FETAL GROWTH RESTRICTION IN PORPHYROMONAS GINGIVALIS-INFECTED PREGNANT RATS

(RESTRIKSI PERTUMBUHAN JANIN PADA TIKUS HAMIL YANG DIINFEKSI PORPHYROMONAS GINGIVALIS)

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Abstract

The periodontopathogens Porphyromonas gingivalis may directly or indirectly attack the fetus by releasing toxins into the blood stream that could reach the placental and influence fetal development. This study aimed to determine the effect of periodontal infection with P. gingivalis on fetal development in pregnant rat model. Female rats were infected with live-P. gingivalis at concentration of 10^9 colony forming unit/ml into subgingival sulcus before and/or during pregnancy. Group I: without P. gingivalis infection; group II: P. gingivalis infection before and during pregnancy; group III: P. gingivalis infection before pregnancy; and group IV: P. gingivalis infection during pregnancy. They were sacrified on gestational day 14 and 20. Fetuses were evaluated for weight and crown-tail length. The results showed that the dams infected with P. gingivalis significantly decrease the mean of fetal weights, lengths and placental weights compared with the control group ($p<0.05$). The percentages of fetal growth restriction at the time of sacrifice were 6.66, 100, 72.97 and 87.09% growth-restricted fetuses in group I, II, III, and IV, respectively. When weights of growth-restricted fetuses of the treated groups were compared with the control group there were significant differences ($p<0.05$). P. gingivalis was detected by API ZYM system in the blood of umbillical cord from the treated groups. In conclusion, in pregnant rat models, periodontal infection with P. gingivalis affects fetal development. The maternal P. gingivalis infection on periodontal tissue can result in P. gingivalis dissemination to umbillical cord and induction of FGR.

Key words: Porphyromonas gingivalis, periodontitis, pregnancy, fetal growth restriction

INTRODUCTION

The role of maternal periodontitis as a potential maternal-fetal stressor that has detrimental effects on the pregnancy outcome is a relatively new field of investigation. Recently, both case-control and cohort studies have reported that maternal oral infection, as indexed by clinical measures of gingivitis and periodontitis may be an independent contributor to abnormal pregnancy outcomes, including preterm births, fetal growth restriction (FGR), and preeclampsia.[1-4] Both gingivitis and periodontitis are relatively common concomitant clinical conditions among pregnancy women, although prevalence estimates during pregnancy varied considerably, there are gingivitis 30-100% and periodontitis 5-20%.[5]

Periodontitis is a common chronic local inflamamatory process characterized by bacterial infection and release of various toxic products (extracellular vesicles and lipopolysaccharides [LPS]) by periodontal pathogens, and by host immune responses comprised of a cellular infiltration of neutrophils, macrophages and lymphocytes, and the release of cytokines such as TNF-α, interleukin-1 (IL-1) and IL-6. The primary etiologic factor for periodontitis is bacteria that it accumulates in the gingival sulcus. *Porphyromonas gingivalis,* a gram-negative, black-pigmented anaerobic rod bacterium, is thought to be

one major periodontal pathogen because of its frequent association with periodontal disease.[6]

It is hypothesized that *P. gingivalis* can disseminate from a local infectious site through the circulatory system and come into remove organs such as uterus and placenta, induce both systemic and placental inflammatory responses, and results in FGR. The purpose of this study was to determine the effect of periodontal infection with *P. gingivalis* on fetal development in a pregnant rat model.

MATERIALS AND METHODS

All procedures were in accordance with the animal welfare guidelines and approved by The Institutional Animal Care and Use Committee, Universitas Gadjah Mada. Adult female Sprague-Dawley rats weighed 150-200 g were used in this study, and maintained under controlled and standardized conditions. Rats were housed in conditions of 12-hour light-dark cycles from 7 a.m. to 7 p.m., and a temperature of 25 ^{o}C. Regular rat diet and water were provided *ad libitum*.

Porphyromonas gingivalis ATCC 33277 was plated on tryptic soy agar containing sheep blood and grown for 5-7 days in anaerobic chamber with 5% H_2, 10% CO_2, and 85% N_2, at 37^{o}C. Bacterial suspensions were prepared from primary cultures at their log phase of growth, and incubated overnight. Bacterial concentration was adjusted to equal of 6.67 McFarland standard, representing 2 x 10^9 CFU/ml. The desired treatment concentrations were diluted with phosphate-buffered saline (PBS).

The female rats were injected with 0.05 ml of 2x10^9 CFU/ml live-*P. gingivalis* ATCC 33277 into the maxillary buccal and palatal gingival between first and second upper molars. The injections were repeated every other day on 3 separate days for 30 days and continued until 20 days after mating. The control group rats received 0.05 ml of PBS injection according to the same schedule as the *P. gingivalis*-injected rats. This study consisted of 4 groups i.e. **g**roup I, no *P. gingivalis* infection; group II, *P. gingivalis* infection before and during pregnancy; group III, *P. gingivalis* infection before pregnancy; and group IV, *P. gingivalis* infection during pregnancy. At least 4 weeks after induction of experimental periodontitis, female rats were mated overnight with male rats of the same strain. The next morning, females were removed from the male cages and examined for vaginal plugs. If a plug was found, that day was recorded as gestational day (GD) 1.

The pregnant rats were sacrificed on GD 14 and 20. Fetuses were removed post-mortem from the uterus and surrounding membranes. Each fetus was removed from its chorioamniotic sac and weighed to the nearest microgram. The resorption site and viable fetuses were counted and recorded for each rat. The viability of each fetus was assessed visibly. Fetuses were evaluated for weight and crown-tail length. Fetal growth restriction (FGR) was defined as fetuses with weight 2 standard deviations (SD) smaller than normal fetal weight (NFW; 3.56±0.19 g)[7]. Blood of umbillical cord was collected from each fetus and pooled per dam.

Blood of umbillical cord was immediately plated on tryptic soy agar containing sheep blood and grown for 5-7 days at 37^{o}C under anaerobic conditions. *P. gingivalis* colonies were identified by their black pigment, Gram staining and API ZYM system, and then were compared to *P. gingivalis* ATCC 33277 for confirmation of organism. The API ZYM colorimetric kit system (bioMérieux SA, Marcy-I'Etoile, France) for detection of enzymes was used according to the direction of the manufacturer. Color reactions were read with a grade scale in which 0 indicated no enzyme activity, 1 or 2 indicated weak activity, and 3 to 5 indicated strong enzyme activity. Key differential tests for oral species of *Bacteroides* described that *P. gingivalis* were very consistent and distinctive for trypsin-like activity, uniformly negative for α-Glucosidase and *N*-Acetyl-β-glucosamidase.

Statistical analysis to compare the mean of fetal weights, fetal lengths and placental weights between groups were performed using one-way analysis of variance. The mean difference is significant at the <0.05 level.

RESULTS

Periodontal infection was found in the trial animals. During the trial of experiment, the trial animals had no febrile, did not exhibit malaise, and did not lose weight as a consequnce of expereiment. However, there was a decrease of weight seen in trial animals. The average weight of dams between GD 0 and GD 14 at the time of sacrification was 206.600±4.219 g (mean±SD) for control group, group II 188.200±12.637 g, group III 156.200-±7.259 g, and group IV 156.600±5.030 g. Whereas at GD 20, group I 220.800±34.953 g, group II 170.400±6.504 g, group III 195.200±10.639 g, and group IV 191.600±9.181 g. When compared to the control, the average weight of the dams from the *P. gingivalis* challenged groups (group II, III, and IV) at GD 14 was significantly lower (p<0.05) than control group. At GD 20, only group II was significantly lower (p=0.004) than control group, and

Tabel 1. Pregnancy development in P. gingivalis-infected pregnant rats observed at GD 14 and GD 20

	Gestational Day	Groups			
		I	II	III	IV
Fetal weight (g)	GD 14	0.172±0.161	0.044±0.078*	0.068±0.163*†	0.073±0.156*
	GD 20	4.079±0.430	0.565±0.168*	2.729±0.500*	2.342±0.582*
Fetal length (mm)	GD 14	10.316±1.232	5.452±0.506*	6.457±0.504*†	6.568±0.502*
	GD 20	48.967±1.751	18.395±1.606*	40.486±2.329*	36.710±2.268*
Placental weight (g)	GD 14	0.199±0.038	0.060±0.005*	0.084±0.195*†	0.089±0.162*
	GD 20	0.615±0.088	0.216±0.033*	0.306±0.040*	0.360±0.071*
FGR/Total fetuses (%)	GD 14	---	---	---	---
	GD 20	2/30 (6.66)	43/43 (100)	27/37 (72.97)	27/31 (87.09)

FGR, fetal growth restriction
*Significantly lower than control group ($p<0.05$)
†No significant difference compared with group IV ($p>0.05$)

Tabel 2. Average fetal weight and presence of P. gingivalis in umbillical cord from fetuses among P. gingivalis-infected dams observed at GD 14 and GD 20

Groups	Presence of *P.gingivalis*	Number of fetuses		Fetal weight (g)	
		GD 14	GD 20	GD 14	GD 20
I	Yes	0	0	0	0
	No	38	30	0.172±0.016	4.079±0.430
II	Yes	17	22	0.040±0.006	0.499±0.157
	No	14	21	0.050±0.002	0.634±0.154
III	Yes	13	12	0.057±0.016	2.380±0.168
	No	22	25	0.073±0.013	2.898±0.522
IV	Yes	15	14	0.063±0.010	2.107±0.571
	No	22	17	0.080±0.015	2.535±0.531

others (group III and IV) showed there was no significant difference when compared to the control.

The recorded the weights, lengths and placental weights at the time of sacrification (GD 14 and 20) were summarized in Table 1. When the mean of fetal weights, compered lengths and placental weights of dams infected with *P. gingivalis* (group II, III, and IV) to the control dams, they were significantly decreasing in the mean of fetal weights, lengths and placental weights compared with the control group ($p<0.05$). How ever, there were no signi-Ficant differences the mean of fetal weights, lengths and placental weights between group III and group IV at GD 14. The percentages of FGR at the time of sacrification (GD 20) are also shown in Table 1. There were 6.66%, 100%, 72.97% and 87.09% growth-restricted fetuses in group I, II, III, and IV, respectively. When weights of growth-restricted fetuses of the treated groups were compared to the control, there were significant differences ($p<0.05$).

In the control group, all blood of umbillical cord samples from normal-weighed fetuses and growth-restricted fetus did not detect *P. gingivalis*. Whereas, blood of umbillical cord samples from the treated groups possesed variable results of enzymatic activities of *P. gingivalis* as measured by the API ZYM system (Table 2). In the treated groups at GD 14 and 20 showed a strong of trypsin-like activities, and uniformly negative for α-Glucosidase and *N*-Acetyl-β-glucosamidase.

DISCUSSION

An animal model is needed in order to investigate the association between local infection and fetal growth, and to ged better understanding of the host-pathogen interactions. Laboratory rats can be a useful model to study the mechanisms of human abnormal pregnancy outcomes[8]. This model of localized chronic infection with *P. gingivalis* is adapted from Offenbacher and coworkers that used a mouse subcutaneous chamber model to study the effect of *P. gingivalis* infection on pregnancy outcomes in hamsters[9] and mice[7] with heat-killed *P. gingivalis* induced a primary immune response. In our experiment, a rat chronic infection model will be infected with live *P. gingivalis* into subgingival sulcus. This model was more closely miniced the chronic infection with periodontal pathogen observed in human patients. Furthermore, it will test the ability of *P. gingivalis* and/or its components to induce FGR when introduced into the bloodstream of pregnant rats.

During periodontal infection, when oral mucosa is injured and inflamed, and the quantities of periodontal pathogens increase dramatically, transient

bacteremia may occur[10]. This can lead to select colonization of undesired sites. In the current study, we proposed initial transmission of organisms from oral cavity into bloodstream and addressed the question of what effects of *P. gingivalis* on pregnancy if it enters the circulation.

We chose to inject subgingival sulcus rats with *P. gingivalis* before and/or during pregnancy. This infection had exposed until on day 20 of gestation, a relatively late stage in the pregnancy, for several reasons. Stress or infection prior to day 14 tends to lead to resorption of the fetuses. Preterm birth in humans also occured during late stages in gestation, and the murine infection was initiated at a gestational age proportional 28 to 32 weeks in humans. Also, the structure of mouse is placenta at late stages of gestation is remarkably similar to that of human placenta.[11]

In a series of studies[7] in which pregnant rodents were infected with *P. gingivalis*, fetal weight was decreased, whereas embryolethality and the percentage of the fetal resorption and FGR increased. Exposure of sheep to LPS from *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Fusobacterium nucleatum* caused much higher rates of fetal lethality than *Escherichia coli* lipopolysaccharides.[12] Moreover, *P. gingivalis*-specific DNA was detected in the placentas and fetuses of subcutaneous *P. gingivalis*-infected rodents.[13]

Our study showed that maternal *P. gingivalis* infection on periodontal tissue can result in *P. gingivalis* dissemination to umbillical cord and induction of FGR, but *P. gingivalis* was not always detected in the umbillical cord from abnornal pregnancies. Several possibilities could explain why *P. gingivalis* was detected in some affected dams but not others. It might be attributed to the technical aspects of the culture technique, mainly the usage of non-specific medium to grow the microorganisms. Alternatively, the effect of *P. gingivalis* on FGR may be mediated by bacterial products or by host mediators, rather than direct dissemination in some *P. gingivalis*-infected dams.

In general, there were significantly more FGR fetuses in the *P. gingivalis*-infected groups than in the control. However, no significant difference percentage of FGR fetuses were observed between the *P. gingivalis*-infected groups. A fetus can be smaller in size due to a general retardation in overall development compared to its littermates, or it can be developmentally normal but lack in weight gain. Our result suggested that FGR fetuses were at the same stage of development as the control fetuses, and the significant difference between the *P. gingivalis* infected groups and the control group in growth, both in weight and length. Futuremore, the average pregnancy weight gain for dams was smaller in the *P. gingivalis*-infected group than the control group. The less weight gain of the treated dams had more FGR than the control.

In mammals, the processes of the placental supply of nutrients to the fetus depend upon the size, morphology, blood flow and transporter abundance of the placenta.[14] Therefore, fetal body weight in late gestation correlates positively with placental weight in many species, both during normal conditions and when placental weight is reduced experimentally either by direct placental manipulations or by indirect alterations of environmental conditions during development.[15] In pregnant sheep and rats, placental efficiency, measured as gram fetus per gram placenta, is increased in late gestation when fetal and placental weight are reduced by maternal heat stress, glucocorticoid administration, under and overnutrition and by restriction of placentation or uterine blood flow.[16,17]

Although longitudinal measurements of the growth of individual feto-placental units were not possible in the present study, the fetal growth trajectory during late gestation appeared to differ from placental size. At GD 20, the fetuses with the lightest placentas were similar in weight to those with the heaviest placentas in a litter, despite being smaller at GD 14. Therefore, the lightest placenta was suppor-ted more growth than the heaviest placenta between GD 14 and GD 20. The positive correlation obser-between placental and fetal weight at GD 14 was also lost by GD 20 (data not shown). These findings are consistent with previous studies in the same strain of mice that showed that fetal weight is positively correlated with placental weight at GD 17 but not later in gestation.[18] Thus, the naturally small placenta is able to support the normal growth spurt of the mouse fetus during late gestation.

This study provided evidence that periodontal disease can produce unfavorable change in the fetal environment and may be a sufficiently infectious challenge to produce FGR. In conclusion, localized infection with *P. gingivalis* is capable of mediating FGR via systemic dissemination of microorganism, and pregnant rats with *P. gingivalis* infection before and during pregnancy possesed very low fetal weight. Therefore, this finding suggests that humans with preexisting periodontal infection might be at a greater risk for pregnancy complications and that the apparently innocuous sustained exposure which occurs via the local periodontal infection might be an

important candidate as a stealth infection.

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