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Table of Content

Endotoxin Levels in the Amniotic Fluid of *Porphyromonas gingivalis*-Infected Pregnant Rats

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Abstract

Background: *Porphyromonas gingivalis* as a major etiology of periodontal disease can produces virulence factors, such as lipopolysaccharide (LPS)/endotoxin, which is potentially involved in tissue destruction and host defenses impairment. *Porphyromonas gingivalis* LPS is expected to play a role in the intrauterine fetal growth. The aims of the present study were to identify endotoxin levels in the amniotic fluid of *Porphyromonas gingivalis*-infected pregnant rats and to determine its effect on fetal growth. **Methods:** Female rats were challenged with live-*Porphyromonas gingivalis* at concentration of $2x10^9$ colony forming unit/ml into subgingival sulcus area of the maxillary first molar before and/or during pregnancy. They were sacrified on gestational day (GD) 14 and 20. Fetuses were evaluated for weight and length. Endotoxin was detected by limulus amebocyte lysate assay on amniotic fluid. **Results:** The mean of LPS concentrations in amniotic fluid on GD 14 and GD 20 was significantly different ($P<0.05$) among the four maternal periodontal infection groups. Fetal endotoxemia on GD 14 and GD 20 influenced ($P<0.05$) placental weight, fetal weight, and fetal length. The increased of LPS concentration in amniotic fluid resulted in the decreased placental weight, fetal weight and fetal length. **Conclusion:** The increased LPS concentration was in accordance to long-term maternal chronic periodontal infection. Periodontitis could serve as a source of bacterial components, such as LPS, thus it triggered the release of inflammatory mediators and resulted the adverse pregnancy outcomes.

Keywords: *Porphyromonas gingivalis,* Periodontitis, Endotoxin, Pregnancy, Fetal Growth Restriction

Introduction

Periodontal disease is a multifactorial chronic infection resulted in periodontal tissue destruction. The primary microorganism which triggers periodontal disease is Gram-negative rod-shaped facultative anaerobes. *Porphyromonas gingivalis*, a periodontal pathogenic microorganism, produces potential virulence factors such as proteolytic enzymes, leucotoxin, endotoxin (lipopolysaccharide or LPS) which able to promote evasion of host responses, invasion of host tissues, and induction of inflammatory mediators [1-3].

Porphyromonas gingivalis is not only exhibit the pathogenic properties on periodontal disease but also on systemic diseases, such as cardiovascular disease and abnormal pregnancies [4, 5]. In humans, Gram-negative bacteria infections are recognized as a cause of fetal death and preterm birth [6]. Lipopolysaccharide has been associated with embryonic resorption, intra-uterine fetal death, intra-uterine growth restriction and preterm birth in rodents [7, 8]. These findings indicate that periodontal pathogens may play a role in the development and progression of systemic disease.

Therefore, we hypothesized that *Porphyromonas gingivalis* and its lipopolysaccharide from periodontal tissue could spread into amniotic fluid through the circulatory system, then induced placental inflammatory response resulting in fetal growth restriction. The aims of the present study were to identify endotoxin levels in the amniotic fluid of *Porphyromonas gingivalis*-infected pregnant rats and determine its effect on fetal growth.

Material and Methods

All procedures were approved by the Health and Medical Research Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada. This study had taken *Sprague-Dawley* primiparous female rats (adult, 2 months, 150-250 g). The rats were maintained on controlled and standardized conditions. The subjects of this study were divided into two blocks of termination: on gestational day (GD) 14 and GD 20. Each block was subdivided into four groups, which consisted of the control group, no *Porphyromonas gingivalis* infection; the Pg-BD group, an infection of *Porphyromonas gingivalis* before and during pregnancy; the Pg-B group, an infection of *Porphyromonas gingivalis* before pregnancy; and the Pg-D group, an infection of *Porphyromonas gingivalis* during pregnancy. Each group consisted of five pregnant rats.

Induction of experimental periodontitis was performed by injection of 0.05 ml live-*Porphyromonas gingivalis* ATCC 33277 with a concentration of $2x10^9$ CFU/ml into the distopalatal and distobuccal gingival sulcus area of maxillary first molar. Injection was repeated every 3 days for 30 days. For infection after pregnancy, it was also performed by a repeated injection every 3 days for 19 days. Control group rats were injected by saline 0.05 ml as the treatment schedule of the treatment group rats. Then, the female rats were mated with the same strain of male rat overnight ratio 2:1. The next morning, female rats were removed from the cages for vaginal plug examination. If the vaginal plug was found, the day was recorded as GD 1.

Each fetus was taken post-mortem from the chorioamniotic sac. Placental weight, fetal weight and fetal length were recorded for each maternal. Amniotic fluid was taken on GD 14 and GD 20 in order to perform endotoxemia test.

Endotoxin in amniotic fluid was tested by limulus amebocyte lysate (LAL) Pyrochrome method according to the manufacturer's instructions (Cape Cod, U.S.). This method is easy to do in a timely, specific, and highly sensitive manner. Pyrochrome was added as soon as possible to all of the negative control samples, endotoxin standards, and specimens, with a ratio 1:1. All samples were then incubated for 30 seconds in 37°C incubator. Furthermore, the reaction was stopped using 0.05 ml sodium nitrite in HCl. Each well was added by 0.05 ml ammonium sulfamate, and 0.05 ml N-(1-Naphthyl)-ethylenediamine (NEDA). Magenta color would be formed quickly. The test was read at 540-550 nm. Standard curve was used to determine the concentration of endotoxin in the specimen. Endotoxin concentrations of the positive control were determined by standard endotoxin dilution consisting of 0.005, 0.05, 0.5, 5 and 50 endotoxin units (EU)/ml. Sensitive detection limit was 0.005 EU/ml.

Numerical variables which consisted of fetal LPS concentration, placental weight, fetal weight, and fetal length were statistically analyzed to identify endotoxin levels in the amniotic fluid of *Porphyromonas gingivalis*-infected pregnant rats, and to determine its effect on fetal growth. One-way analysis of variance (ANOVA) with post hoc test was performed to compare the endotoxin levels of maternal periodontal infection. Linear regression analysis was performed to determine the linear relationship between numerical variables. Value of significance was determined as $P<0.05$. Numerical data were presented in mean ± standard deviation.

Results

Endotoxemia test result showed that the mean of LPS concentrations in amniotic fluid was significantly different ($P<0.05$) on GD 14 and GD 20 in the four maternal periodontal infection groups. On GD 14, the LPS concentration of control group was significantly different ($P<0.05$) with Pg-BD and Pg-B groups, but was not significantly different ($P>0.05$) with Pg-D group. The mean of LPS concentration in amniotic fluid of Pg-BD group was not significantly different ($P>0.05$) with Pg-B and Pg-D groups. Similarly, the mean of LPS concentration in amniotic fluid from Pg-B group was not significantly different ($P>0.05$) with Pg-D group. On GD 20, the LPS concentration of control group was significantly different ($P<0.05$) with Pg-BD, Pg-B, and Pg-D groups. Similarly, the mean of LPS concentration in amniotic fluid from Pg-B group was significantly different ($P<0.05$) with Pg-D group. However, the mean of LPS concentration in amniotic fluid from Pg-BD group was significantly different ($P<0.05$) with Pg-B group, and was not significantly different ($P>0.05$) with Pg-D group (Figure 1). It indicated that LPS concentration in amniotic fluid was affected by the severity of maternal periodontal disease.

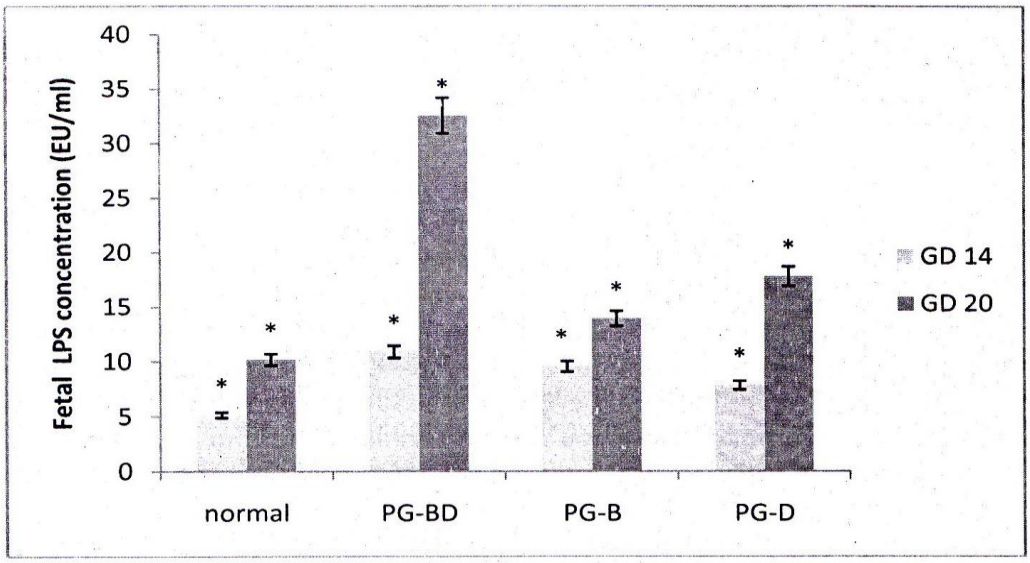

Figure 1. Fetal endotoxemia caused by *Porphyromonas gingivalis* infection in maternal periodontal tissues. Amniotic fluid samples were taken 24 hours after the bacteria exposure. Data were presented in mean ± SEM and were compared with ANOVA (* $P<0.05$) by the maternal periodontal infection.

The linear regression analysis showed that fetal endotoxemia on GD 14 and GD 20 influenced ($P<0.05$) the placental weight, fetal weight, and fetal length. The increased LPS concentration in amniotic fluid resulted in the decreased fetal weight and fetal length. The results can be seen in Table 1.

TABLE 1. EFFECT OF FETAL ENDOTOXEMIA ON PLACENTAL WEIGHT, FETAL WEIGHT, AND FETAL LENGTH ON GD 14 AND 20

Variable	Fetal endotoxemia							
	GD 14				GD 20			
	N	R^2	B	*P*	N	R^2	B	*P*
Placental weight, gram	141	0.175	-0.004	0.001	141	0.234	-0.007	0.000
Fetal weight, gram	141	0.194	-0.004	0.001	141	0.321	-0.062	0.000
Fetal lenght, mm	141	0.191	-0.163	0.001	141	0.402	-0.562	0.000

Data were analized by the linear regression ($P<0.05$)

Discussion

This study identified that the endotoxin was contained in amniotic fluid of the control group. The mean of LPS concentration in amniotic fluid was 5.15 ± 5.05 EU/ml on GD 14 and 10.19 ± 7.86 EU/ml on GD 20. However, fetal endotoxemia in the control group was not adversely affect fetal growth. In accordance to our study, previous studies showed that *Escherichia coli* LPS intravenously at a concentration of 0.003 mg and 0.3 mg per 100 g body weight resulted in the increased fetal weight, but when the concentration was increased to 10 mg per 100 gram body weight, it can decrease the fetal weight. Similarly, the *Porphyromonas gingivalis* LPS at concentration of 30 ng to 300 ng per 100 g body weight resulted in the increased fetal weight, and when the concentration was increased to 3 mg to 10 mg per 100 gram body weight, it can decrease the fetal weight [9].

The LPS concentration of treatment groups were higher than control group. The increased LPS concentration was directly proportional to the severity of periodontal disease. Previous studies showed that the LPS concentrations in the blood serum of patients with periodontitis were higher than healthy individuals and patients with post-treatment of periodontal disease [10, 11].

Porphyromonas gingivalis LPS plays an important role in the induction of innate and acquired immune responses. Differential cytokine response to live-*Porphyromonas gingivalis* indicates that live-*Porphyromonas gingivalis* and its components play different roles. Live-*Porphyromonas gingivalis* can lead to a relatively minor inflammatory infiltration and less intense antigen-specific immune responses [12, 13]. A reduced immune response can cause excessive bacterial growth which previously confined subgingival bacteria in the early stages of infection. Meanwhile, an overgrowth of bacteria in subgingival tissue can stimulate the expression of IL-1, IL-6, and TNF-α, which leads to local tissue destruction. In the late stages of infection, LPS released by bacteria cell walls breakage can penetrate gingival tissue and moves into the blood circulation, further contribute to the systemic inflammatory response [14, 15].

Porphyromonas gingivalis from maternal periodontal tissue can spread into the placenta [16], it is assumed that *Porphyromonas gingivalis* can also achieve chorio-decidual space and then penetrate through amnion into the amniotic fluid. Finally, fetus can be infected if amniotic fluid enters the fetal lungs and gastrointestinal tract. Therefore, infection can occur in the membrane chorio-decidual, amniotic fluid, umbilical cord or fetus itself. These factors affect the incidence of maternal periodontal infection. When maternal periodontal infection is more severe, it can lead to higher LPS concentrations in amniotic fluid.

This study showed that the increased LPS concentration in amniotic fluid resulting from *Porphyromonas gingivalis* infection on maternal periodontal tissues can result in decreased placental weight, fetal weight, and fetal length. The increasing LPS concentration was in accordance to long-term maternal chronic periodontal infection. Periodontitis could serve as a source of bacterial components, such as LPS, thus it triggered the release of inflammatory mediators and resulted the adverse pregnancy outcomes.

The decreased fetal weight and fetal length were caused by decreasing placental weight. This suggests that placental growth is essential for proper fetal growth. The placenta provides a better intrauterine environment, in particular the specific changes in immune responses leading to environmental change pro-inflammatory and anti-inflammatory. It will affect the activity of nutrient delivery to the fetus from the mother, resulting in decreased fetal weight and fetal length. Thus, changes in placental morphologic condition caused by exposure to toxic agents can play a role as markers of intrauterine environmental disturbance.

Conclusion

This finding further confirms that the maternal periodontal health affects maternal status during pregnancy. Therefore, it can be concluded that maternal periodontal inflammation before and/or during pregnancy is highly contribute to impaired fetal growth, in which characterized by endotoxemia resulting in decreased placental weight, fetal weight, and fetal length.

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