scientific reports

OPEN

Periodontitis associated with *Porphyromonas gingivalis* infection is a risk factor for infertility through uterine hypertrophy

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Periodontitis has recently been recognized as a potential risk factor for infertility due to its adverse effect on conception, although the underlying mechanisms remain unclear. This study investigated serum IgG antibody titers against periodontopathogenic bacteria in women with unexplained infertility and investigated how periodontal inflammation affects pregnancy and uterine function using a ligature-induced periodontitis mouse model infected with *Porphyromonas gingivalis* (Pg). IgG antibody titers against seven periodontopathogenic bacteria strains were measured by ELISA in 76 spontaneously pregnant women and 70 women undergoing infertility treatment. In the in vivo study, periodontitis mice were bred four weeks after periodontitis induction. Birth numbers, newborn weights, and gestation periods were assessed. To evaluate periodontal inflammation, alveolar bone, serum, and uterus was collected before mating. Uterine tissue was evaluated through histological and immunohistochemical staining. Women receiving infertility treatment were significantly older and had higher IgG titers against three Pg strains. Periodontitis mice had fewer births, lower newborn weights, and increased uterine cross-sectional areas. Additionally, elevated estrogen receptor α and progesterone receptor expression levels were observed in endometrial and stromal tissues. These results suggest that periodontitis may cause uterine hypertrophy and hormone receptor changes, potentially impairing pregnancy.

Keywords Infertility, Periodontitis, *Porphyromonas gingivalis*, Chronic inflammation, Uterus, Sex hormone receptor

The World Health Organization (WHO) defines infertility as "a disease of the male or female reproductive organs that prevents pregnancy despite having regular unprotected sexual intercourse for more than 12 months." Approximately 17.5% of adults experience infertility[1]. In Japan, more than one in three couples are reported to be suffering from infertility. Furthermore, 22.7% of couples have undergone infertility testing or treatment, and 6.7% of those married for less than five years have pursued testing or treatment[2]. In recent years, the number of individuals seeking infertility treatment in Japan has been steadily increasing. A report by the Japan Society

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of Obstetrics and Gynecology indicates that 77,206 babies were born through assisted reproductive therapy (ART) in 2022[3]. Considering that the Ministry of Health, Labour and Welfare reported a total of 770,747 births in the same year[4], this suggests that approximately one in ten babies born in Japan was conceived through ART. Additionally, for couples undergoing fertility treatment, infertility represents a significant social issue, leading to physical pain, mental distress, and financial strain[5].

Both male and female factors contribute to infertility. According to WHO, male factors account for 24%, female factors for 41%, and both male and female factors for 24%[6]. Male infertility issues include testicular dysfunction, ejaculation problems, and sperm passage difficulties. In women, causes encompass ovarian dysfunction, polycystic ovary syndrome, endometriosis, uterine fibroids, endometrial polyps, and chronic endometritis. There are various risk factors associated with infertility for both men and women, including hypogonadism, hyperprolactinemia, ciliary dysfunction, cystic fibrosis, infections, systemic diseases, and lifestyle choices[7]. Even after tests such as ovulation function, fallopian tube patency, and semen analysis, it has been reported that the cause of infertility remains unidentified in 10–30% of couples undergoing infertility treatment[8,9]. This type of infertility is termed unexplained infertility, as no clear cause can be identified even after a thorough evaluation[10]. The regulation of the uterine environment is crucial for establishing pregnancy, with estrogen, progesterone, and their respective receptors serving as essential factors. During the menstrual cycle, estrogen levels rise as the follicle develops, peak at ovulation, and then decline. Simultaneously, as estrogen levels decrease, progesterone levels increase, prompting the decidualization of the endometrium during the luteal phase and creating a receptive window for implantation[11]. Once implantation occurs and pregnancy advances, progesterone levels remain elevated. These processes are mediated by the expression of hormone receptors. Notably, estrogen receptor (ER)-α knockout mice exhibit a hypoplastic endometrium and infertility, indicating the critical role of ER-α in implantation[12]. Similarly, progesterone receptor (PR) knockout mice show a significant decrease in ovulation, uterine enlargement, and defective decidualization, ultimately leading to infertility[13].

Age, smoking, obesity, being overweight, and even stress are recognized risk factors for infertility[14]. Additionally, recent study has linked periodontitis, a chronic inflammatory disease of the mouth, to infertility[15]. Periodontitis occurs due to an imbalance in the normal oral bacterial flora, which shifts due to an increase in periodontopathogenic bacteria, such as *Porphyromonas gingivalis* (Pg). This bacterium infects and proliferates in mature gingival sulci, triggering an immune response that prompts immune cells and gingival fibroblasts to produce various inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. These cytokines further activate immune cells, resulting in inflammation[16]. Such effects prolong the immune and inflammatory response[17]. Consequently, the destruction of periodontal tissue extends to deeper layers, with alveolar bone resorption progressing[18]. Regarding the relationship between female infertility and periodontitis, studies show that women with high levels of immunoglobulin (Ig)A and IgG antibodies to periodontal bacteria in their saliva have a lower pregnancy rate than those with lower levels[19]. Additionally, women with periodontitis take longer to conceive. Reports indicate that the time from conceptualization to actual pregnancy is significantly longer for women with periodontitis compared to those without[14]. Moreover, the incidence of caries and periodontitis is higher in patients experiencing unexplained infertility[20]. In contrast, regarding male infertility and periodontitis, male patients with periodontitis, characterized by calculus buildup and gingival bleeding, demonstrate a higher rate of symptoms related to decreased reproductive function, such as oligospermia and asthenospermia[21]. Although several clinical epidemiological studies have identified a link between periodontitis and infertility, the actual prevalence of periodontitis, including the level of periodontal pathogen infection (serum IgG antibody titer) among patients undergoing infertility treatment, remains unknown. Furthermore, the underlying mechanisms connecting infertility and periodontitis are still unclear.

In the clinical study, we examined the relationship between infertility and periodontitis by comparing serum IgG antibody titers against periodontal pathogens in women experiencing infertility with those who conceived naturally. In an in vivo study, we assessed the impact of infection and inflammation in periodontal tissues on pregnancy outcomes and the uterus using a mouse model of periodontitis induced by Pg-infected silk ligature and analyzed histologically and immunologically.

Results

High serum IgG antibody titers against periodontopathogenic bacterial strains in women with unexplained infertility

In this clinical study, 101 spontaneously pregnant women and 100 patients with unexplained infertility were enrolled. In the spontaneously pregnant group, 99 subjects were included after excluding one subject under 30 years of age and one subject who did not consent to the study. Next, one subject with an unknown delivery outcome, one subject with an insufficient serum sample, and two subjects with unknown delivery status were excluded. The delivery methods were classified as follows: 76 pregnant women had vaginal deliveries, 17 delivered via cesarean section, and 2 delivered via aspiration. In the infertility treatment group, 98 patients were included, excluding one subject under 30 years of age and one subject whose information was insufficiently collected due to interrupted hospital visits. Patients were classified based on whether the cause of infertility was obvious or unexplained. One participant with an insufficient serum sample was also excluded. We analyzed 76 pregnant women who had vaginal deliveries, a more natural form of childbirth (natural pregnancy group), and 70 patients with unexplained infertility (infertility treatment group) (Fig. 1).

The backgrounds of spontaneously pregnant women and patients with unexplained infertility were analyzed (Table 1). The average age of patients with unexplained infertility was significantly higher than that of naturally pregnant women ($P < 0.05$). Among the 70 patients with unexplained infertility, 28.6% (20 patients) had been infertile for less than 1 year, 18.6% (13 patients) for 1 to 2 years, and 41.4% (29 patients) for more than 2 years. The stages of infertility treatment included the timing method in 45.7% (32 patients), artificial insemination in 22.9% (16 patients), and assisted reproductive technology in 28.6% (20 patients).

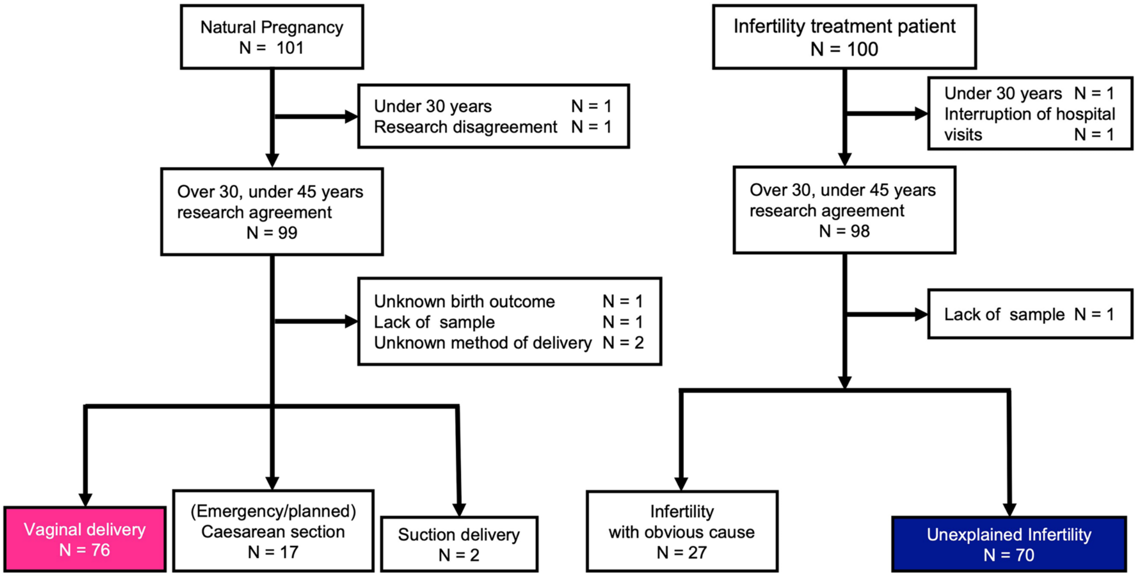


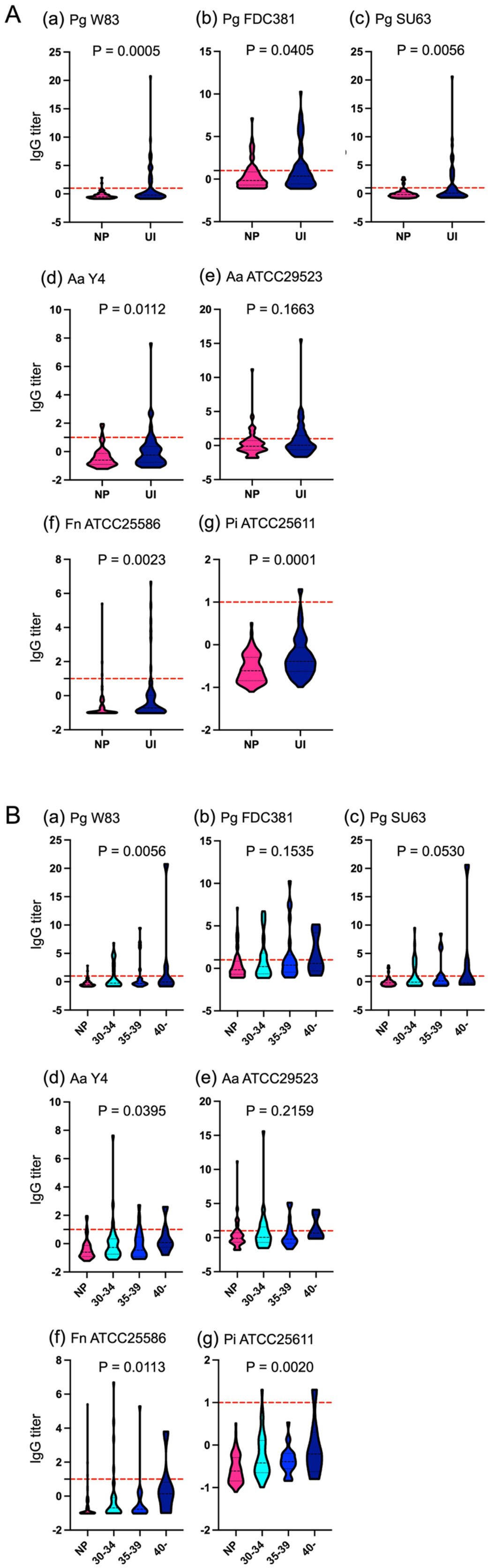
Fig. 1. Patient selection in clinical research Spontaneously pregnant women (n = 101) and patients with infertility (n = 100) were recruited. In the spontaneous pregnancy group, one woman under 30 years old and one woman who did not agree to participate in the study were excluded. Of the 99 women aged 30–45 who consented to the study, 95 were categorized into three groups based on the mode of delivery, excluding one woman whose delivery outcome was unknown, one woman whose serum sample was insufficient for measurement, and two women for whom the delivery status was unknown. Among the patients undergoing infertility treatment, one patient under 30 years old and one patient who had stopped visiting the clinic, with incomplete information, were excluded. The remaining 98 patients were divided into two groups based on whether the cause of infertility was known or unknown, excluding one woman whose serum sample was insufficient for measurement.

Items	Natural pregnancy (n = 76)	Unexplained infertility (n = 70)	*P* value
Age (years)	33	35	0.0251
Age distribution (years)			
30–34	52	39	
35–40	22	24	
> 40	2	7	
Duration of infertility (years)			
< 1 year		20	
> 1 but < 2 years		13	
> 2 years		29	
Period unknown		8	
Infertility treatment Stage			
Timing method		32	
Artificial insemination		16	
Assisted reproductive medicine		20	
Interruption of hospital visits		2	

Table 1. Patient background.

Serum antibody titers against periodontopathogenic bacterial strains were measured and compared. Serum IgG antibody titers against Pg W83 ($P = 0.0005$), Pg FDC381 ($P = 0.0405$), Pg SU63 ($P = 0.0056$), *Aggregatibacter actinomycetemcomitans* (Aa) Y4 ($P = 0.0112$), *Fusobacterium nucleatum* (Fn) ATCC25586 ($P = 0.0023$), and *Prevotella intermedia* (Pi) ATCC25611 ($P = 0.0001$) were significantly higher in patients with unexplained infertility than in spontaneously pregnant women (Fig. 2A).

Furthermore, we compared the antibody titers of unexplained infertility based on age and duration of infertility treatment. When analyzed by age, serum IgG antibody titers against Pg W83 ($P = 0.0056$), Aa Y4 ($P = 0.0395$), Fn ATCC25586 ($P = 0.0113$), and Pi ATCC25611 ($P = 0.0020$) were significantly elevated in patients with unexplained infertility compared to those in spontaneously pregnant women (Fig. 2B). When



evaluated by duration of infertility, serum IgG antibody titers against Pg W83 ($P = 0.0041$), Pg SU63 ($P = 0.0446$), Fn ATCC25586 ($P = 0.0049$), and Pi ATCC25611 ($P = 0.0031$) were significantly elevated in patients with unexplained infertility compared to spontaneously pregnant women (Fig. 2C).

◀**Fig. 2**. Serum IgG antibody levels against periodontopathogenic bacteria: comparison between spontaneously pregnant and unexplained infertility and comparison by age and duration of infertility in patients with unexplained infertility (**A**) Serum IgG antibody titers against seven periodontopathogenic bacterial strains (**a**) Pg W83, (**b**) Pg FDC381, (**c**) Pg SU63, (**d**) Aa Y4, (**e**) Aa ATCC29523, (**f**) Fn ATCC25586, and (**g**) Pi ATCC25611 were measured in pregnant women (n = 76) who had a vaginal delivery and in patients with unexplained infertility (n = 70). Mann–Whitney *U* test was used for statistical analyses. (**B**) Serum IgG antibody titers against seven periodontopathogenic bacterial strains, (**a**) Pg W83, (**b**) Pg FDC381, (**c**) Pg SU63, (**d**) Aa Y4, (**e**) Aa ATCC29523, (**f**) Fn ATCC25586, and (**g**) Pi ATCC25611, were measured in pregnant women (n = 76) who delivered vaginally and in patients with unexplained infertility (n = 70). Patients with unexplained infertility were grouped by age, and statistical analysis was conducted using the Kruskal–Wallis test. (**C**) Serum IgG antibody titers against the seven periodontopathogenic bacteria strains (**a**) Pg W83, (**b**) Pg FDC381, (**c**) Pg SU63, (**d**) Aa Y4, (**e**) Aa ATCC29523, (**f**) Fn ATCC25586, and (**g**) Pi ATCC25611 were measured in pregnant women (n = 76) who delivered vaginally and in patients with unexplained infertility (n = 70). Patients with unexplained infertility were categorized based on the duration of their infertility, and statistical analysis was conducted using the Kruskal–Wallis test.

Effects of periodontitis associated with PG infection on pregnancy and delivery in a mouse model

High resorption of alveolar bone in mice with periodontitis
The in vivo experiment was conducted according to the schedule shown in Fig. 3. No teeth were lost at the time of sample collection in either group; however, 6 out of 9 mice in the 2-week periodontitis group and 14 out of 16 mice in the 4-week group experienced one or more tooth losses during the bone sample preparation. Additionally, alveolar bone resorption progressed significantly in all teeth of the periodontitis mouse group compared to the healthy group (2 weeks: first molar $P = 0.0026$, second molar $P < 0.0001$, third molar $P < 0.0001$; 4 weeks: first molar $P < 0.0001$, second molar $P < 0.0001$, third molar $P < 0.0001$; Fig. 4a).

Elevated serum IgG antibody levels against Pg in mice with periodontitis
Serum IgG antibody titers against Pg were significantly higher in the periodontitis mouse group compared to those in the healthy group at 2 weeks ($P = 0.0070$) and 4 weeks ($P = 0.0012$) after periodontitis induction (Fig. 4b).

Decreased birth rates and lower body weight of newborn mice in mice with periodontitis
The pregnancy and delivery outcomes of periodontitis mice and healthy mice were compared. Relative to the healthy group, the periodontitis mouse group exhibited a significantly lower number of births (median: 8 vs. 5, $P = 0.0019$, Fig. 5a), a significantly decreased number of live births (median: 8 vs. 4, $P < 0.0001$, Fig. 5b), a significantly increased number of fetal deaths (median: 0 vs. 1, $P = 0.0005$, Fig. 5c), and a significantly lower mean weight of newborn mice (median: 1.3 g vs. 1.2 g, $P = 0.0345$, Fig. 5d). However, there was no difference in gestational length (median: 21 days vs. 20 days, $P = 0.8858$, Fig. 5e).

Increased cross-sectional area of the uterus in mice with periodontitis
Two weeks after the induction of periodontitis, the uterus of the periodontitis mice did not differ significantly in size or cross-sectional area compared to that of the healthy group (median: 6.32 mm^2 vs. 6.70 mm^2, $P = 0.8633$). In contrast, four weeks after periodontitis induction, the uterus of the periodontitis mice was enlarged, showing a significant increase in cross-sectional area compared to the healthy group (median: 6.56 mm^2 vs. 12.21 mm^2, $P = 0.0093$, Fig. 6).

Increased expression of estrogen receptor alpha and progesterone receptor in the uterus of mice with periodontitis
Immunohistochemical staining showed that in control mice, ERα expression was primarily observed as DAB signals in the nuclei of stromal cells. In contrast, in periodontitis mice, strong DAB signals were detected in the nuclei of both endometrial and stromal cells (Fig. 7a–h). Similarly, PR expression was markedly increased in the nuclei of endometrial and stromal cells in periodontitis mice compared to controls, as evidenced by stronger DAB signals (Fig. 7i–p).

Discussion

In this study, we examined the serum IgG antibody titers against periodontal pathogens in women with unexplained infertility who are undergoing infertility treatment. We also explored the immunological and histological impacts of periodontal tissue infection and inflammation on pregnancy, delivery, and uterine tissue, using a ligature-induced mouse periodontitis model.

In a clinical study, serum IgG antibody titers against seven strains of periodontopathogenic bacteria were measured in patients with unexplained infertility undergoing treatment and in spontaneously pregnant women. It was found that patients with unexplained infertility had higher levels of serum IgG titers with three Pg strains, one Aa strain, Pi, and Fn compared to spontaneously pregnant women (Fig. 2A). In comparing the backgrounds of the subjects, the age of patients with unexplained infertility was significantly higher than that of spontaneously pregnant women (Table 1). Additionally, when comparing patients with infertility by age, differences were observed between the groups for one Pg strain, one Aa strain, Fn, and Pi (Fig. 2B). When comparing the groups based on the duration of infertility treatment, differences were noted for two Pg strains, Fn, and Pi (Fig. 2C). Since patients seeking infertility treatment are generally older, the impact of aging on fertility decline in those with unexplained infertility cannot be disregarded[22–24]. Furthermore, it has been reported that the incidence

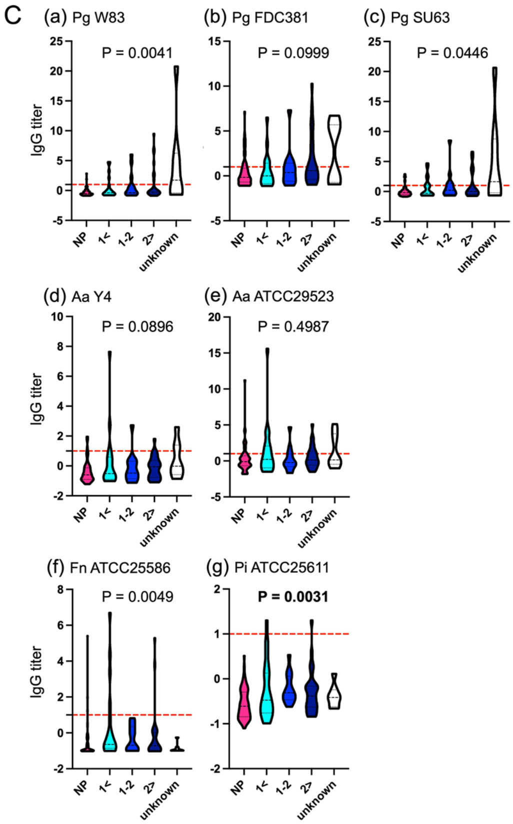


Fig. 2. (continued)

of periodontal disease increases with age[25], and it cannot be ruled out that the age-related increase in infection by periodontal pathogens may be involved in the elevated serum antibody levels observed in women with unexplained infertility. Therefore, we investigated the correlation between the participants' age and serum IgG antibody titers against seven periodontopathogenic bacterial strains. As a result, no significant correlations were observed between age and serum IgG antibody titers for any of the seven bacterial strains (supplemental Table 1). In addition, a multivariate logistic regression analysis was performed to identify clinical and immunological factors associated with infertility, using infertility (coded as 1) as the dependent variable. Age and serum IgG antibody titers against seven periodontopathogenic bacterial strains were included as independent variables. The results revealed that increasing age was significantly associated with higher odds of infertility ($\beta = 0.1495$, OR = 1.161, 95% CI 1.022–1.328, $P = 0.0242$). Among the antibody titers, elevated levels of Pg W83 ($\beta = 1.197$, OR = 3.310, 95% CI 1.304–11.17, $P = 0.0294$) and Pi ATCC25611 ($\beta = 1.505$, OR = 4.503, 95% CI 1.548–15.08, $P = 0.0091$) were significantly associated with increased odds of infertility. In contrast, higher titers against Pg FDC381 were inversely associated with infertility ($\beta = -0.5992$, OR = 0.5492, 95% CI 0.312–0.863, $p = 0.0203$). No statistically significant associations were found for Pg SU63, Aa Y4, Aa ATCC29523, or Fn ATCC25586 (supplemental table 2). These findings suggest that specific antibody responses to certain periodontal pathogens -particularly Pg W83 and Pi—may be positively associated with infertility, whereas others, such as Pg FDC381, may play a protective role. The variability in associations across bacterial strains highlights the importance of microbial specificity in periodontal-systemic interactions.

Serum IgG antibody titers against the three Pg strains were significantly elevated in patients with unexplained infertility (Fig. 2A). A clinical study conducted in Finland reported a low pregnancy rate in women with high salivary IgG and IgA antibodies against Pg[19], which aligns with our findings. *Fusobacterium nucleatum*, another periodontopathogenic bacterium besides Pg, has been suggested to be linked with preterm delivery[26] and endometriosis[27]. In addition to Pg, serum IgG antibody titers against Pi and *Streptococcus oralis* are also found to be increased in patients with polycystic ovary syndrome[28]. Thus, recent studies have suggested that periodontopathogenic bacteria may negatively influence female reproductive tract disorders and pregnancy outcomes. The results of the present study indicated that the antibody titers of the three Pg strains in women with infertility were significantly higher than the standard values when compared to the other strains, suggesting that Pg infection adversely affects pregnancy.

Based on the results of clinical studies, we hypothesized that periodontal inflammation associated with Pg infection may adversely affect pregnancy, prompting us to conduct in vivo research using a ligature-induced mouse periodontitis model. An association between Pg infection, preterm delivery, and low birth weight has also been reported in a mouse model[29]. However, to the best of our knowledge, no study has investigated the effects of Pg infection on pregnancy and delivery in a ligature-induced mouse periodontitis model. Therefore, after

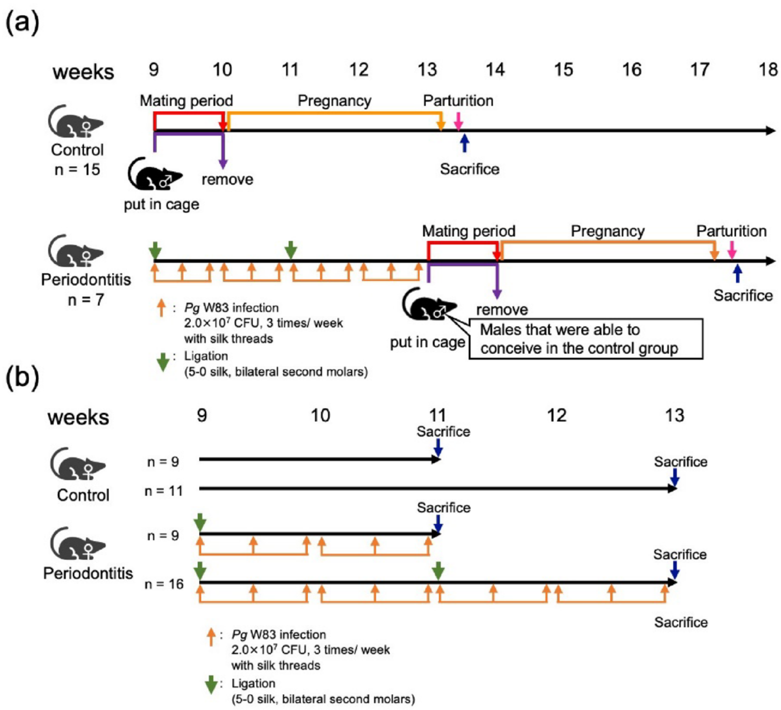


Fig. 3. Schedule for time and animal experimentation protocol A 5–0 silk thread was ligated to the cervix of the bilateral maxillary second molars of 9-week-old C57BL/6 J wild-type female mice, and a bacterial solution of Pg W83 strain was infiltrated into the silk thread to induce periodontal tissue inflammation. (**a**) Examination of the effects of periodontitis on pregnancy and parturition. Four weeks after periodontitis induction, male mice that had previously mated with other female mice to ensure reproductive fitness were placed in a cage and allowed to mate for one week before being removed from the cage, and female mice were euthanized the day after delivery. After euthanasia, the number of births, body weights of newborn mice, and gestation periods were recorded. (**b**) Examination of the effects of periodontitis on uterine tissue. Two and four weeks after periodontitis induction, female mice were euthanized, and various tissues were collected for analysis. Simultaneous to periodontitis induction, a control group was established and compared with the periodontitis group.

confirming alveolar bone resorption and Pg infection (Fig. 4), we examined whether periodontal inflammation impacts pregnancy and delivery outcomes. In periodontitis mice, the number of births, live births, and weights of newborn mice were significantly lower than those in the healthy group, while the number of fetal deaths was significantly higher compared to the healthy group (Fig. 5). We focused particularly on the decreased number of births and hypothesized that some changes occurred in the uteri of mother mice with periodontitis during the implantation stage.

We histologically and immunologically investigated the changes in uterine tissue following periodontitis induction. Two weeks after the induction of periodontitis, no changes in the uterine tissue were observed; however, four weeks later, the uterus was enlarged (Fig. 6). Hematoxylin–Eosin (H&E) staining of the uterus revealed a significant increase in the cross-sectional area four weeks after periodontitis induction. Additionally, we evaluated the expression of sex hormone receptors in the uterus through immunostaining (Fig. 7). In the uterus of periodontitis mice, increased expression of ER-α and PR was observed in both the endometrium and stroma compared to control mice. Uterine enlargement has been observed in both a rat model of *Escherichia coli*-induced endometritis and a mouse model of *Streptococcus aureus*-induced endometritis, as compared to healthy controls, which is consistent with the findings of the present study[30,31]. Chronic endometritis is considered one of the causes of infertility, and it has been reported that treatment of chronic endometritis improves the outcome of in vitro fertilization in patients with recurrent implantation failure[32]. Additionally, previous studies have reported elevated expression of ERα in the endometrium of patients with endometrial hyperplasia[33]. In this study, we also observed upregulation of ERα, consistent with these findings. ER and PR play crucial roles in regulating the female menstrual cycle, and an imbalance in their expression may disrupt the cyclical changes of the endometrium. Estrogen binds to ER and promotes endometrial proliferation during the proliferative phase, while also inducing the synthesis of PR, thereby facilitating the transition to the secretory phase. Excessive expression of ERα may affect PR expression and disturb the normal hormonal cycle, potentially leading to an endometrial environment that is unfavorable for embryo implantation.

We hypothesize that dysbiosis of the microbiota may be involved in the pathogenesis of conditions such as chronic endometritis and endometrial hyperplasia. In patients with chronic endometritis, a distinct endometrial microbiota has been identified compared to that of healthy individuals[31]. Furthermore, in a mouse model of

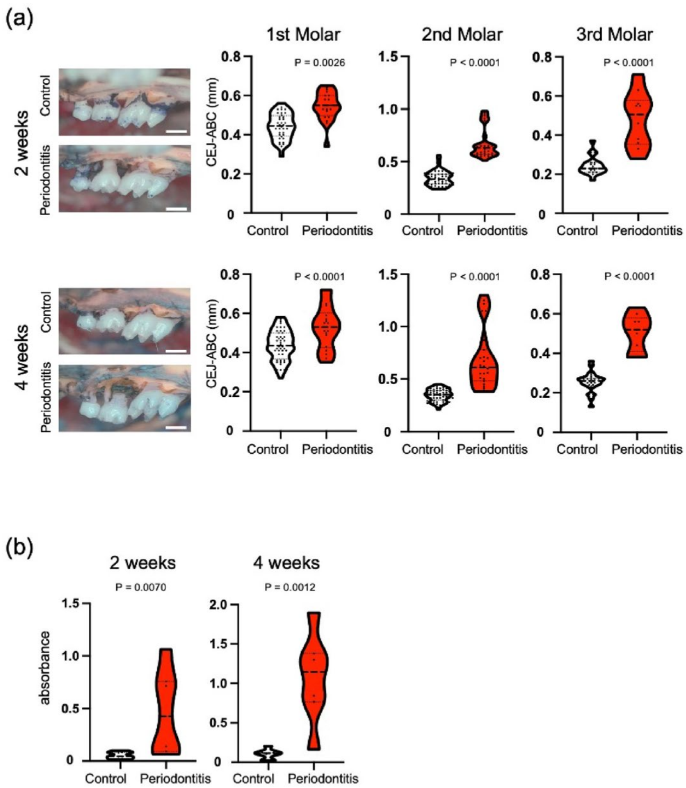


Fig. 4. Comparison of alveolar bone resorption and serum IgG antibody levels against Pg Bone resorption and serum IgG antibody titers against Pg were assessed in healthy mice and mice with periodontitis. The Mann–Whitney *U* test was applied for statistical analysis. (**a**) Comparison of bone resorption. The palatal side of the maxillary right first to third molars is depicted, measured using the method described by Abe and Hajishengallis (2013) with slight modifications. White bar: 1 cm. The graph illustrates the amount of alveolar bone resorption per tooth in each group (2-week sample: healthy group, n = 18 and periodontitis group, n = 10; 4-week sample: healthy group, n = 22 and periodontitis group, n = 9). (**b**) Comparison of anti-Pg serum IgG antibody titers. Serum IgG antibody titers are presented as absorbance (2-week samples: healthy group, n = 7 and periodontitis group, n = 7; 4-week samples: healthy group, n = 7 and periodontitis group, n = 7).

Streptococcus aureus-induced endometritis, administration of *Clostridium tyrobutyricum* has been reported to alleviate endometrial inflammation[31]. Furthermore, in patients with chronic endometritis, increased expression of estrogen and progesterone receptors has been reported to slow endometrial maturation and affect receptivity, contributing to one of the causes of infertility[34]. Also, previous reports have indicated that human endometrial hyperplasia samples exhibit a microbiota composition distinct from that of healthy controls, suggesting a potential involvement of microbial dysbiosis in the disease pathology[35]. Ye et al.[36] conducted a study using a mouse model of periodontitis induced by oral administration of multiple periodontal pathogens (Pg, *Treponema denticola*, *Tannerella forsythia*, and *Fusobacterium nucleatum*), in which they examined the microbiota of the oral cavity, heart, aorta, and reproductive organs. Compared to healthy mice, the periodontitis model exhibited detectable bacterial DNA from these pathogens in all examined tissues, with Pg DNA significantly elevated in both the oral cavity and uterus. Correspondingly, pro-inflammatory cytokines IL-6 and TNF-α were also upregulated in the uterus. These findings suggest that periodontal pathogens may reach the uterus, modulate the local microbiota, and induce cytokine expression, potentially impairing female reproductive function. Taken together, these studies suggest that microbial dysbiosis may contribute to uterine hypertrophy and altered sex hormone receptor expression. Additionally, recent evidence has highlighted the role of exosomes —small membrane-bound vesicles (30–100 nm in diameter) containing functional biomolecules such as proteins, lipids, RNA, and DNA—in the pathogenesis and organ-specific metastasis of cancer[37], as well as in the progression of neurodegenerative[38] and cardiovascular diseases[39]. It is plausible that a similar exosome-mediated mechanism may be involved in the crosstalk between the oral cavity and reproductive organs. Based on these findings, we hypothesize that both microbial dysbiosis and exosome signaling may play critical roles in the oral–uterine axis. Further investigation into this inter-organ connection is warranted.

A limitation of this study is that while the clinical study confirmed an increase in serum antibody titers against Pg in patients with unexplained infertility, the oral conditions of the study subjects were not evaluated in detail (e.g., probing pocket depth measurements and X-ray examinations). The association between periodontitis and infertility has yet to be recognized in actual infertility treatment settings, and patients undergoing infertility treatment have not typically consulted dentists as part of their care. Thus, in this study, we assessed serum IgG

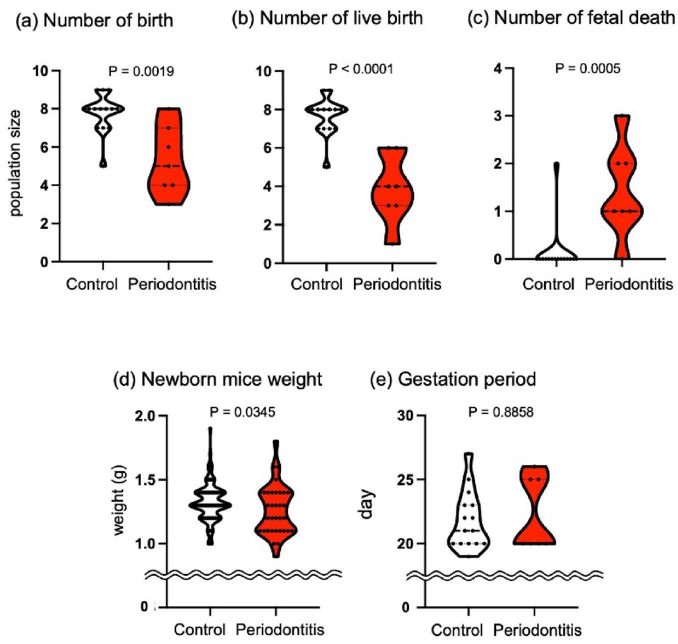


Fig. 5. Comparison of changes in pregnancy and delivery status in mice with periodontitis. Pregnancy and delivery outcomes in healthy mice and those with periodontitis were compared. The Mann–Whitney *U* test was used for statistical analyses. (**a**) Total number of births (healthy group: n = 15; periodontitis group: n = 7) (**b**) Total number of live birth (healthy group: n = 15; periodontitis group: n = 7) (**c**) Total number of fetal death (healthy group: n = 15; periodontitis group: n = 7) (**d**) Body weights of newborn mice (healthy group: n = 117; periodontitis group: n = 37) (**e**) Gestational period (healthy group: n = 15; periodontitis group: n = 7).

antibody titers against periodontopathogenic bacteria through a relatively straightforward blood test. There is a correlation between serum IgG antibody titers against periodontopathogenic bacteria and the progression of periodontitis[40,41], and patients receiving infertility treatment benefit from having these tests included in their blood work. However, clinical periodontal parameters such as probing pocket depth, bleeding on probing, and X-ray examinations cannot be fully substituted by the evaluation of serum IgG antibody titers alone. Therefore, this represents a methodological limitation of the present study. In the future, it is anticipated that opportunities will arise for dentistry to become integrated into the infertility treatment system, such as referring patients with elevated IgG antibody titers against periodontopathogenic bacteria for comprehensive examinations at dental clinics. Also, in addition to age, other known risk factors for infertility include obesity, underweight status, and smoking[14]. Since these factors were not evaluated in the present pilot study, their potential influence on both periodontal disease and infertility cannot be excluded. We also note this as a point for improvement in future studies involving larger cohorts and more comprehensive data collection.

In our basic research, we focused on factors related to female infertility. However, in humans, approximately half of infertility cases are attributed to male factors[6], and recent reports have pointed out a link between periodontal disease and male infertility, such as a decline in semen quality and erectile dysfunction[42–45]. Male mice used for mating were paired with a female mouse to test for reproductive abnormalities. However, since the male mice were housed with mice exhibiting periodontitis during the mating period, the possibility of transmission of periodontal pathogens to the males through grooming or other interactions cannot be ruled out, which may lead to changes in their reproductive function. In the future, it will be essential to investigate the presence or absence of periodontopathogenic bacterial infections in male mice after mating with those showing periodontitis. Furthermore, the potential impact of experimentally induced periodontitis in male mice on pregnancy and birth outcomes warrants further investigation.

In conclusion, serum IgG antibody titers against Pg were elevated in women with unexplained infertility compared to pregnant women who underwent vaginal delivery. In a mouse model of ligature-induced periodontitis with Pg W83 infection, uterine tissue showed enlargement and the expression of ER-α and PR was increased, suggesting that pregnancy and delivery may be negatively impacted.

Methods
Study subjects
As a pilot study, serum IgG antibody titers against periodontopathogenic pathogens were initially measured in 30 women with natural conception and 30 women undergoing infertility treatment. Based on the results of this preliminary analysis, we calculated the required sample size for the clinical study. For the IgG titer analysis using clinical blood samples, the significance level (α) was set at 0.05 and the statistical power (1-β) at 0.8 (β = 0.2). Using preliminary data for IgG titer against Pg FDC 381 —selected for its clinical relevance in daily practice—,

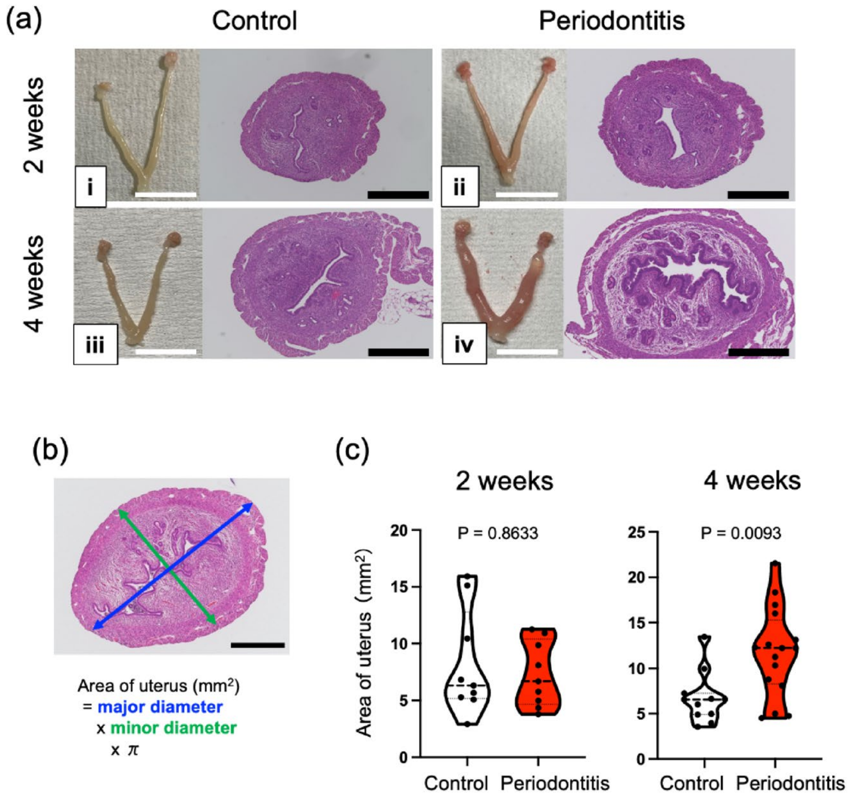


Fig. 6. Changes in the uterus of mice with periodontitis. Changes in the uterus were observed through hematoxylin-eosin (H&E) staining. (**a**) Image of the mouse uterus at the time of sampling and the H&E-stained image of the uterine cross-section. White bar: 1.0 cm; black bar: 500 µm. (**b**) Method for measuring uterine cross-sectional area, approximated using the equation shown in the figure. Black bar: 500 µm. (**c**) Comparison of uterine cross-sectional area where the Mann–Whitney *U* test was employed for statistical analyses (2-week sample: healthy group, n = 9; periodontitis group, n = 9; 4-week sample: healthy group, n = 11; periodontitis group, n = 16).

we assumed a mean difference of 1.42 between the two groups (natural conception: 0.88; infertility patients: 2.30), a pooled standard deviation of 3.525 (natural conception: 2.75; infertility patients: 4.30), and an equal allocation ratio (1:1). Based on these assumptions, the required sample size was estimated to be 98 participants per group. To account for potential dropouts, we increased the final sample size to 100 participants per group.

The study involved 99 spontaneously pregnant women aged 30 to 45 and 98 patients undergoing infertility treatment who visited a reproductive clinic in Okayama, Japan, from January 2020 to April 2022 and consented to participate. The study was explained to the participants before its initiation, and written informed consent was obtained from all participants by the Okayama University Ethics Committee (approval number #1909-023). After obtaining consent, 3.0 mL of peripheral blood was collected; serum was separated by centrifugation (4 °C, 1,710×*g*, 5 min) and subsequently stored at – 30 °C.

Serum antibody titers against periodontopathogenic bacteria using enzyme-linked immunosorbent assay (ELISA)

Serum IgG antibody titers against periodontopathogenic bacteria were measured using ELISA, following the method detailed by Ohyama et al.[46]. Seven bacterial strains—Pg W83, Pg FDC381, Pg SU63, *Aggregatibacter actinomycetemcomitans* (Aa) Y4, Aa ATCC29523, *Fusobacterium nucleatum* (Fn) ATCC25586, and *Prevotella intermedia* (Pi) ATCC25611—were subjected to ultrasonic disruption extraction to prepare bacterial antigens, as outlined by Murayama et al.[47]. The dried material of these antigens was mixed with phosphate-buffered saline containing polyoxyethylene (20) sorbitan monolaurate (Tween 20) (PBST, pH 7.4), 0.8% (w/v) sodium chloride, 0.02% (w/v) potassium dihydrogen phosphate, 0.29% (w/v) disodium hydrogen phosphate dodecahydrate, 0.02% (w/v) potassium chloride, 0.02% (w/v) sodium azide, and 0.05% (v/v) Tween 20 to achieve a final concentration of 1.0 mg/mL; this was further diluted using a 50 mM carbonate-bicarbonate buffer solution (pH 9.6; 0.17% (w/v)

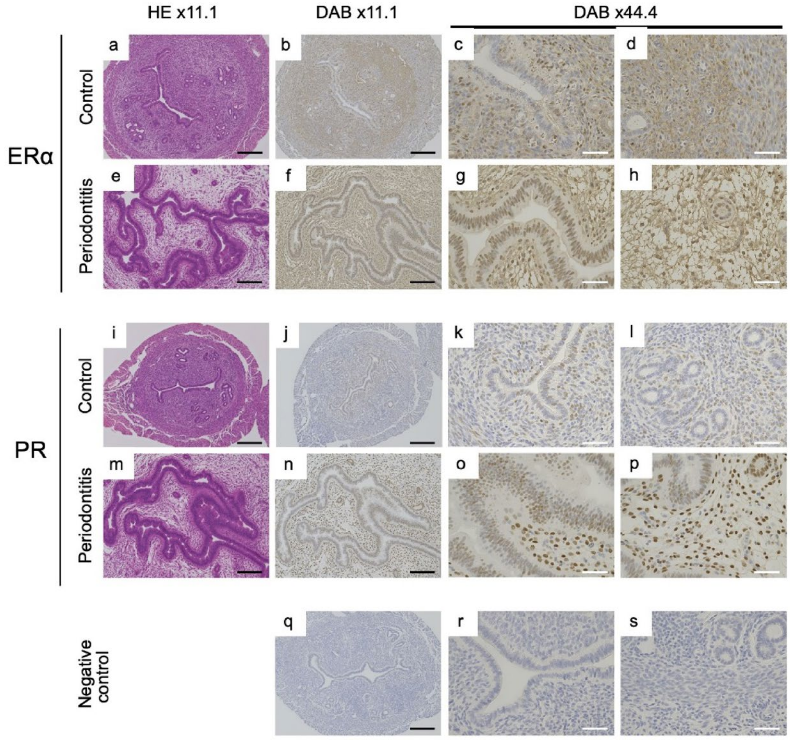


Fig. 7. Changes in the expression of sex hormone receptors in the uterus of mice with periodontitis. Expression of sex hormone receptors (estrogen receptor (ER)-α and progesterone receptor (PR)) in the uterus was observed after immunostaining (healthy group: n = 3, periodontitis group: n = 3). (**a**, **e**, **i**, **m**): H&E-stained image of the uterine cross-section. Black bar: 200 µm. (**b**, **f**, **j**, **n**): 3,3′-Diaminobenzidine Tetrahydrochloride (DAB)-stained images of the uterine cross-section. Black bar: 200 µm. (**c**, **g**, **k**, **o**) High-magnification images of the mouse uterine epithelium. White bar: 50 µm. (**d**, **h**, **i**, **p**) High-magnification images of the mouse uterine stoma. White bar: 50 µm. (**q**, **r**, **s**): Images of the negative control with no primary antibody added. Black bar: 200 µm, white bar: 50 µm.

sodium carbonate, 0.3% (w/v) sodium bicarbonate) to reach a final concentration of 10 µg/mL. One hundred microliters of each antigen solution were added to the wells of a 96-well ELISA microplate (Greiner Bio-One, Kremsmünster, Austria) and kept at 4 °C for 6–8 h to ensure proper solidification. Standard sera comprised equal volumes of mixed sera from five participants aged 25–29 years, with no systemic diseases and no history of smoking. The sera underwent four-fold serial dilution, ranging from 12.5-fold to 51,200-fold using PBST. Sera collected from participants were diluted 3200-fold in PBST. After washing the antigen-coated plates three times with PBST, 100 µL of both standard and test sera were allowed to react at 4 °C for 6–8 h. Alkaline Phosphatase AffiniPure F(ab') Fragment Goat Anti-Human IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA), diluted 5,000-fold in PBST, served as the secondary antibody. After three additional washes with PBST, 100 µL of the secondary antibody was added to each well and allowed to react for 2 h at 37 °C. The chromogenic substrate, p-nitrophenyl phosphate disodium hexahydrate (Fujifilm Wako Pure Chemicals, Osaka, Japan), was dissolved in diethanolamine buffer (pH 9.8; 10% (v/v) 2,2′-iminodiethanol, 0.01% (w/v) magnesium chloride hexahydrate, 0.12% (w/v) sodium azide). The plates were washed three times with PBST, and 100 µL of substrate was added to each well, allowing it to react at 20–25 °C. Absorbance was then measured using an iMark Microplate Reader and Microplate Manager Software 6.3 (both from Bio-Rad, Hercules, CA, USA). The absorbance of each well was recorded once it reached 1.6 at 405/490 nm. Absorbance values were converted into ELISA units (EU) based on a calibration curve (4-parameter logistic curve) derived from the serial dilution of standard serum. The EU of the test serum with the same absorbance as that of the standard serum diluted 3200-fold was assigned a value of 100, while the EU of the test serum exceeding the standard serum range of EU 25,600–6.25 (12.5–51,200-fold dilution) was capped at EU 25,600 for samples above 25,600 and at EU 6.25 or less for those below it. The EU 6.25 marked sera below the threshold of EU 6.25. Additionally, antibody titers were standardized as ((EU of each sample)–(mean of EU of healthy sera))/(2 × (standard deviation of healthy sera)).

Confirmation of reproductive function in male mice
Nine-week-old male and female C57BL/6 J wild-type mice (CELA Japan, Tokyo, Japan) were housed in pairs for a one-week mating period. One week after mating, the male mice were removed from the cage. Males that mated with females displaying signs of pregnancy, indicated by abdominal enlargement and considered to have no reproductive issues, were paired with female mice with periodontitis, as described in the next section. Pregnant female mice were euthanized with CO_2 gas one day after delivery. The newborn mice were euthanized by decapitation after weighing and counting. Pregnant (normal) female mice served as the healthy group and negative controls for the periodontitis mice. The day the male mice were placed in their cages was designated as day 0 of pregnancy (Fig. 3a). Mice were maintained in a specific pathogen-free environment. For the birth rate comparison experiment in Experimental Schedule (a) (Fig. 3a), to eliminate potential male infertility factors, each male mouse was pre-mated with a healthy female to confirm the ability to achieve pregnancy. As a result, the number of healthy control mice was larger. In contrast, for the analysis using periodontitis-induced female mice, a significance level of alpha = 0.05 and a statistical power of 1-beta = 0.8 (beta = 0.2) were set. Based on the preliminary experiment results, assuming a mean difference between the two groups of 3.0 (healthy group: 8.0, periodontitis group: 5.0), a standard deviation of 1.75 (healthy group: 1.5, periodontitis group: 2.0), and an allocation ratio of 1:1, the estimated required sample size was calculated to be n = 5.38. Considering dropouts and other risks, such as suture detachment, the sample size was set to at least 7.

Establishment of a ligature-induced mouse periodontitis model
Nine-week-old female C57BL/6 J wild-type mice were used to establish a mouse model of periodontitis, following the method described by Abe and Hajishengallis[48] with slight modifications. Briefly, the mice were anesthetized intraperitoneally with 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride diluted in PBS, then ligated with 5–0 silk sutures around the cervical area of the bilateral maxillary second molars. Additionally, 200 µL of a periodontopathogenic bacterial solution of Pg W83 strain (cultured anaerobically at 37 °C and adjusted to OD600 = 0.8 (1.0×10^8 CFU/mL)) was infiltrated into the silk sutures three times a week to induce infection and inflammation of the periodontal tissues.

After four weeks of inducing periodontitis, male mice were bred. In the mating group, following periodontitis induction (4 weeks), male mice confirmed to have normal fertility were paired 1:1 in the same cage, as detailed in Fig. 3a. One week after gestation (day 0), male mice were removed from the cage. Female mice were euthanized using CO_2 gas one day after giving birth. The newborn mice were euthanized by decapitation after being weighed and counted. Mice showing no signs of pregnancy were deemed non-pregnant and euthanized with CO_2 gas one week after the last delivery date in the same group.

Groups that were not mated following the induction of periodontitis were euthanized using CO_2 gas at the same time as the negative control, the healthy group, as well as at 2- and 4-weeks post-periodontitis induction (Fig. 3b). After euthanasia, serum, maxilla, and uterus were collected. Mice that died during the experimental period were excluded from the analysis.

For the uterine size comparison experiment in Experimental Schedule (b) (Fig. 3b), under the same assumptions of a significance level of alpha = 0.05 and a statistical power of 1-beta = 0.8 (beta = 0.2), preliminary data indicated a mean difference of 4.5 between the two groups (healthy group: 6.5, periodontitis group: 11.0), with a standard deviation of 3.5 (health group:2.5, periodontitis group:4.5). The estimated sample size was calculated as n = 9.67. Considering dropouts and other risks, such as suture detachment, the number of animals per group was set to at least 11.

Confirmation of alveolar bone resorption due to periodontitis
Bone specimens were prepared, and alveolar bone resorption was analyzed according to the method described by Abe and Hajishengallis[48]. Mouse heads were autoclaved (LSX-500, Tommy Seiko, Tokyo, Japan) at 121 °C and 2 atm for 20 min to remove soft tissues. After bleaching with 30% hydrogen peroxide (Nacalai Tesque, Kyoto, Japan) and neutralization with sodium hypochlorite solution (Nacalai Tesque), the heads were stained with eosin (Muto Chemical Company, Tokyo, Japan) for 5 min and methylene blue (Merck KGaA, Darmstadt, Germany) for 10 s. After drying, bone resorption was measured using a microscope (SZ-LW61 T2; Olympus, Tokyo, Japan). Bone resorption was measured at six points on the centrolingual fissure and centro-lingual cusp of the first molar; proximal palatal cusp, palatal fissure, and centrolingual cusp of the second molar; and palatal cusp of the third molar by measuring the vertical distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC), which was measured at six points on the palatal cusp of each third molar, and the average vertical distance for each tooth was used to calculate the degree of bone resorption. One sample was taken per jaw, and an alveolar bone sample with a lost tooth was excluded from the measurement.

Measurement of murine serum IgG antibody titer against Pg W83
The ELISA method described by Ohyama et al.[46] was utilized for this assay with slight modifications. The antigen solution was prepared as detailed in Sect. 2.2; 100 µL of this solution was added to each well of a 96-well ELISA microplate (Greiner Bio-One) and incubated at 4 °C for 6–8 h to allow the antigen to solidify on the plate. Mouse serum was diluted tenfold with phosphate buffered saline with Tween 20 (PBST). The antigen-coated plates were washed three times with PBST, after which 100 µL of mouse serum was added and allowed to react at 4 °C for 8 h. The secondary antibody, alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG (H + L) (Proteintech Group, Inc., Rosemont, IL, USA), was diluted 1000-fold in PBST. The plates were washed three times with PBST, then 100 µL of the secondary antibody was added to each well and allowed to react at 37 °C for 2 h. The chromogenic substrate used was p-nitrophenyl phosphate, disodium salt, hexahydrate (Fujifilm Wako Pure Chemicals), dissolved in diethanolamine buffer (pH 9.8; 10% (v/v) 2,2′-iminodiethanol, 0.01% (w/v) magnesium chloride hexahydrate, 0.12% (w/v) sodium azide). After washing the plate three times with PBST,

100 μL was added to each well and allowed to react for 20 min at 20–25 °C. The absorbance was measured using the iMark Microplate Reader and Microplate Manager Software 6.3 (both from Bio-Rad). Measurements were performed in duplicates, and serum IgG antibody titers were expressed as absorbance values.

Histological analysis of the uterus

A portion of the uterus from the healthy, non-pregnant periodontitis mice group was immersed in a 4% paraformaldehyde solution (pH 7.4; Fujifilm Wako Pure Chemicals) for 24 h for tissue fixation. The fixed tissues were dehydrated using a graded ethanol series and then paraffin-embedded to create blocks, which were sliced thinly to obtain 5 μm-thick paraffin sections. These sections were deparaffinized with xylene (Nacalai Tesque) and rehydrated by immersing glass slides in graded alcohol, progressing stepwise from 70% ethanol to anhydrous ethanol (Fujifilm Wako Pure Chemicals). H&E staining was performed, followed by dehydration by immersing the glass slides in graded alcohol, also progressing stepwise from 70% ethanol to anhydrous ethanol. The slides were then immersed in xylene and coverslipped with Mount-Quick (Daido Sangyo Co., Ltd., Tokyo, Japan). After drying, histological analysis of the uterine tissue was performed using a light microscope (BX-50; Olympus).

Observation of the localization of sex hormone receptors in uterine tissue using immunohistochemistry

Uterine tissues were collected from mice four weeks after periodontitis induction, and immunohistochemistry was used to determine the localization of sex hormone receptors, including ER-α and PR, in the uterine tissue. The collected uterine tissue was processed into paraffin sections, deparaffinized, and rehydrated as described in Sect. 2.7. Endogenous peroxidase activity in the tissues was inhibited using H_2O_2 and methanol. Antigen retrieval was achieved by immersing the sections in a 10 mM solution of 1 M citrate buffer (pH 6.0; Muto Pure Chemicals, Tokyo, Japan) diluted with distilled water, followed by incubation at 95 °C for 22 min. After cooling to 20–25 °C, the sections were immersed in normal rabbit serum for 10 min to block them. Subsequently, primary antibodies were added: rabbit polyclonal antibody to Estrogen Receptor-alpha (Affinity Biosciences, Cincinnati, OH, USA) diluted 1000-fold with normal rabbit serum, and rabbit monoclonal antibody to Progesterone Receptor (Abcam, Cambridge, UK) diluted 400-fold with normal rabbit serum. The reaction was allowed to proceed overnight at 4 °C. After washing with Tris Buffered Saline with Tween 20 (TBST), biotinylated goat anti-rabbit secondary antibody diluted to 5 μg/mL with TBST was added, and the reaction was allowed to proceed for 1 h at room temperature. After an additional wash with TBST, peroxidase-labeled streptavidin was added and incubated for 30 min at room temperature. Normal rabbit serum, biotinylated goat anti-rabbit secondary antibody, and peroxidase-labeled streptavidin were used from the VECTASTAIN Elite ABC Rabbit IgG Kit (VECTOR). Following another wash with TBST, the sections were stained brown using the DAB Substrate Kit (VECTOR). Mayer's hematoxylin (Wako Pure Chemicals) was used for counterstaining, and the sections were mounted using 60% xylene. After drying, tissue images were observed under an optical microscope (BZ-X810; Keyence). Histological and immunohistochemical evaluations were conducted by a board-certified pathologist (TO), who analyzed the stained tissue sections in a blinded manner.

Ethical approval

In this clinical study, after obtaining approval from the Okayama University Ethics Committee, the study was thoroughly explained to the participants prior to initiation, and written informed consent was obtained from all participants (Okayama University Ethics Committee approval number #1909-023). This study was conducted in accordance with the ethical principles based on the Declaration of Helsinki. All animal experiments adhered to the ARRIVE guidelines and were conducted under the Okayama University Animal Experiment Approval (OKU-2021680, OKU-2023532).

Statistical analysis

Statistical analysis was performed using Mann–Whitney *U* test to compare human serum IgG antibody titers against periodontal pathogenic bacterial strains between the natural pregnancy and infertility treatment groups. Kruskal–Wallis test was used for comparisons of human serum IgG antibody titers by age group and duration of infertility within the infertility treatment group. Spearman's rank correlation coefficient investigated the correlation between the participants' age and serum IgG antibody titers against seven periodontopathogenic bacterial strains. A multivariate logistic regression analysis was performed to identify clinical and immunological factors associated with infertility, using infertility (coded as 1) as the dependent variable. In the basic research analysis, normality was tested using Shapiro–Wilk test, and Mann–Whitney *U* test was used for comparisons between two groups when normality was not met. Spearman's rank correlation coefficient was calculated using NCSS 2021 (NCSS, LLC), while all other statistical analyses were performed with GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), and a significance level was set at $P < 0.05$.

Data availability

The datasets supporting the conclusions of this study are included within the manuscript. All relevant data are available from the corresponding author on reasonable request.

Received: 18 March 2025; Accepted: 4 September 2025
Published online: 07 October 2025

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Acknowledgements
This study was supported by the Grant-in-Aid for Scientific Research (B) (Grant No. 23K27773 to KO), the Grant-in-Aid for Challenging Research (Exploratory) (Grant No. 24K22249 to KO), and the Grant-in-Aid for Early-Career Scientists (Grant No. 21K16992 to HS) from the Japan Society for the Promotion of Science. The authors thank Dr. Riko Takayoshi, Dr. Aya Ito, Dr. Tomoyoshi Takata, Dr. Toshiyuki Hata, and the staff of the Miyake Clinic and Miyake Hello Dental Clinic, Pediatric Dentistry and Orthodontics for their support in clinical research procedures. The authors would like to thank Dr. Hidetaka Ideguchi for providing technical support during in vivo experiments, and Dr. Ayaka Tsuboi-Yoshida for providing statistical analysis support. We would like to thank Editage (www.editage.com) for the English language editing.

Author contributions
CKN and KO contributed to the conception, design, data acquisition, analysis, and interpretation of the data and drafted and critically revised the manuscript. HS, KS, MN, TO, HM, MKT, FK, and IT acquired, analyzed, and interpreted the data. KH, AI, KTH, YSI, and TaY interpreted the data. MTT, AS, MK, ToY, TO, and MT contributed to clinical research. TM contributed to the conception, data analysis, and interpretation and critically revised the manuscript. ST contributed to the conception, design, data analysis, and interpretation and critically revised the manuscript. All authors have approved the final manuscript and agreed to be accountable for all aspects of this study.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-18992-x.

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