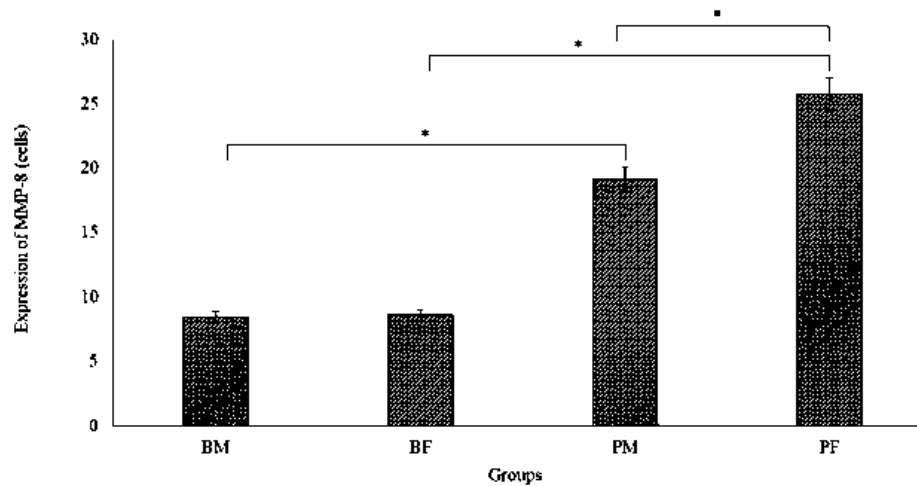


Figure 1. MMP-8 Expression in periodontal tissue of animal models.

Figure presented the mean and standard error of each group analysed by multiple comparison test ($p<0.05$); BM, male baseline; BF, female baseline; PM, male periodontitis models; PF, female periodontitis models; *, significant difference ($p<0.05$)

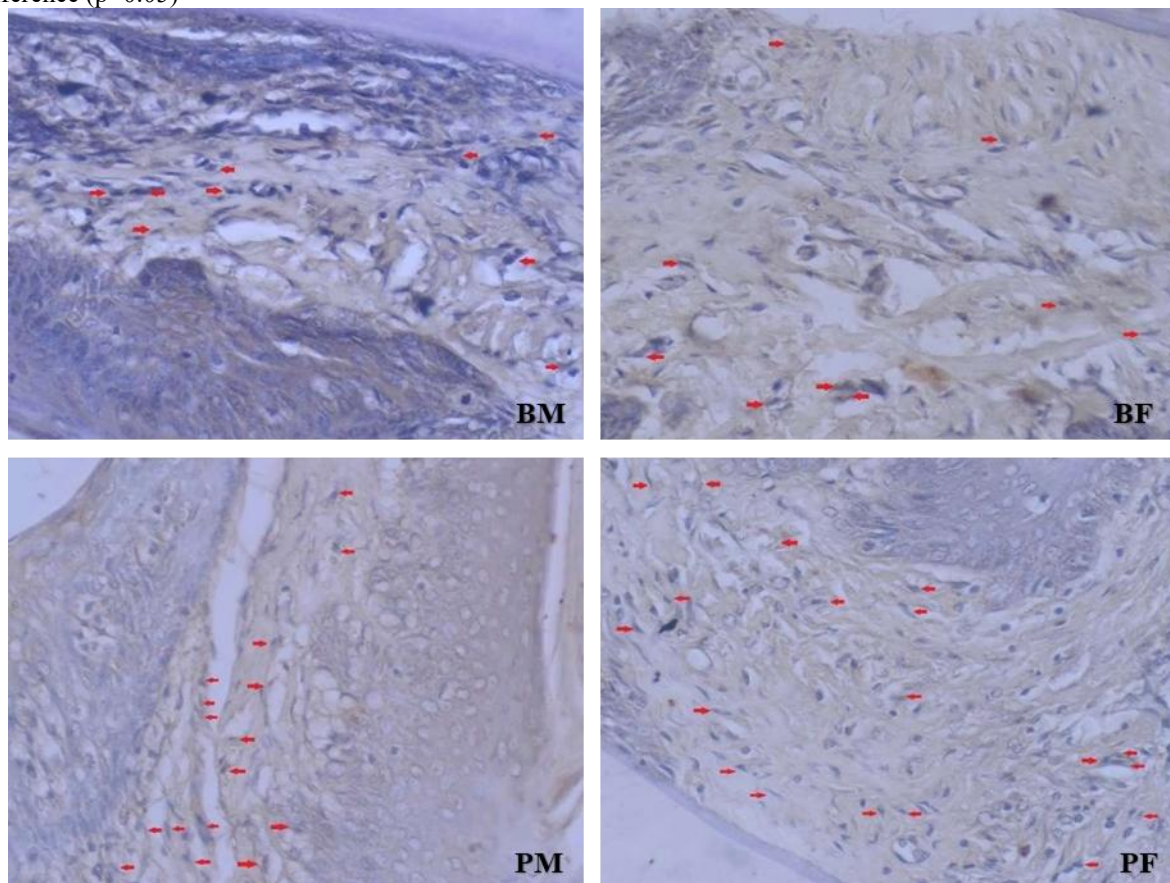


Figure 2. MMP-8 Expression in periodontal tissue of animal models.

Activated expression of MMP-8 was shown as brown in the cytoplasm of fibroblasts of periodontal tissue (red arrow) at 400× magnification; BM, male baseline; BF, female baseline; PM, male periodontitis models; PF, female periodontitis models

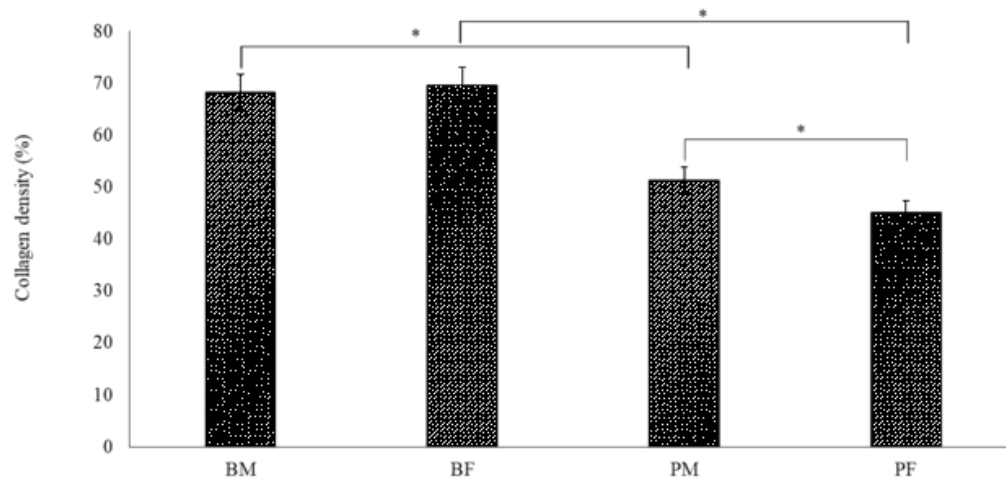


Figure 3. Collagen density of periodontal tissue of animal models.

Figure presented the mean and standard error of each group analysed by multiple comparison test ($p < 0.05$); BM, male baseline; BF, female baseline; PM, male periodontitis models; PF, female periodontitis models; *, significant difference ($p < 0.05$)

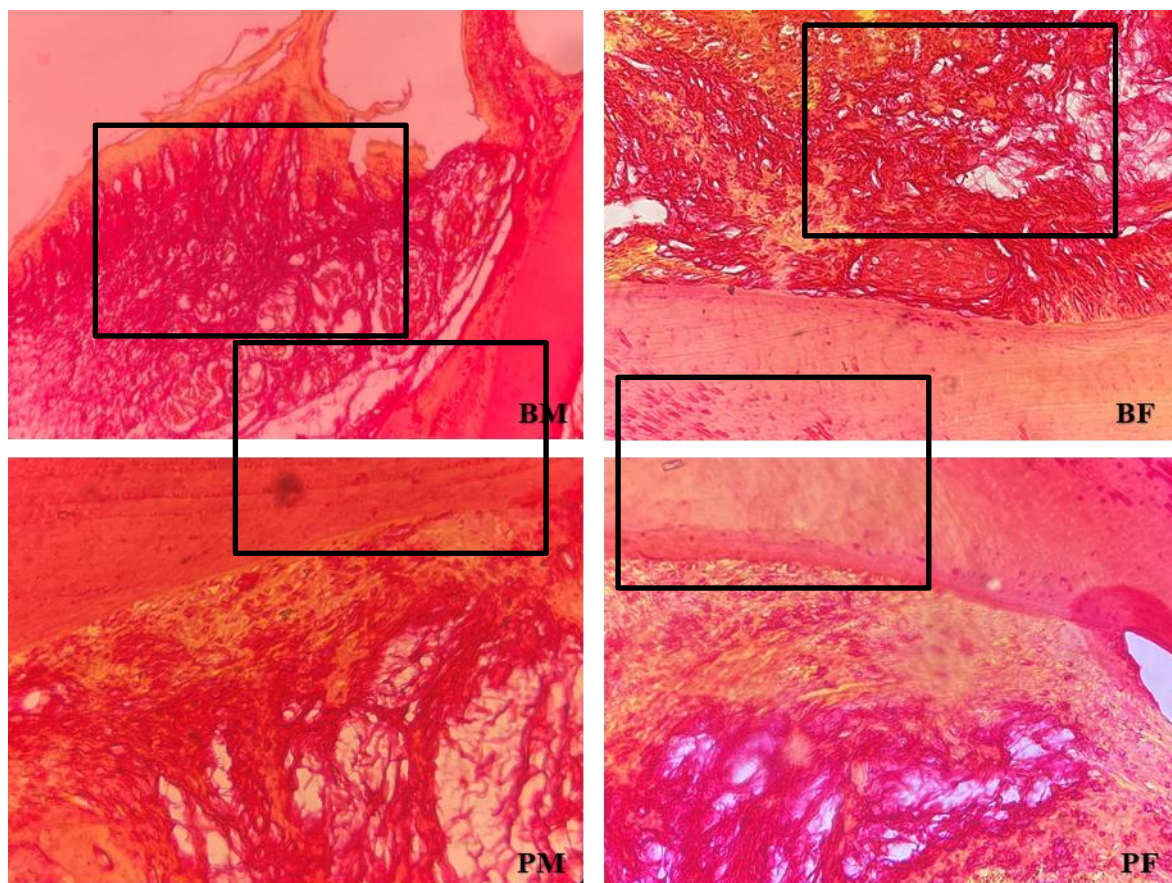


Figure 4. Collagen density in periodontal tissue of animal models.

Percentage of collagen density in black box, at 400× magnification; BM, male baseline; BF, female baseline; PM, male periodontitis models; PF, female periodontitis models

The results of the multiple comparison analysis showed a significant difference in the average expression of MMP-8 between the healthy mouse model group and the periodontitis model group ($p < 0.05$). In the periodontitis group, the female periodontitis model exhibited a significantly higher average MMP-8 expression than the male periodontitis model ($p < 0.05$) (Figures 1 and 2).

Similarly, when measuring the percentage of collagen density, the healthy mouse model group had a significantly higher collagen density than the periodontitis model in both males and females. In the periodontitis group, the percentage of collagen density in the male periodontitis model was significantly higher than that in the female periodontitis model ($p < 0.05$) (figures 3 and 4).

The periodontitis model in this study used *P. gingivalis* induction once every three days for 19 days in female and male rats as an animal model. This study used two sexes of experimental animals to determine whether the progression of periodontitis was influenced by sex. The results indicated that *P. gingivalis* induction triggered inflammation and periodontal tissue deterioration in both female and male periodontitis models, which was marked by increase MMP-8 expression and decreased collagen density. The induction of *P. gingivalis* might trigger the adhesion and colonization of this bacterium in periodontal tissue so that *P. gingivalis* and its virulence factors provoked periodontal tissue damage and an excessive immune response in periodontal tissue. *P. gingivalis* is the dominant periodontal pathogen in chronic periodontitis and triggers microbiome dysbiosis in the gingival sulcus area, forming complex pathogen colonies. Complex bacterial colonies stimulate inflammation in periodontal tissue to trigger periodontal tissue damage, including connective tissue [30,31].

The collagen density percentage was higher in the control group than in the periodontitis group in both sexes. Healthy periodontal tissue maintains a balance between collagen synthesis and degradation, resulting in a relatively dense and organized collagen matrix. However, in disease conditions, such as periodontitis, exposure to periodontopathogenic bacteria can trigger an immune response characterized by increased pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-17), which in turn triggers the increased expression of matrix metalloproteinases (MMPs) and collagenase enzymes. This condition causes an imbalance, resulting in the degradation of collagen in the periodontal ligament and gingival lamina

propria. Consequently, histological examination revealed a decrease in collagen density in the periodontitis group [32,33].

Moreover, *P. gingivalis* virulence factors, such as lipopolysaccharide (LPS), presumably reduced sexual hormone levels in female-periodontitis models, especially estrogen and progesterone. A decrease in these hormones increases susceptibility to infection, inflammation and descent of periodontal tissue. Several studies revealed that LPS treatment declines the production of estrogen and progesterone [34–37]. These two hormones play a significant role in the remodelling and regeneration of periodontal tissue [23,38,39].

These hormonal changes will affect the immune system and inflammation in periodontal tissue, where there is an increase in leukocyte activity and their migration to periodontal tissue to fight infection and inflammation [39,40]. Besides, while there are bacteria, the host responds by increasing immunocompetent inflammatory cells. The proliferation and infiltration of leukocytes in periodontal tissue are signs of periodontitis lesions. Bacterial metabolic products trigger functional epithelial cells to produce cytokines and cause local vascular vasodilation [41,42]. This vasodilation facilitates leukocytes to leave the blood vessels and migrate to the site of inflammation. The increase in leukocytes begins with neutrophil infiltration in connective tissue accompanied by macrophages, lymphocytes, plasma cells and mast cells [41,43].

This study showed periodontal tissue deterioration, including the connective tissue. Periodontal tissue damage in the female-periodontitis model was more severe than in the male-periodontitis model. Induction of *P. gingivalis* might induce loss of attachment. Periodontitis lesions are also characterized by attachment loss. The attachment loss is caused by proteolytic enzymes in periodontal tissue, such as MMP-8, which also decrease collagen density [42,44]. It was proven that the expression of MMP-8 in the connective tissue of female-periodontitis models was higher than that of male periodontitis models. This result aligns with the decrease in collagen density in connective tissue, which is also more in the female periodontitis model than in the male periodontitis model. MMP-8 expression increases due to increased neutrophil activity in periodontal tissue due to the host response to *P. gingivalis* induction and hormonal changes. Increased MMP-8 secretion causes connective tissue damage and decreases collagen density in periodontal tissue [14,45,46].

Estrogen plays a crucial role in periodontal tissue homeostasis, promoting tissue differentiation-remodeling and supporting collagen and tissue formation. However, estrogen fluctuations or deficiencies can disrupt the remodeling balance, increasing bone resorption and periodontal tissue destruction. Animal studies on estrogen deficiency have shown more severe periodontal damage, with greater bone loss and matrix degradation. This condition may explain the lower collagen density in females in the periodontitis model. In addition, females tend to generate more intense immune and inflammatory responses, including higher expression of proinflammatory cytokines, which accelerate collagen and bone destruction in periodontitis [33,47,48].

The limitation of this study was only to compare periodontal tissue damage (MMP-8 expression and collagen density) between male and female mice but did not examine estrogen and progesterone levels or other contributing hormones. Consequently, a direct causal relationship between hormonal changes and differences in periodontal tissue damage cannot be established and can only be indirectly inferred from the literature.

Conclusion

Briefly, *P. gingivalis*, the primary periodontal pathogen, influenced periodontal tissue damage in female and male periodontitis models. However, the severity of inflammation and periodontal tissue damage in the two periodontitis models differed, whereas the female periodontitis model experienced more severe tissue damage. However, the study did not detail the relationship between periodontal tissue damage and hormone levels. In addition, this study can be the basis for further research to explore the involvement of sex hormones in periodontitis. The implication is periodontitis in women needs specific awareness because it may pose a risk of triggering reproductive system disorders. Women need to maintain their oral health and visit the dentist regularly to control plaque, especially during their each phases.

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