Regulation of caspase-3 expression to maintain fetal growth in porphyromonas gingivalis-infected pregnant rats

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Abstract

Objective: Periodontal disease has been involved in a variety of systemic disorders and suspected as a potential risk factor for fetal growth restriction. This study aimed to analyze the role of TNF- α , IL-10 and caspase-3 to maintain fetal growth in porphyromonas gingivalis-infected pregnant rats.

Material and Methods: The subjects of the study were two blocks, they were sacrificed on gestational day (GD)-14 and GD20. Each block of gestational day was subdivided into two groups, which consisted of the healthy group with no porphyromonas gingivalis infection and the periodontitis group which experienced infection of porphyromonas gingivalis before and during pregnancy.

Results: The expression TNF- α , IL-10 and caspase-3 in macrophages and trophoblast cells were detected by immunohistochemistry. GD14

TNF- α ($R^2=0.416$; $P=0.000$) and IL-10 ($R^2=0.187$; $P=0.012$) had an important role to increase the expression of caspase-3 in the placenta, however only TNF- α ($R^2=0.393$; $P=0.000$) was able to increase the expression of caspase-3 on GD20. TNF- α and caspase-3 also had an important role ($P<0.000$) to decrease fetal weight, fetal length and placental weight on GD14 and GD20, but it was not the case with IL-10 ($P>0.000$). The increasing expressions of TNF- α and IL-10 did not only enhance immune protection, but also maintained the trophoblast cells survival by regulating expression of caspase-3.

Conclusion: Porphyromonas gingivalis infection in maternal periodontal tissue can lead to a decrease in placental weight, fetal weight and fetal length which are mediated by increasing expression of TNF- α , IL-10 and caspase-3 in the placenta.

Keywords: Caspase-3, Fetal growth, IL-10, Periodontal disease, TNF- α Article History

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Introduction

Periodontal disease, as a source of subclinical and persistent infection, can induce a systemic inflammatory response that increases the risk of premature birth, low birth weight, miscarriage and preeclampsia.¹⁻⁵

Periodontal disease can burden pregnant women with systemic endotoxemia, inflammatory cytokines and oxidative stress in the maternal-fetal interface.⁶⁻⁸ In addition, periodontal bacteria often enter the maternal circulation and spread to vital organs, including the placenta.^{4,9}

Placental infection is a major cause of maternal and fetal disease. Pathogen transmission from mother to fetus can occur at two direct contact locations between maternal cells and trophoblast cells in the placenta. Porphyromonas gingivalis as the primary etiology of periodontal disease may affect pregnancy.¹⁰ Histopathological analysis of the placenta of pregnant rats with periodontal tissue infections showed that there was a decrease in the density of trophoblast cells in the junctional zone and the labyrinth zone. This will affect the activity of the transfer of nutrients from mother to fetus, which affects the weight and length of the fetus. In addition, more nucleated erythrocytes were also found in the

fetal blood vessels in the late stage of pregnancy. It should be in the fetal blood vessels, erythrocytes are nonnucleated. Because if nucleated erythrocytes are found, then it indicates that the placenta is undergoing developmental disorders. Nevertheless fetus is still trying to produce erythrocytes to prevent fetal hypoxia.¹¹ Thus, changes in the morphology of the placenta is the result of exposure to the conditions of toxic/infectious agents and may be a marker of the intrauterine environment disorders.

The placenta gives a better overview of the intrauterine environment, particularly the specific changes on immune response that causes the pro-inflammatory and anti-inflammatory environmental changes. Maternal periodontal infection may also increase the activation of TLR2 and TLR4, and this ligation can stimulate the production of TNF- α in macrophages and trophoblast cells resulting in fetal growth restriction.^{7,12,13}

The increased expression of TNF- α appears to be accompanied by the increased expression of IL-10, thus the placenta and fetal pathological conditions induced by porphyromonas gingivalis could be inhibited by the expression of IL-10 in macrophages and trophoblast cells. The dominance

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of anti-inflammatory cytokines that was more than the pro-inflammatory cytokines in the placenta was considered important to determine the success and failure of pregnancy.^{14,15} Based on this fact, this research is required to analyze the role of TNF- α and IL-10 expressions to regulate caspase-3 expression in macrophages and trophoblast cells of the maternal periodontitis and also to analyze the role of TNF- α , IL-10 and caspase-3 expressions to maintain fetal weight, fetal length and placental weight.

Material and Methods

All procedures were approved by the Ethics Committee, Faculty of Dentistry, University of Gadjah Mada, Yogyakarta Indonesia. This study had taken female Sprague-Dawley rats, 2 months old adult, 150–250 g and primiparous. The rats were maintained under the controlled condition and were standardized on their conditions. The subjects of the study were two blocks, they were sacrificed on gestational day (GD)-14 and GD20. Each block of gestational day was subdivided into two groups, which consisted of the healthy group with no porphyromonas gingivalis infection and the periodontitis group which experienced infection of porphyromonas gingivalis before and during pregnancy. Each group consisted of five pregnant rats.

Before injecting porphyromonas gingivalis, all female rats were given antibiotic to depress the “natural” resident flora. Rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days. Induction of experimental periodontitis was performed by injection of 0.05 ml live porphyromonas gingivalis ATCC 33277

(MediMark, France) with a concentration of 2×10^9 cells/ml that was dissolved in saline 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 treated group rats. Then, the female rats were mated with the same strain of male rat overnight in the ratio 2:1. The next morning, female rats were removed from the cages and examined for the vaginal plug. If the vaginal plug was found, the day was recorded as GD1.

Each fetus was taken post-mortem from the chorioamniotic sac on GD14 and GD20. The placental weight, fetal weight and fetal length were recorded for each maternal rat. Furthermore, the placental immunohistochemistry was undertaken to determine the expression of TNF- α , IL-10 and caspase-3. The samples were incubated overnight at 4°C with primary antibody, rabbit polyclonal antibody anti-TNF- α dilution 1:1000 (Abbiotec, San Diego, CA, 1:1000-1:2000), anti-IL-10 dilution 1:400 (Abbiotec, San Diego, CA, 1:200-1:500) and anti-caspase-3 dilution 1:500 (Abbiotec, San Diego, CA, 1:100-1:500), whereas negative control was incubated with secondary antibody as a substitute for the primary antibody. TNF- α , IL-10 and caspase-3 were expressed in the cytoplasm. The data were presented as mean number of cells expressing TNF- α , IL-10 and caspase-3 in each type of cell. These specimens were evaluated in macrophages and trophoblast cells of labyrinth zone, junctional zone, decidua zone and were examined at X400 magnification.

The effect of maternal periodontitis to fetal growth was analyzed with independent sample T-test. Furthermore, a linear regression analysis was performed to predict the role of TNF- α and IL-10 expressions to regulate caspase-3 expression in macrophages and trophoblast cells of the healthy and periodontitis groups also to analyze the role of TNF- α , IL-10, caspase-3 expressions to maintain fetal weight, fetal length and placental weight. Value of significance was determined as $p < 0.05$. The numerical data were presented in mean $\pm$ standard deviation.

Results

Porphyromonas gingivalis infection in maternal periodontal tissue can lead to a decrease in placental weight, fetal weight and fetal length ($P = 0.000$) both on GD14 and GD20 table 1. Maternal periodontitis group had placental weight, fetal weight, and fetal length two-thirds lower than the healthy.

Table 1 The effect of maternal periodontitis toward fetal weight, fetal length and placental weight on GD14 and GD20

Variable	Maternal periodontal infection		p
	Healthy	Periodontitis	
GD14:			
Placental weight [g], n(x±SD)	38(0.20±0.039)	31(0.06±0.004)	0.000
Fetal weight [g], n(x±SD)	38(0.17±0.016)	31(0.04±0.007)	0.000
Fetal length [mm], n(x±SD)	38(10.32±1.232)	31(5.45±0.501)	0.000
TNF-α	23(1.87±0.344)	10(3.60±0.516)	0.000
IL-10	23(2.91±0.668)	10(3.20±0.422)	0.221
Caspase-3	23(2.43±0.507)	10(3.50±0.527)	0.000
GD20:			
Placental weight [g], n(x±SD)	30(0.62±0.088)	43(0.22±0.033)	0.000
Fetal weight [g], n(x±SD)	30(4.08±0.430)	43(0.56±0.169)	
Fetal length [mm], n(x±SD)	30(48.97±1.752)	43(18.39±1.606)	0.000
TNF-α	23(2.83±0.778)	10(3.60±1.075)	0.000
IL-10	23(4.22±0.902)	10(4.40±0.843)	0.026
Caspase-3	23(3.09±0.848)	10(4.20±0.784)	0.590

p: mean difference was significant at 0.05

On GD14, the expression of TNF- α ($P=0.000$) and IL-10 ($P=0.012$) had a significantly important role to increase the expression of caspase-3 in macrophages and trophoblast cells but only TNF- α ($P=0.000$) was able to increase the expression of caspase-3 in macrophages and trophoblast cells [table 2](#). The expression of TNF- α is closely related to the expression of caspase-3 both on GD14 and GD20. Each additional macrophage or trophoblast cell which expresses TNF- α would increase the expression of caspase-3 in trophoblast cells or macrophages on GD14 and GD20. Likewise, the events happened on the role of IL-10 in regulating the expression of caspase-3 on GD14.

The expression of TNF- α and caspase-3 also had a significantly important role ($P<0.000$) to decrease the fetal weight, fetal length and placental weight on GD14 and GD20, but the expression of IL-10 did not play a role ($P>0.000$) to decrease the fetal weight, fetal length and placental weight on GD14 and GD20 [table 3](#). The expression of TNF- α and caspase-3 was closely linked with the fetal weight,

fetal length and weight of the placenta on GD14 and GD20. Each additional trophoblast cell or macrophage which expresses TNF- α would decrease for 0.059 g fetal weight, 2.173 mm fetal length and 0.061 g placental weight on GD14, as well as it will decrease for 0.766 g fetal weight, 6.395 mm fetal length and 0.089 g placental weight on GD20. Accordingly the case, each additional trophoblast cell or macrophages which expresses caspase-3 would decrease for 0.058 g fetal weight, 2.160 mm fetal length and 0.058 g placental weight on GD14 and it would also reduce 1.016 g fetal weight, 8.247 mm fetal length and 0.103 g placental weight on GD2.

Discussion

Evaluation of fetal morphology visually on GD14, the period of embryogenesis showed morphological differences between fetuses of periodontitis and healthy groups. The shape of the head, legs and tail of a fetus on periodontitis group had not been fully developed. Likewise, the weight and length of the fetus were lower than the healthy group. During this stage, the majority of organogenesis had occurred thus the fetus was very vulnerable to the effects of exposure to *porphyromonas gingivalis* that could lead to impaired fetal growth. However, this study only observed morphological defects of the fetus shortly after taking the fetus out of the chorioamniotic sac on GD20, because dysfunction such as central nervous system disorders could not be detected immediately after birth. On GD14 and GD20, morphological defects were not found in all groups, but fetal weight was lower in the periodontitis group than the healthy group. This finding further confirmed that active inflammation before and/or during pregnancy may contribute to a decrease in placental weight, fetal weight and fetal length.

Table 2 The role of TNF- α and IL-10 expressions to regulate caspase-3 expression in macrophages and trophoblast cells on GD14 and GD20

Variable	Expression of Caspase-3			
	R ²	B	Beta	p
GD14:				
TNF- α	0.416	0.508	0.645	0.000
IL-10	0.187	0.500	0.432	0.012
GD20:				
TNF- α	0.393	0.651	0.627	0.000
IL-10	0.006	0.089	0.080	0.657

p: mean difference was significant at 0.05

R²: coefficient of determination

B: coefficient of regression

Beta: standardized coefficients

Table 3 The role of TNF- α , IL-10, and caspase-3 expressions to maintain fetal weight, fetal length and placental weight on GD14 and GD20

Variable	Fetal weight				Fetal length				Placental weight			
	R ²	B	Beta	p	R ²	B	Beta	p	R ²	B	Beta	p
GD14:												
TNF- α	0.778	-0.059	-0.882	0.000	0.638	-2.173	-0.779	0.000	0.634	-0.061	-0.796	0.000
IL-10	0.024	-0.015	-0.155	0.388	0.053	-0.917	-0.229	0.119	0.010	-0.011	-0.098	0.589
Caspase-3	0.467	-0.058	-0.683	0.000	0.391	-2.160	-0.626	0.000	0.347	-0.058	-0.0589	0.000
GD20:												
TNF- α	0.178	-0.766	-0.422	0.014	0.172	-6.395	-0.415	0.016	0.175	-0.089	-0.418	0.015
IL-10	0.006	-0.145	-0.075	0.678	0.003	-0.926	-0.056	0.755	0.004	-0.014	-0.063	0.729
Caspase-3	0.339	-1.016	-0.582	0.000	0.308	-8.247	-0.555	0.001	0.256	-0.103	-0.506	0.003

p: mean difference was significant at 0.05

R²: coefficient of determination

B: coefficient of regression

Beta: standardized coefficients

Immunohistochemical analysis of the placenta showed that TNF- α was not only expressed on periodontitis group but also on healthy group. Its role in healthy pregnancy is very speculative but is thought to be involved in the early growth of the fetus. The TNF- α is produced to regulate trophoblast proliferation and differentiation, cell adhesion, tissue remodeling, apoptosis of villous trophoblast and trophoblast hormone production.¹⁶ Therefore, it appears that the expression of TNF- α were less useful for pregnancy, whereas the increase in expression was very detrimental. The consequence is that early implantation and successful pregnancy is dependent on tight regulation of the expression of TNF- α in placental tissue. Thus, the presence of TNF- α on trophoblast showed that the trophoblast was also a source and target of cytokines. These data indicate the complexity of the regulation of TNF- α on fetomaternal interface.

The balance of pro-inflammatory and anti-inflammatory cytokines is essential for placental development. The role of TNF- α to induce apoptosis and the ability of IL-10 to attenuate the inflammatory response may be a viable mechanism underlying fetal growth restriction. This study identified that IL-10 was highly expressed in the placenta on GD14 and GD20 because IL-10 was allegedly involved in a variety of important events, which included the formation of placental and fetal growth. IL-10 has a protective effect on the unit as it may inhibit secretion of fetoplacental pro-inflammatory cytokine TNF- α . These findings supported the notion that the synthesis of IL-10 is a major determining factor of vulnerability or resistance to fetal pathological conditions induced by porphyromonas gingivalis. Thus, it is clear that the protective effects of IL-10 in the placenta will be deployed through inhibitory effects on macrophages and trophoblast cells to dampen the consequences of synergistic activation mediated by TNF- α . This extends the understanding of the role of anti-inflammatory IL-10 in the placenta by demonstrating the role of regulators in the cytokine network that not only regulated macrophages but also trophoblast cells.

The profile shift of TNF- α and IL-10 was alleged to have not only enhanced the immune protection but also regulated trophoblast cells survival by regulating the expression and/or function of components of the apoptotic cascade. This study had also observed the expression of caspase-3 in the placenta of healthy and periodontitis groups on GD14 and GD20. The expression of caspase-3 as a component of the apoptotic cascade differentially expressed in trophoblast cells was thought to be relate with different stages of apoptosis. Apoptosis during pregnancy was thought to be associated with the regulation of growth and turnover of trophoblast cells. On a healthy pregnancy, trophoblast

cells apoptosis was thought to be a physiological process that occurred during pregnancy.

However, in pathological conditions characterized incidents of apoptosis by caspase-3 expression in trophoblast cells and macrophages were greater.

Furthermore, it was also found that placental weight and fetal growth could be attributed to the stimulation of TNF- α released by macrophages and placental trophoblasts in response to maternal periodontal tissue infections which mainly occur before and during pregnancy. The increased expression of TNF- α could be expected to lead to placental dysfunction and impaired placental perfusion.

The increased expression of TNF- α on GD14 and GD20 seemed to be accompanied by the increased IL-10 expression so that the placental and fetal pathologic conditions induced by porphyromonas gingivalis can be inhibited by IL-10 in macrophages and trophoblast cells. The dominance of anti-inflammatory cytokines more than the pro-inflammatory cytokines in the placenta was considered important to determine the success and failure of pregnancy.

Decreased placental weight, fetal weight, and fetal length in the periodontitis group was accompanied by an increased incidence of trophoblast apoptosis characterized by the expression of caspase-3. Apoptosis is known to be associated with the stage of development of the placenta, including attachment and invasion of trophoblast cells,¹⁷ the transformation of the spiral arteries,¹⁸ as well as differentiation and turnover of trophoblast cells.¹⁹ In addition, apoptosis has also been shown to play an important role in improving maternal immune tolerance to paternal antigens expressed by trophoblast cells.²⁰ This event had been supported by this study demonstrating the absence of a dead fetus. The fetuses stayed alive although porphyromonas gingivalis infection had occurred during the fetal growth in the uterus.

Conclusion

It can be concluded that the increase of TNF- α and IL-10 expressions are not only enhancing immune protection but also maintaining the trophoblast cells survival by regulating the expression of caspase-3. Porphyromonas gingivalis infection in maternal periodontal tissue can lead to a decrease in placental weight, fetal weight and fetal length which is mediated by the increased expression of TNF- α , IL-10 and caspase-3 in the placenta.

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Conflict of Interest

The authors report no conflict of interest.

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