Volume 45 Number 3 September 2012 ISSN 1978 - 3728

# Dental Journal

Majalah Kedokteran Gigi

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Printed by: *Airlangga University Press*. (151/11.12/AUP-B5E). Kampus C Unair, Jln. Mulyorejo Surabaya 60115, Indonesia. Telp. (031) 5992246, 5992247, Telp./Fax. (031) 5992248. E-mail: aupsby@rad.net.id. Ijin penerbit: No. 0787/SK/Dir. PK/SIT/1969. *Accredited* No. 48/DIKTI/Kep/2006.

Dental Journal
Majalah Kedokteran Gigi
Volume 45 Number 3 September 2012

Research Report

# Maternal Endotoxin-induced Fetal Growth Restriction in Rats: Fetal Responses in Toll-like Receptor

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***ABSTRACT***

***Background:*** *Porphyromonas gingivalis as a major etiology of periodontal disease can produce virulence factor, lipopolysaccharide/LPS, which is expected to play a role in the intrauterine fetal growth. Trophoblast at the maternal-fetal interface actively participates in response to infection through the expression of a family of natural immune receptors, Toll-like receptor (TLR).* ***Purpose:*** *This study was aimed to identify endotoxin concentration in maternal blood serum of Porphyromonas gingivalis-infected pregnant rats, to characterize the TLR-4 expression in trophoblast cells, and to determine its effect on fetal growth.* ***Methods:*** *Female rats were infected with live-Porphyromonas gingivalis at concentration of 2 x $10^9$ colony forming unit/ml into subgingival sulcus area of the maxillary first molar before and/or during pregnancy. They were sacrified on gestational day 14 and 20. Fetuses were evaluated for weight and length. Endotoxin was detected by limulus amebocyte lysate assay in the maternal blood serum. The TLR-4 expression in trophoblast cells was detected by immunohistochemistry.* ***Results:*** *The mean of LPS concentrations in maternal blood serum was significantly different ($p<0.05$) among the four maternal periodontal infection groups. The TLR-4 expressions in syncytiotrophoblast, spongitrophoblast and trophoblastic giant cells from Porphyromonas gingivalis-infected periodontal maternal groups were significantly higher than the control group ($p<0.05$). Maternal endotoxemia affected ($p<0.05$) the fetal weight and fetal length.* ***Conclusion:*** *The increased LPS concentration in maternal blood serum resulted in the decreased fetal weight and fetal length. Syncytiotrophoblast, spongitrophoblast and trophoblastic giant cell were able to recognize Porphyromonas gingivalis LPS through the TLR-4 expression. This findings strengthened the link between periodontal disease and fetal growth restriction.*

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

***ABSTRAK***

***Latar belakang:*** *Porphyromonas gingivalis sebagai etiologi utama penyakit periodontal dapat menghasilkan faktor virulensi, lipopolisakarida/LPS, yang diharapkan dapat berperan dalam pertumbuhan janin intrauterin. Trofoblas pada antarmuka maternal-janin aktif berpartisipasi dalam respon terhadap infeksi melalui ekspresi suatu famili reseptor imun alamiah, Toll-like receptor (TLR).* ***Tujuan:*** *Penelitian ini bertujuan untuk mengidentifikasi konsentrasi endotoksin dalam serum darah maternal dari tikus hamil yang terinfeksi Porphyromonas gingivalis, mengkarakterisasi ekspresi TLR-4 pada sel trofoblas, dan menentukan efeknya pada pertumbuhan janin.* ***Metode:*** *Tikus betina diinfeksi dengan Porphyromonas gingivalis hidup pada konsentrasi 2x109 colony forming unit/ml ke area sulkus subgingiva molar pertama maksilaris sebelum dan/atau selama kehamilan. Tikus tersebut dikorbankan pada hari kehamilan ke 14 dan 20. Janin dievaluasi untuk berat dan panjang janin. Endotoksin dideteksi dengan uji limulus amebocyte lysate dalam serum darah maternal. Ekspresi TLR-4 pada sel trofoblas dideteksi secara imunohistokimiawi.* ***Hasil:*** *Rerata konsentrasi LPS dalam serum darah maternal berbeda nyata ($p<0,05$) di antara empat kelompok infeksi periodontal maternal. Ekspresi TLR-4 pada sinsitiotrofoblas, spongitrofoblas dan giant cell trofoblas dari kelompok periodontal maternal yang terinfeksi Porphyromonas gingivalis secara signifikan lebih tinggi daripada kelompok kontrol ($p<0,05$). Endotoksemia maternal mempengaruhi ($p<0,05$) berat janin dan panjang janin.* ***Kesimpulan:*** *Peningkatan konsentrasi LPS meningkat dalam serum darah maternal mengakibatkan penurunan berat janin dan panjang janin. Sinsitiotrofoblas, spongitrophoblast dan giant cell trofoblas mampu mengenali LPS Porphyromonas gingivalis melalui ekspresi TLR-4. Temuan ini memperkuat hubungan antara penyakit periodontal dan restriksi pertumbuhan janin.*

***Kata kunci:*** *Porphyromonas gingivalis, periodontitis, endotoksin, kehamilan, restriksi pertumbuhan janin*

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## INTRODUCTION

Periodontal disease is a multifactorial chronic infection resulted that make destruction of the periodontium. The primary microorganism which caused of periodontal disease is Gram-negative rod-shaped facultative anaerobes. *Porphyromonas gingivalis* is a periodontal pathogenic bacteria which has potential virulence factors such as proteolytic enzymes, leucotoxin, endotoxin (lipopolysaccharide/LPS), evasion of host responses, invasion of host tissues, and induction of inflammatory mediators.[1,2]

*Porphyromonas gingivalis* is not only exhibit the pathogenic properties on periodontal disease but also on systemic diseases such as cardiovascular disease and abnormal pregnancies.[3,4] In humans, Gram-negative bacteria infections have been recognized as a cause of stillbirth and perinatal death[5] whereas LPS has been associated with embryonic resorption, intra-uterine fetal death, intrauterine growth restriction and preterm birth in rodents.[6,7] These findings indicate that periodontal pathogens may play a role in the development and progression of systemic disease.

Placental growth and development relies highly on trophoblast cells. Apoptosis and the factors involved in the regulation are associated with almost all stages of development and trophoblast differentiation.[8] One of the factors that can lead to the increased apoptosis of trophoblast during pregnancy is intrauterine infection.[9] The exact mechanism of infection resulted the progression of this disorder is not clearly understood, but recent studies suggested that bacterial products have a direct effect on the trophoblast. Trophoblast can respond to infection through the expression of a family of natural immune receptors, Toll-like receptor (TLR). TLR capable of recognizing conserved sequences on the surface of microorganisms.[10] Ligation of TLR-4 with LPS led to the first trimester human trophoblast cells to produce cytokines, including tumor necrosis factor-$\alpha$ (TNF-$\alpha$), which induces apoptosis of trophoblast cells.[11]

Therefore, we hypothesized that Porphyromonas gingivalis and its lipopolysaccharide from periodontal tissue can spread into the uterus through the circulatory system, then induces placental inflammatory response resulting in fetal growth restriction. The aims of the present study were to identify endotoxin level in maternal blood serum of Porphyromonas gingivalis-infected pregnant rats, to characterize the TLR-4 expression in trophoblast cells, and to determine its effect on fetal growth.

## MATERIALS AND METHODS

All procedures were approved by the Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia. This study had taken female Sprague-Dawley rats, adult, 2 months, 150-250 g and primiparous. The rats were maintained on the controlled and standardized conditions. The subjects of study were consisted of two blocks, they were sacrificed 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 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 and examined the vaginal plug. If the vaginal plug was found, the day was recorded as GD 1.

Each fetus was taken post-mortem from the chorioamniotic sac on GD 14 and GD 20. Placental weight, fetal weight and fetal length were recorded for each maternal. Furthermore, the placental immunohistochemically was undertaken to determine the expression of TLR-4. Samples were incubated overnight at 4 °C with primary antibody, rabbit polyclonal anti-TLR4 antibody dilution 1:500 (Abbiotec, San Diego, CA, 1:100-1:500), while negative control was incubated with secondary antibody as a substitute for the primary antibody. TLR-4 is expressed on the cell wall and cytoplasm. Data were presented as mean number of cells expressing TLR-4 in each type of cell. These specimens were evaluated in macrophages of labyrinth zone (LM), junctional zone (SM) and decidua zone (DM), as well as syncytiotrophoblast (LS), spongitrophoblast (ST) and trophoblastic giant cell (DG).

Maternal blood serum was taken on GD 13 and GD 19, and it was performed to endotoxemia test. Endotoxin in maternal blood serum 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. Pyrochrome was added as soon as possible to all of the negative control samples, endotoxin standards and specimens with a ratio 1:1 and was incubated 37 °C for 30 seconds in an incubator. Furthermore, the reaction was stopped with 0.05 ml sodium nitrite in HCl, and it

was added 0.05 ml ammonium sulfamate, and 0.05 ml N-(1-Naphthyl)-ethylenediamine (NEDA) to each well. 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 maternal LPS concentration, TLR-4 expression, placental weight, fetal weight and fetal length were performed by statistical analyzes to identify endotoxin level in maternal blood serum of Porphyromonas gingivalis-infected pregnant rats, to characterize the TLR-4 expression in trophoblast cells, and to determine its effect on fetal growth. One-Way ANOVA with post hoc test was performed to compare the endotoxin levels of maternal periodontal infection. Linear regression analysis was to analyze 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 showed that the mean of LPS concentration in maternal blood serum from control group was 1.11±0.62 EU/ml on GD 14 and 4.19 ± 2.45 EU/ml on GD 20. Furthermore, the mean of LPS concentration in maternal blood serum on GD 14 and GD 20 were significantly different ($p<0.05$) in the four maternal periodontal infection groups. The control group was significantly different ($p<0.05$) with the Pg-BD, Pg-B and Pg-D groups. The mean of LPS concentration in the maternal blood serum from Pg-BD group was significantly different ($p<0.05$) with Pg-B group, but was not significantly different ($p>0.05$) with Pg-D group. Similarly, the mean of LPS concentration in maternal blood serum from Pg-B group was significantly different ($p<0.05$) with Pg-D group (Figure 1).

This study also showed that TLR-4 expressions in the labyrinth zone, junctional zone and decidual zone (Figure 2) were significantly different ($p<0.05$) from the control, Pg-BD, Pg-B and Pg-D groups on GD 14. However, TLR-4 expressions of the control, Pg-BD, Pg-B and Pg-D groups on GD 20 were significantly different only in the macrophages, syncytiotrophoblasts and trophoblastic giant cells. Both GD 14 and GD 20, the Pg-BD, Pg-B and Pg-D groups had a higher TLR-4 expression than control group (Table 1).

The linear regression analysis showed that maternal endotoxemia on GD 14 and GD 20 affected ($p<0.05$) the placental weight, fetal weight and fetal length. The increased LPS concentration in maternal blood serum resulted in the decreased fetal weight and fetal length. The results can be seen in Table 2.

## DISSCUSION

The LPS concentration in *Porphyromonas gingivalis*-infected periodontal maternal groups were higher than control group. The increased LPS concentration was directly proportional to the severity of periodontal disease. The increased LPS concentration was in accordance to long-term maternal chronic periodontal infection. LPS concentration in the maternal blood serum was 2-fold higher than in amniotic fluid. The dynamic changes of LPS were correlated with disease severity and suggested LPS causing secondary hepatic injury.[12] Much evidence indicates that bacterial LPS (endotoxin) is removed from the bloodstream mainly by the liver, yet the hepatic

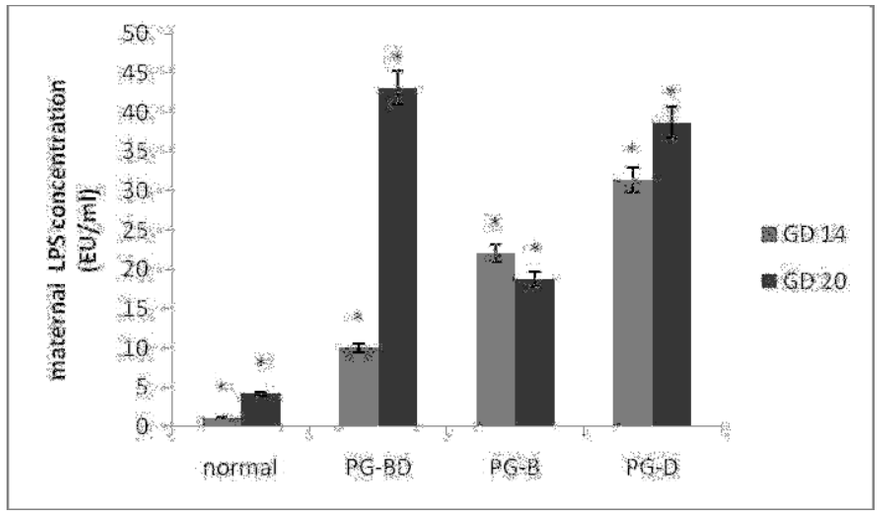


**Figure 1.** Maternal endotoxemia caused by *Porphyromonas gingivalis* infection in maternal periodontal tissues. Maternal blood serum samples were taken 40 minutes after the bacteria exposure. Data were presented in mean±SEM and were compared with ANOVA (* $p<0.05$) by the maternal periodontal infection.

**Table 1.** TLR-4 expression in the rat placenta of Porphyromonas gingivalis-infected periodontal maternal on GD 14 and GD 20

| Variable | Maternal periodontal infection | | | | p |
|---|---|---|---|---|---|
| | Control (n = 23) | Pg-BD (n = 10) | Pg-B (n = 13) | Pg-D (n = 10) | |
| GD 14: | | | | | |
| TLR4-LM | 1.13 ± 0.92*†§ | 2.50 ± 1.84*† | 2.62 ± 1.19*§ | 2.50 ± 1.08* | 0.001 |
| TLR4-LS | 1.57 ± 0.79*†§ | 3.30 ± 1.16*† | 2.92 ± 1.04*§ | 3.30 ± 1.95* | 0.001 |
| TLR4-SM | 1.48 ± 0.79*†§ | 2.60 ± 0.97*† | 2.15 ± 0.38*§ | 2.20 ± 1.03* | 0.002 |
| TLR4-ST | 1.65 ± 1.03*†§ | 6.70 ± 1.89*†§ | 4.92 ± 1.85*†§ | 4.80 ± 1.48*† | 0.001 |
| TLR4-DM | 1.35 ± 0.65*†§ | 2.60 ± 1.65*† | 2.46 ± 0.66*†§ | 1.90 ± 1.10 | 0.002 |
| TLR4-DG | 1.74 ± 0.92*†§ | 3.90 ± 0.88*†§ | 3.08 ± 0.64*†§ | 3.60 ± 1.35*† | 0.001 |
| GD 20: | | | | | |
| TLR4-LM | 2.00 ± 0.95*† | 3.70 ± 1.70*†§ | 2.31 ± 1.18†§ | 3.60 ± 2.07*§ | 0.003 |
| TLR4-LS | 2.78 ± 1.09*†§ | 7.50 ± 1.72*†§ | 3.85 ± 1.41*†§ | 6.90 ± 1.73*§ | 0.001 |
| TLR4-SM | 2.22 ± 1.04* | 2.60 ± 1.17 | 2.23 ± 0.83§ | 3.30 ± 0.95*§ | 0.037 |
| TLR4-ST | 2.96 ± 1.07 | 3.80 ± 1.39 | 3.38 ± 1.33 | 3.40 ± 0.52 | 0.249 |
| TLR4-DM | 2.35 ± 1.03 | 2.50 ± 1.18 | 2.00 ± 0.71 | 2.90 ± 1.19 | 0.224 |
| TLR4-DG | 2.26 ± 1.05*†§ | 3.40 ± 0.69*† | 2.85 ± 0.69*§ | 3.30 ± 0.48* | 0.001 |

Macrophages of labyrinth zone (LM), junctional zone (SM) and decidua zone (DM); syncytiotrophoblast (LS); spongitrophoblast (ST); trophoblastic giant cell (DG)

Periodontal infection before and during pregnancy (Pg-BD); periodontal infection before pregnancy (Pg-B); periodontal infection during pregnancy (Pg-D)

*, †, § : mean difference was significant at 0.05

**Table 2.** Effect of maternal endotoxemia to placental weight, fetal weight and fetal length on GD 14 and GD 20

| Variable | Maternal endotoxemia | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GD 14 | | | | GD 20 | | | |
| | N | $R^2$ | B | p | N | $R^2$ | B | p |
| Placental weight, gram | 141 | 0.562 | -0.003 | 0.001 | 141 | 0.434 | -0.006 | 0.001 |
| Fetal weight, gram | 141 | 0.626 | -0.003 | 0.001 | 141 | 0.548 | -0.056 | 0.001 |
| Fetal length, mm | 141 | 0.584 | -0.099 | 0.001 | 141 | 0.526 | -0.478 | 0.001 |

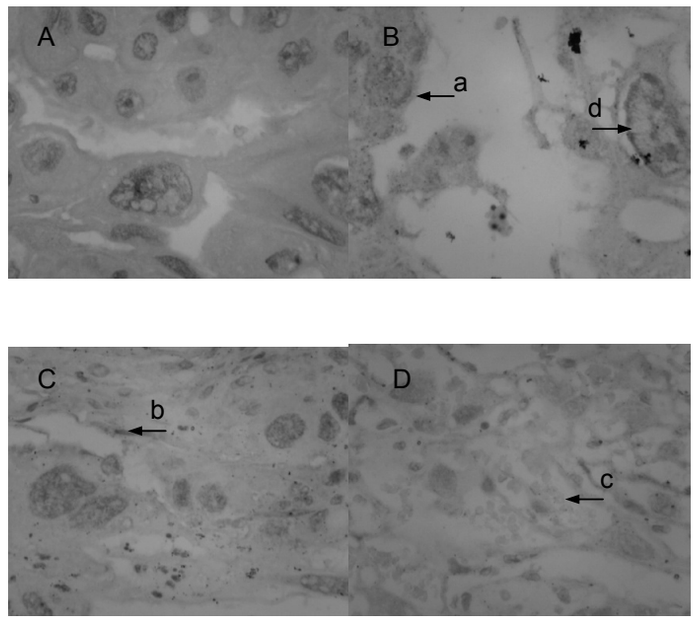


**Figure 2.** TLR-4 expression on placenta of control A) Pg-BD; B) Pg-B; C) and Pg-D; D) groups at GD 20. TLR-4 was expressed weakly A) and strong; B-D) on the cell wall and cytoplasm of macrophage (a), syncytiotrophoblast (b), spongiotrophoblast (c) and trophoblastic giant cell (d). 400× magnification

uptake mechanisms remain uncertain and controversial. In plasma, LPS can be either 'free' (as aggregates, bacterial membrane fragments or loosely bound to albumin, CD14, or other proteins) or 'bound' (complexed with lipoproteins). Whereas most free LPS is taken up by Kupffer cells (KCs), lipoprotein-bound LPS has seemed to be cleared principally by hepatocytes.[13]

This study identified that the endotoxin was contained in the maternal blood serum from the control group. The mean of LPS concentration in the maternal blood serum from the control group was 1.11 ± 0.62 EU/ml on GD 14 and 4.19 ± 2.45 EU/ml on GD 20. However, the maternal endotoxemia in the control group was not adversely affect fetal growth. In accordance to our study, previous study showed that low-dose LPS pretreatment greatly attenuated LPS-induced increases in TNF-$\alpha$ protein in fetal liver and fetal brain. Taken together, these results indicate that perinatal exposure to low-dose LPS induces a reduced sensitivity to subsequent LPS challenge.[14]

*Porphyromonas gingivalis* LPS plays an important role in the induction of inate 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 in antigen-specific immune responses.[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 the amnion into the amniotic fluid. Finally, the fetus can be infected if the amniotic fluid enters the fetal lungs and gastrointestinal tract. On GD 14 and GD 20, TLR-4 were expressed by syncytiotrophoblasts and spongiotrophoblasts. This indicated that TLR-4 in syncytiotrophoblasts and spongiotrophoblasts were able to respond *Porphyromonas gingivalis* that previously have been through the decidual compartment. Thus, *Porphyromonas gingivalis* will only pose a threat to fetus if the syncytiotrophoblast layer was breached, so that *Porphyromonas gingivalis* can enter the fetal blood vessels.

Toll-like receptor-4 were strong expressed in placental on GD 14 and 20, especially the trophoblasts in labyrinth zone and junctional zone which are a frontal barrier between maternal and fetus. Trophoblast was expected to have important functions in regulating the host immune response against bacterial infection. Stimulation of TLR-4 is required for the reliable signaling to synergize LPS binding with TLR-4 in TNF release from macrophages and trophoblasts. Thus, expression of TLR-4 in macrophages and trophoblast of the labyrinth and junctional zone could potentially be a security against destructive infection. It is assumed that expressions of TLR-4 are increased their regulation in placenta as a defense mechanism that can be easily mobilized to protect the fetus from infection during pregnancy. TLR-4 may be an important regulator of the immune system from placental infection, but also may be required for the maturation of fetal immune response.

*Porphyromonas gingivalis* exposure in trophoblastic giant cell can increase the expression of TLR-4. Trophoblastic giant cell has been reported as a differentiated trophoblast precursor cells. It is possible that *Porphyromonas gingivalis* can cause cells of maternal-fetal interface to degenerate and die, thus it stimulates trophoblasts to perform phagocytosis and eliminate damaged cells. Phagocytosis act as a biological mechanism for the elimination of dead or degenerated cells. It was expected that the degeneration and deterioration of trophoblastic giant cells eventually lead to reduced trophoblasts. Therefore, TLR-4 serve as an important sensor for macrophages and trophoblasts cells, which makes it possible to coordinate the local immune response and to enhance cell invasion and placenta formation. TLR-4 also can provide a bridge for the introduction of placenta to the danger signal, and the produced type of response can have harmful consequences for the pregnancy.

This study also analyzed that the increasing concentration of LPS in the maternal blood serum resulting from *Porphyromonas gingivalis* infection on maternal periodontal tissues can result in decreased placental weight, fetal weight and length of the fetus. The decreased fetal weight and fetal length were caused by a decrease placental weight.

The placenta provides a better characterization about the 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 transport to fetus from maternal, 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.

In conclusion, the present study indicates that the increased LPS concentration in maternal blood serum resulted in the decreased fetal weight and fetal length. Syncytiotrophoblast, spongitrophoblast and trophoblastic giant cell were able to recognize *Porphyromonas gingivalis* LPS through the TLR-4 expression. This findings strengthened the link between periodontal disease and fetal growth restriction.

**ACKNOWLEDGEMENT**

We would like to thank Dr. Retno Pujirahayu, Sitarina Widyarini, Ph.D., Tri Wibawa, Ph.D., Dr. Totok Utoro and Dr. Indwiani Astuti, and Prof. Al. Supartinah, each of whom has made invaluable contributions to our work. Their efforts, collaborations, insights, and discussions are greatly appreciated. This study was supported by grant no. 481/SP2H/PP/DP2M/VI/2010 from Directorat General of Higher Education, Ministry of National Education – Indonesia.

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