ISSN. 2088-9739

Proceeding

Anniversary Faculty of Dentistry University of Sumatera Utara

RDM&E-V 2011

THE 5th REGIONAL DENTAL MEETING AND EXHIBITION

@ TIARA CONVENTION CENTER, MEDAN
NOVEMBER 11 - 12, 2011

Theme
Improving Competence and Professionalism for Global Challenge in Dentistry

Fakultas Kedokteran Gigi
Universitas Sumatera Utara

PROCEEDING

Regional Dental Meeting&Exhibition-V 2011
Medan, 11 – 12 November 2011

EDITOR:

Prof. Trimurni Abidin, drg.,M.Kes.,Sp.KG (K)
Prof. Sondang Pintauli, drg.,Ph.D
Dr. Ameta Primasari, drg.,MDSc.,M.Kes
Dr. Trelia Boel,drg.,M.Kes.,Sp.RKG (K)
S. Hamzah Daliemunthe.,drg.,Sp.Perio (K)
Nurhayati Harahap, drg.,Sp.Ort (K)
Lasminda Syafiar, drg.,M.Kes
Shaukat Osmani Hasbi, drg.,Sp.BM
Syafrinani, drg.,Sp.Pros (K)
Sayuti Hasibuan, drg.,Sp.PM
Essie Octiara, drg.,Sp.KGA

FAKULTAS KEDOKTERAN GIGI
UNIVERSITAS SUMATERA UTARA
MEDAN

ISSN: 2088 – 9739

Proceeding RDM&E-V 2011

Daftar Isi (*Contents*)

Porphyromonas gingivalis BACTEREMIA INDUCES INTRAUTERINE GROWTH RESTRICTION IN PREGNANT RATS

Banun Kusumawardani[*], Marsetyawan HNE. Soesatyo[§], Djaswadi Dasuki[†], Widya Asmara[‡]

[*]Doctorate Program of Medical and Health Science, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia

[§]Departement of Histology and Cell Biology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia

[†]Departement of Obstetric and Gynecology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia

[‡]Departement of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta-Indonesia

Abstract

During periodontal infection, when the oral mucosa is injured and inflamed, and the quantities of periodontal pathogens increase dramatically, transient bacteremia may occur. This can lead to selective colonization of undesired sites. The aim of this study was to examine the potential effect of *Porphyromonas gingivalis* bacteremia to intrauterine growth restriction in pregnant rats. Female rats were challenged with live *P. gingivalis* at concentration of 10^9 colony forming unit/ml into subgingival sulcus before and/or during pregnancy. This study was consisted of 4 groups i.e. group 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. They were sacrified on gestational day 20. Fetuses were evaluated for weight. *P. gingivalis* was detected by API–ZYM system in the maternal blood of the retro-orbital venous plexus and the umbillical cord. The percentages of IUGR at the time of sacrifice were 6.66% growth-restricted fetuses in group I, and 100%, 72.97% and 87.09% growth-restricted fetuses in group II, III, and IV, respectively. When weights of growth-restricted fetuses of the treated groups were compared to the control, there were significant differences ($P<0.05$). *P. gingivalis* was first detected in the maternal blood, and the bacteria finally spread to the umbilical cord. This study represents that *P. gingivalis* may be transmitted hematogenously to the umbilical cord, and cause IUGR. The results strengthen the link between periodontal disease and adverse pregnancy outcomes.

Key words: *Porphyromonas gingivalis*; periodontitis; bacteremia; pregnancy; intrauterine growth restriction

Correspondence: Banun Kusumawardani, Departement of Periodontology, Faculty of Dentistry, Jember University. Address: Jl. Kalimantan 37 Jember 68121, Indonesia. Email: kusumawardani_banun@yahoo.co.id

INTRODUCTION

Epidemiological studies have linked intrauterine growth restriction (IUGR) and the increased risk of developing preterm low birth weight (PLBW) babies. Although 25% to 50% of PLBW deliveries occur without any known etiology, there is increasing evidence that infection may play a significant role in preterm delivery[1]. Several inflammatory disease are associated with reduced fetal growth, including rheumatoid arthritis[2], and periodontal disease[3,4]. In addition, elevated maternal serum[5] or placental inflammatory cytokines have been associated with IUGR[6]. Women with active inflammatory arthritis during pregnancy had smaller neonates at birth compared with health control women or women whose disease was in remission[7], suggesting that active inflammation during pregnancy may contribute to a reduction in fetal growth.

An animal model is needed in order to investigate the association between local infection and fetal growth. Laboratory rats can be a useful model to study the mechanisms of human abnormal pregnancy outcomes[8-10]. This model of localized chronic infection with *Porphyromonas 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 hamster[11] and mice[12] with heat-killed *P. gingivalis* induced a primary immune response. In addition, Han and coworkers injected *Fusobacterium nucleatum* into the tail vein of pregnant mice to study the ability of *F. nucleatum* to induce preterm birth or other adverse pregnancy outcomes when introduced into bloodstream of pregnant mice[13].

In the present study, a rat chronic infection model will be challenged with live *P. gingivalis* into subgingival sulcus. This model more closely mimics the chronic infection with periodontal pathogen observed in human patients. Furthermore, will be tested the ability of *P. gingivalis* to induce IUGR when introduced into the bloodstream of pregnant rats. The aim of this study was, therefore, to examine the potential effect of *P. gingivalis* bacteremia to intrauterine growth restriction in pregnant rats.

MATERIALS AND METHODS

Animals and treatments. 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 °C. Regular rat diet and water were provided *ad libitum*. 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 was consisted of 4 groups i.e. group 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. The protocols and procedures were in accordance with the animal welfare guidelines and approved by The Institutional Animal Care and Use Committee, Universitas Gadjah Mada.

Timed mating. 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.

Sample collection. The pregnant rats were sacrificed on GD 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. IUGR was defined as fetuses with weight 2 standard deviations (SD) smaller than normal fetal weight (NFW; 3.56±0.19 g)[12,14]. Blood of umbillical cord was collected from each fetus and pooled per dam. Maternal blood was bled from the retro-orbital venous plexus, and collected. All samples were stored at -80°C until analysis.

Detection of *P. gingivalis* bacteremia. Maternal blood of retro-orbital venous plexus and blood of umbillical cord was immediately plated on tryptic soy agar containing sheep blood and grown for 5-7 days at 37°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 (bioMéricux SA, Marcy-l'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[15].

Statistical analysis. *The fetal weights were presented as mean values ± the SD of the mean. Statistical analysis of compared mean fetal weights between groups were performed using one-way analysis of variance. The mean difference was significant at the <0.05 level.*

RESULTS

Periodontal infection were found in the challenged animals. During the course of experiment, the challenged animals had no febrile, did not exhibit malaise, and did not lose weight as a consequnce of challenge. When comparing the mean fetal weights from dams challenged with *P. gingivalis* (group II, III, and IV) to the control dams, they significantly resulted in a decrease in the mean fetal weights than the control group ($P<0.05$). The percentages of IUGR at the time of sacrifice 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 ($P<0.05$).

Tabel 1. Pregnancy outcomes in *P. gingivalis*-infected pregnant rats observed at GD 20

Groups	Mean fetal weight ± SD (g)	IUGR/Total fetuses (%)
I	4.079±0.430	2/30 (6.66%)
II	0.565±0.168*	43/43 (100%)
III	2.729±0.500*	27/37 (72.97%)
IV	2.342±0.582*	27/31 (87.09%)

*Significantly lower than control group ($P<0.05$)

In the control group, all maternal blood of retro-orbital venous plexus and umbillical cord samples from normal-weighed fetuses and growth-restricted fetus were not detected *P. gingivalis*. Whereas, maternal blood of retro-orbital venous plexus and umbillical cord samples from the treated groups possesed variable results of enzymatic activities of *P. gingivalis* as measured by the API-ZYM system. In the treated groups at GD 20 showed a strong of trypsin-like activities, and uniformly negative for α-Glucosidase and *N*-Acetyl-β-glucosamidase (Table 2 and 3).

Tabel 2. Average fetal weight and presence of *P. gingivalis* in maternal blood of retro-orbital venous plexus and umbillical cord from fetuses among *P. gingivalis*-infected dams observed at GD 20

Groups	Presence of *P. gingivalis*	Number of fetuses	Mean fetal weight ± SD (g)
I	Yes	0	0
	No	30	4.079±0.430
II	Yes	22	0.499±0.157
	No	21	0.634±0.154
III	Yes	12	2.380±0.168
	No	25	2.898±0.522
IV	Yes	14	2.107±0.571
	No	17	2.535±0.531

Tabel 3. Enzymatic activities of *P. gingivalis* from blood of retro-orbital venous plexus and umbillical cord observed at GD 20

Groups	Test result								
	Trypsin			α-Glucosidase			*N*-Acetyl-β-glucosamidase		
	Neg.	Weak	Strong	Neg.	Weak	Strong	Neg.	Weak	Strong
I	18	12	0	6	6	18	6	12	6
II	15	4	22	22	11	8	24	9	8
III	14	11	12	16	14	7	14	11	12
IV	7	10	14	23	5	3	18	7	6

Neg.: negative

DISCUSSIONS

During periodontal infection, when the oral mucosa is injured and inflamed, and the quantities of periodontal pathogens increase dramatically, transient bacteremia may occur[16]. This can lead to selective 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* has on pregnancy if it enters the circulation.

Porphyromonas gingivalis reaches high proportions in plaques associated with advanced periodontitits but are rarely detected in health. The trypsin-like activity seems to be unique to this bacteria as more than 25 other oral species are known[17]. The presence of this trypsin activity primarily in periodontophatic organisms suggests that this enzyme may be an important determinant of their virulence in periodontal disease. Such proteolitic activity may have a direct effect upon the junctional epithelium in the periodontal pocket as trypsin has been shown in vitro to disrupt cell-cell or cell-substratum adhesions[18]. In addition, trypsin seems to activate latent gingival tissue collagenase by destruction of a collagenase inhibitor present in serum[19]. Finally, this bacterial enzyme could activate the alternate pathway of complement fixation causing the release of leukotactic factors C3a and C5a, as has been demonstrated with trypsin and certain bacterial proteinases[20]. In fact, this trypsin-like enzyme could be the factor in periodontal plaque[21] and pure cultures of *P. gingivalis*[22] which has been found to be chemotactic for polymorphonuclear leukocytes. These trypsin-like activities acting singly or in concert could effect significant pathology on the periodontium.

Porphyromonas gingivalis also have been shown to resist phagocytosis even in the presence of specific antibodies and factor C3 of the complement system[23]. Recently, Sundqvist[24] showed that *P. gingivalis* W83 was able to degrade complement proteins C3 and C5 from guinea pig serum both in vitro and in vivo. By degrading complement and immunoglobulins, *P. gingivalis* may evade the phagocytic host defense.

Blood was sampled immediately following *P. gingivalis* injection in subgingival sulcus. Animal studies have shown that peak bacteremia occurs quickly within the first minute when human oral microorganisms are injected into the bloodstream[25], and systemic dissemination has been reported within 40 minutes of commencing dental procedures[26]. The timing may be an important factor in determining the amount of

bacteria recovered from the bloodstream and whether a bacteremia can be detected at all. The reduction bacteremia over several minutes after dental instrumentation is due to the effectiveness of host defence system in rapidly clearing microorganism from the blood[27]. In our study, the bacteria were usually cleared within 10 minutes and always within 20 minutes.

Our study showed that maternal *P. gingivalis* infection on periodontal tissue can result in *P. gingivalis* dissemination to umbillical cord and induction of IUGR, but *P. gingivalis* was not always detected in the umbillical cord from abnormal 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 IUGR 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 IUGR fetuses in the *P. gingivalis*-infected groups than in the control, and difference percentage of IUGR 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.

The role of bacterial infections in pregnancy complications is well known. Bacterial vaginosis and chorioamnionitis can lead to spontaneous preterm birth, especially in early gestation[28,29]. Studies showed that intrauterine infection were common among women who gave birth prematurely[30,31]. Four possible mechanism exist for microbes to spread to the uterus, which otherwise is a sterile environment: 1) organisms from vagina and the cervix ascend to the uterus; 2) the organisms originate elsewhere in the body and infect placental tissues as a result of hematogcous spread; 3) organisms from the peritoneal cavity translocate retrogradely through the fallopian tube; and 4) organisms are inoculated accidentally in uterine tissues during invasive procedures, such as amniocentesis or chorionic villous sampling[31]. Hence, the putative link between periodontal disease and pregnancy complications might be attributable to repeated exposures of the decidual tissues to periodontal pathogens through transient bacteremia.

In humans, oral microorganisms, including *Fusobacterium nucleatum* and *Capnocytophaga sputigena*, were detected in the amniotic fluid of women with intact membranes[32-34] and those with preterm labor[35], thus supporting the possibility that oal bacteria or bacteria products can spread through the bloodstream to the placenta. Oral pathogens presumbly gain access to the systemic circulation via local tissue inflammation and breakdown, as demontrated in animal models, and might cause damage by affecting the placenta and possibly the fetus itself.

The evidence of *P. gingivalis* in blood of umbillical cord of dams with IUGR may support a possible role of this microorganism in the pathogenesis of IUGR, making maternal oral infectious exposure a crucial phenomenon throughout pregnancy.

Once our present finding are established further and corroborated, future research will be focused on preventive strategies aimed at reducing oral bacterial load, which hopefully would decrease the incidence of IUGR.

ACKNOWLEDGMENTS

We would like to thank Drs. Sitarina Widyarini, Tri Wibawa, Totok Utoro and 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.

REFERENCES

1. Kramer MS. Determinants of low birth weight: Methodological assessment and meta-analysis. Bull World Health Organ 1987;65:663-737.
2. Skomsvoll JF, Baste V, Irgens LM, Ostensen M. The recurrence risk of adverse outcome in the second pregnancy in women with rheumatic disease. Obstet Gynecol 2002;100:1196-202.
3. Offenbacher S, Lieff S, Boggess KA, Murtha AP, Madianos PN, Champagne CME, *et al.* Maternal periodontitis and prematurity. Part I: obstetric outcome of prematurity and growth restriction. Ann Periodontol 2001;6:164-74.
4. McGaw T. Periodontal disease and preterm delivery of low birth-weight infants. J Can Dent Assoc 2002;68:165-69.
5. Dasanayake AP, Boyd D, Madianos PN, Offenbacher S, Hills E. The association between *Porphyromonas gingivalis*-specific maternal serum IgG and low birth weight. J Periodontol 2001;72:1491-97.

6. Hahn-Zoric M, Hagberg H, Kjellmer I, Ellis J, Wennergren M, Hanson LA. Aberration in placental cytokine m RNA related to intrauterine growth retardation. Pediatr Res 2002;51:201-06.
7. Bowden AP, Barret JH, Fallow W, Silman AJ. Women with inflammatory polyarthritis have babies of lower birth weight. J Rheumatol 2001;28:355-59.
8. Parr EL, Tung HN, Parr MB. Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats. Biology of Reproduction 1987;36:211-25.
9. Rivera DL, Olister SM, Liu X, Thompson JH. Zhang X-J, Pennline K, *et al.* Interleukin-10 attenuates experimental fetal growth restriction and demise. FASEB J 1998;12:189-97.
10. Cong J, Diao H, Zhao Y, Ni H, Yan Y, Yang Z. Differential expression and regulation of cyclooxygenases, prostaglandin E synthases and prostacyclin cynthase in rat uterus during the peri-implantation period. Reproduction 2006;131:139-51.
11. Collins JG, Windley III HW, Arnold RR, Offenbacher S. Effect of a *Porphyromonas gingivalis* infection on inflammatory mediator response and pregnancy outcome in hamsters. Infect Immun 1994;62:4356-61.
12. Lin D, Smith MA, Champagne C, Elter J, Beck J, Offenbacher S. *Porphyromonas gingivalis* infection during pregnancy increases maternal tumor necrosis factor alpha, suppresses maternal interleukin-10, and enhances fetal growth restriction and resorption in mice. Infect Immun 2003;71:5156-62.
13. Han YW, Redline RW, Li M, Yin L, Hill GB, McCormick TS. *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. Infect Immun 2001;72:2272-79.
14. ARTA and MTA. Historical control data (1992-1994) for developmental and reproductive toxicity studies using the Crl:CD®(SD)BR rat. Charles River Laboratories; 1996. p. 1-32.
15. Laughon BE, Syed SA, Loesche WJ. API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp., and Spirochetes of oral origin. J Clin Microbiol 1982;15:97-102.
16. Daly CG, Mitchell DH, Highfield JE, Grossberg DE, Stewart D. Bacteremia due to periodontal probing: a clinical and microbiological investigation. J Periodontol 2001;72:210-14.
17. Hofstad T. Evaluation of the API-ZYM system for identification of *Bacteroides* and *Fusobacterium* species. Med Microbiol Immunol 1980;168:173-77.
18. Britch M, Allen TD. The modulation of cellular contractility and adhesion by trypsin and EGTA. Exp Cell Res 1980;125:221-31.
19. Birkendall-Hansen H, Cobb CM, Taylor RE, Fullmer HM. Trypsin activation of latent collagenase from several mammalian sources. Scand J Dent Res 1975;83:302-05.
20. Ward PA, Chapitis J, Conroy MC, Lepow IH. Generation by bacterial proteases of leukotactic factors from human serum, and human C3 and C5. J Immunol 1973;110:1003-09.
21. Okada H, Silverman MS. Chemotactic activity in periodontal disease. II. The generation of complement-derived chemotactic factors. J Periodontal Res 1979;14:147-52.
22. Lindhe J, Socransky SS. Chemotaxis and vascular permeability produced by human periodontopathic bacteria. J Periodondal Res 1979;14:95-184.

23. Sundqvist GK, Bloom GD, Enderg K, Johansson E. Phagocytosis of *Bacteroides melaninogenicus* and *Bacteroides gingivalis* in vitro by human neutrophils. J Periodontal Res 1982;17:113-21.
24. Sundqvist GK, Carlsson J, Herrmann B, Holding JF, Vaatainen A. Degradation in vivo of the C3 protein of guinea-pig complement by a pathogenic strain of *Bacteroides gingivalis*. Scand J Dent Res 1984;92:14-24.
25. Silver J, Martin I, McBridge B. Recovery and clearance rates of oral microorganism following experimental bacteraemias in dogs. Arch Oral Biol 1975;20:675-79.
26. Nieves MA, Hartwig P, Kinyon JM, Riedesel DH. Bacterial isolate fro plaque and from blood during and after routine dental procedures in dog. Veterin Surg 1997;26:26-32.
27. Pallasch T, Slots J. Antibiotic prophylaxis and the medically compromised patient. Periodontal 2000 1996;10:107-38.
28. Chaim W, Mazor M, Leibermen JR. The relationship between bacterial vaginosis and preterm birth. A review. Arch Gynecol Obstet 1997;259:51-58.
29. Goldenberg RL, Andrews WW, Hauth JC. Choriodecidual infection and preterm birth. Nutr Rev 2002;60:S19-S25.
30. Goncalves LF, Chaiworapongsa T, Romeo R. Intrauterine infection and prematurity. Ment Retard Dev Disabil Res Rev 2002;8:3-13.
31. Romero R, Avila C, Brekus CA, Morotti R. The role of systemic and intrauterine infection in preterm parturition. Ann N Y Acad Sci 1991;622:355-75.
32. Chaim W, Mazor M. Intraamniotic infection with fusobacteria. Arch Gynecol Obstet 1992;251:1-7.
33. Hill GB. Preterm birth: Associations with genital and possibly oral microflora. Ann Periodontol 1998;3:222-32.
34. Bearfield C, Davenport ES, Sivapathasundaram V, Allaker RP. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. BJOG 2002;109:527-33.
35. McDonald H, Gordon DL. Capnocytophaga species: A cause of amniotic fluid infection and preterm labour. Pathology 1988;20:74-76.