

HUMORAL IMMUNE RESPONSE TO PORPHYROMONAS GINGIVALIS IN POSTPARTUM WOMEN AND NEWBORNS

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ABSTRACT

AIMS: The aim of this study was to evaluate the presence of *Porphyromonas gingivalis* (Pg) in the subgingival biofilm, as well as to compare the immune response of postpartum women and their newborns to *Porphyromonas gingivalis* using antibody serum levels.

METHODS: For this cross-sectional study, a total of 43 postpartum women and 24 newborns were selected in the municipal maternity hospital of Alagoinhas, Bahia, Brazil between February and December 2003, for this cross-sectional study. The presence of Pg was verified using polymerase chain reaction (PCR). The immunoglobulin serum levels: IgG1, IgG2, IgG3, IgG4 and IgA, reactive to Pg ATCC33277 were tested using Enzyme ImmunoAssay (ELISA).

RESULTS: 39,3% of the samples from postpartum women biofilm were positive for Pg. The incidence of this pathogen in women with periodontitis (70%) was higher than in the group of women without disease (30,30%). Among the newborns, high serum levels of IgG4 anti-Pg were observed ($p < 0.001$).

CONCLUSION: These findings confirm that postpartum women with periodontitis present remarkable incidence of *Porphyromonas gingivalis* which may influence systemic response, as represented by high levels of serum antibody levels against this pathogen in women and newborns. Furthermore, evidence suggests that some antibodies crossed the placental barrier in newborns.

Keywords: periodontitis, gingivitis, pregnancy, immunoglobulins.

R E S U M O

OBJETIVO: Avaliar a presença de *Porphyromonas gingivalis* (Pg) no biofilme subgengival de puérperas e comparar a sua resposta imune e a de seus recém-nascidos contra este patógeno por meio dos níveis séricos de anticorpos.

MÉTODO: Para este estudo transversal, um total de 43 puérperas e 24 recém-nascidos foi selecionado na maternidade municipal da cidade de Alagoinhas, Bahia, Brazil entre fevereiro e dezembro de 2003. A presença de Pg foi verificada por meio da reação em cadeia da polimerase (PCR). Os níveis séricos das imunoglobulinas IgG1, IgG2, IgG3, IgG4 e IgA, reativas a Pg ATCC33277, foram testados por imunoenensaio enzimático (ELISA).

RESULTADOS: Foi observado que 39,3% das amostras de biofilme das puérperas foram positivas para Pg. A incidência do patógeno nas mulheres com periodontite (70%) foi superior à encontrada no grupo de mulheres sem a doença (30,30%). Entre os recém-nascidos, foram observados altos níveis séricos de IgG4 anti-Pg ($p < 0.001$).

CONCLUSÃO: Os achados confirmam que puérperas com periodontite apresentam uma alta incidência de *Porphyromonas gingivalis*, o que pode influenciar a resposta sistêmica, representada pelos altos níveis séricos de anticorpos contra este patógeno em mulheres e recém-nascidos. Além disso, as evidências sugerem que alguns anticorpos atravessam a barreira placentária, alcançando os recém-nascidos.

Palavras-chave: periodontite, gengivite, gravidez, imunoglobulinas

INTRODUCTION

Indications of distant repercussions relating to periodontal disease can be seen to be a potential determinant for adverse gestational outcomes¹. It is known that, in the relationship between bacterial aggression and host defense, a serie of reactions translated as local alterations takes place. All the indications are that distant alterations also occur.

Periodontal infection may serve as a chronic reservoir for microbial products that can affect other parts of the body through the blood circulation. Inflammatory mediators like PGE₂ and tumor necrosis factor- α present increasing concentration during pregnancy, thereby reaching the critical levels that are needed for triggering delivery labor². These mediators may be produced locally in the periodontium, in response to microbial challenge. Because of the high level of vascularization in periodontium, high levels of these substances may become established, thereby representing a systemic source of cytokines that are toxic to the fetus¹.

Some studies^{3,4} found that periodontal and bacteriological conditions of pregnant women and the immunological profiles relating to periodontal disease are risk indicators in the relationship with adverse gestational outcomes. Other authors suggest that successful periodontal therapy in pregnant women with periodontitis is a protective factor promoting the birth of children with normal weight⁵. On the other hand, there are studies that do not corroborate this relationship^{6, 7, 8, 9, 10}. One possible explanation for these divergent findings lies in the response of the organism to periodontal pathogenic bacteria, since this reflects an intricate network of events that is started by contact between the host's immune system and the bacteria¹¹.

The high serum levels of antibodies against important periodontal pathogenic bacteria reflect the intensity of periodontal infection, for example by *Porphyromonas gingivalis* (Pg). This anaerobic bacterium has been implicated as one of the most important agents of periodontal disease¹². It can induce immune response profile imbalance, which switch to different isotypes of immunoglobulins, which can disturb the maternal-fetal balance.

Given the controversy surrounding this topic, the present pilot study sought to evaluate the presence of periodontal pathogens in the subgingival biofilm and the humoral immune response to sonicated

extract of Pg from puerperae and to compare the serum levels of Pg antibodies between these women and their newborns.

MATERIAL AND METHODS

Subjects

Forty-three puerperae whose delivery had taken place at the municipal maternity hospital of Alagoinhas, Bahia, between February and December 2003, participated of this study. In 24 women, blood from the umbilical cord of their newborns had also been collected to evaluate the humoral immune response.

Exclusion criteria included diabetes, cardiovascular disease, or any other systemic disease that could alter the course of periodontal disease, pregnancy or immune response. Former smoking, previous consumption of systemic antimicrobials or anti-inflammatory drugs (six or two months before, respectively), and periodontal therapy during the last twelve months also served as exclusion criteria. The eligible subjects were interviewed through a structured questionnaire to obtain socio-demographics, medical and lifestyle characteristics. The women's periodontal condition was evaluated by a single calibrated dentist (ECR; kappa 0.832). The probing depth of the sulcus/pocket, gingival recession or hyperplasia, clinical attachment loss and bleeding on probing were evaluated. These clinical parameters made it possible to determine each individual's periodontal condition¹³. Post-partum women were considered to have periodontitis if they had four or more teeth with one or more sites presenting a probing depth greater than or equal to 4 mm, a clinical attachment loss greater than or equal to 3 mm, as well as bleeding on probing at the same site(s).

Biofilm collection from post-partum women

Samples of subgingival dental biofilm were collected from each site with the greatest probing depth in all sextants, using one sterile paper point (Dentsply, York, PA, USA) at each site, after drying and isolating the surrounding area using sterile gauze. Next, all six paper points from an individual's sextants were stored together in a single DNase-free and RNase-free microcentrifuge tube (Eppendorf) in a sterile solution of phosphate-buffered saline (PBS)

at -20°C until time of analysis.

DNA extraction

Bacterial DNA was extracted from the supernatant obtained by heating the tubes for 10 min at 100°C , followed by thermal shock in an ice bath and centrifugation at 1300 rpm for one minute [14]

PCR for detection of bacterial DNA

Amplification using the polymerase chain reaction (PCR) for detection of bacterial DNA was performed in volumes of 50 μl containing 1X PCR/Mg⁺⁺ buffer (Boehringer Mannheim, Indianapolis, IN, USA), 0.2 mM of dNTP (Pharmacia Biotech, Piscataway, NJ, USA), 0.5 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA), 0.4 μM of each pair of primers and 10 ng of the base. The amplification was performed in a thermocycler (Perkin Elmer, GeneAmp PCR System 2400, Norwalk, CT, USA), set at 94°C (five minutes) and accompanied by 30 cycles with a suitable annealing temperature for each pair of primers¹⁵. After 30 cycles, a temperature of 72°C was used for five minutes, for complete DNA extension. The amplification products were compared by means of electrophoresis on 1% agarose gel in 1XTBE buffer (1M Tris, 0.9M boric acid, 0.01M EDTA; pH 8.4) (Gibco BRL, Life Technologies Ltda, Bethesda, MD, USA), with ethidium bromide staining (0.5 $\mu\text{g}/\text{ml}$), and were photographed under a transilluminator with ultraviolet light (Kodak Digital Science System 120, Eastman Kodak Company, NY, USA). A standard of molecular mass 1 kb (kilobase) was included (Gibco BRL, Life Technologies Ltda, Bethesda, MD, USA).

Blood Collection

Samples of 5 ml of blood were collected from antecubital fossa of the puerperae and from umbilical cord of the newborns, using tubes without anticoagulant (Vacutainer, BD, Indianapolis, IN, USA), for subsequent centrifugation. The serum was collected and stored at -20°C .

Immunological evaluation

The levels of IgG subclass and IgA antibodies were evaluated using the Enzyme Linked Immunosorbent Assay (ELISA) method. High adsorption polystyrene plates (Costar, Corning Life Science, Lowell, MA, USA) were sensitized with 10 $\mu\text{g}/\text{ml}$ of protein from the sonicated extract of *Porphyromonas gingivalis* (ATCC 33277), diluted in 0.05M carbonate-bicarbonate

buffer (pH 9.6). Diluted antigen was applied at the rate of 50 $\mu\text{l}/\text{well}$ and the plates were incubated for 15 hours at 4°C , in a damp chamber. The washing buffer used at all stages of the tests was PBS, containing 0.05% Tween-20 detergent (PBS-T) and blocking was performed using a 2% BSA solution in STF-T at a rate of 200 $\mu\text{l}/\text{well}$ for two hours at 37°C , in a damp chamber.

For each test, different dilutions of the serum in PBS containing 0.5% BSA were made: 1:50 (IgA), 1:500 (IgG1), 1:100 (IgG2), 1:50 (IgG3) and 1:100 (IgG4). The plates were incubated for one hour at 37°C , in a damp chamber, and were then washed five times. The following immunoglobulin conjugates were added at the rate of 50 $\mu\text{l}/\text{well}$: goat anti-human IgA conjugated with peroxidase (Bethyl Laboratories, Montgomery, TX, USA), at a dilution of 1:50,000; mouse anti-human IgG1 conjugated with biotin (Southern Biotechnology Associates Inc., Birmingham, AL, USA), at a dilution of 1:2000; mouse anti-human IgG2 conjugated with biotin (Southern Biotechnology Associates Inc., Birmingham, AL, USA), at a dilution of 1:1000; mouse anti-human IgG3 conjugated with biotin (Southern Biotechnology Associates Inc., Birmingham, AL, USA), at a dilution of 1:2000; and mouse anti-human IgG4 conjugated with biotin (Southern Biotechnology Associates Inc., Birmingham, AL, USA), at a dilution of 1:1000. After incubation for one hour at 37°C , the conjugates were discarded and the plates were washed five times with PBS-T.

To perform ELISA on the IgG subclasses, a signal amplification system using biotin-streptavidin was needed, thus adding a further step: incubation with streptavidin diluted in PBS at 1:7:500, followed by another five washes. The reactions were developed by means of 50 $\mu\text{l}/\text{well}$ of tetramethylbenzidine (TMB) developing solution (Zymed Laboratories, San Francisco, CA, USA), in accordance with the manufacturer's instructions. After incubation for 15 minutes at room temperature, shaded from bright light, the reactions were halted by adding 2N H₂SO₄ at the rate of 25 $\mu\text{l}/\text{well}$. Following this, the optical densities were measured using an ELISA reader (ELx 800; Bio Tek, Winooski, VT, USA) adjusted for wavelengths in the range from 450- 630 nm. The optical densities were corrected between plates for each type of standardized ELISA.

Statistical Analysis

Simple frequencies and central trend measurements

were obtained regarding the socio-demographics, medical and lifestyle characteristics and statistical differences between groups were evaluated using the Chi-Square test for categorical variables and t test for continuous variables, with a significance level of 5%.

For comparisons between groups regarding the central trends of the data, the Mann-Whitney test was used. All the data analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 17.0 for Windows.

RESULTS AND DISCUSSION

Characterization of the subjects

It was observed that there were no differences regarding the socio-demographics, medical and life style characteristics evaluated in this study among the groups: puerperae with periodontitis (n=10/23.26%), and puerperae without periodontitis (n=33/76.74%). The age of participants ranged from 21 to 36 years old and it was composed mainly by low socio-economic level.

Microbiological evaluation of the subgingival biofilm

The microbiological data presented below relate to detection of periodontal pathogens in each puerperae examined, since the samples collected from six different sites were homogenized to

reflect the individual's overall response. The results from PCR shown DNA fragments in agarosis gel: E.c.: Escherichia coli; P.g.: Porphyromonas gingivalis (Figure 1). It was demonstrated that the incidence of Porphyromonas gingivalis in the evaluated sample was 39.53%. The incidence of Pg in ten post-partum women with periodontitis was remarkable: seven (70%) were positive to the pathogen. In the group without periodontitis ten of 33 women showed Porphyromonas gingivalis in biofilm samples (30.30%).

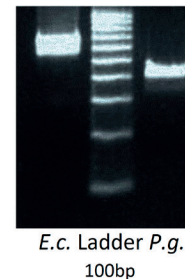


Figure 1: PCR for bacterial detection and identification.

Evaluation of the humoral immune response of puerperae and newborns

Prior quality control was performed on the ELISA tests by using the coordinates of ROC curves on data from healthy individuals and from individuals with periodontal disease without any systemic impairment (including in relation to pregnancy). From this, the cutoff point, sensitivity, specificity and positive and negative predictive values were established, as shown in Table 1.

Table 1: Quality control analysis on ELISA tests, by means of ROC curves.

Immunoglobulin assay	Sensitivity	Specificity	Cutoff point	Positive predictive value	Negative predictive value
IgA	73.3	84.6	0.076	80.8%	70.0%
IgG	82.8	80.8	0.206	82.8%	80.8%
IgG1	69.0	69.2	0.128	67.7%	66.7%
IgG2	62.1	57.7	0.186	67.7%	66.7%
IgG3	65.5	57.8	0.142	61.3%	58.3%
IgG4	93.1	76.9	0.198	81.8%	90.9%

Anti-*Porphyromonas gingivalis* IgG1 levels were found to be low in both groups (Figure 2A), Puerperae had statistically significant higher IgG2 (Figure 2B) levels than newborns ($p < 0.05$). IgG3 levels were low in both groups (Figure 2C). IgG4

levels were found to be high in the serum of both the puerperae and their newborns (Figure 2D). Puerperae had statistically significant higher IgG total (Figure 2E) and IgA (Figure 2F) levels than newborns ($p < 0.001$).

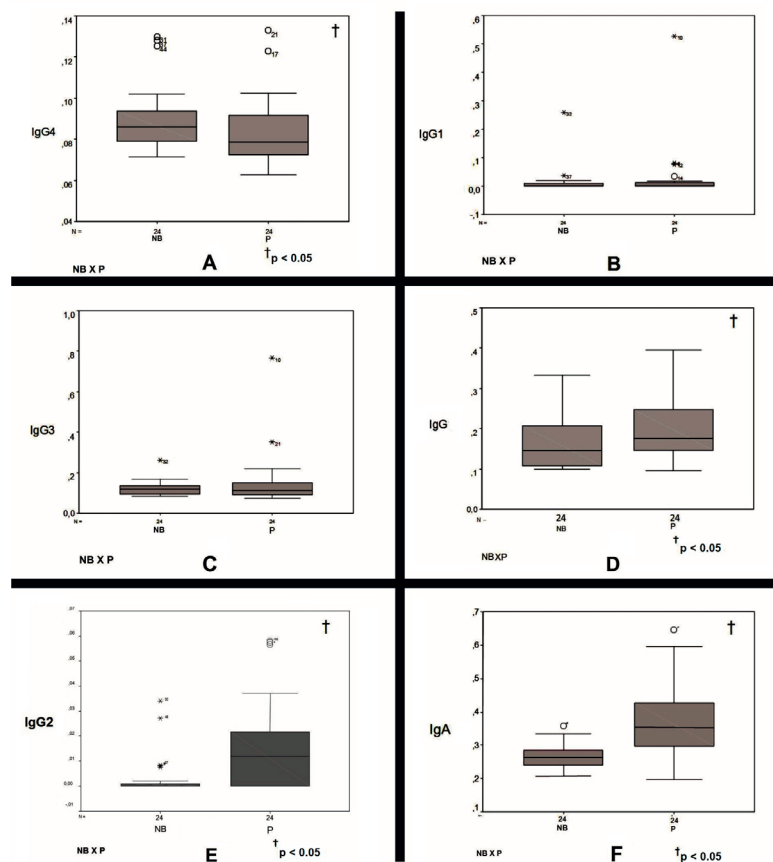


Figure 2: Serological reactivity among the postpartum women (P) and newborns (NB) against *Porphyromonas gingivalis*'s antigens. † $p < 0.001$.

According to the analyses performed in the present pilot study, the occurrence of *Porphyromonas gingivalis* was high, mainly in the post-partum women with periodontitis when compared with ones without this disease. This was also found among others individuals with periodontitis, compared with individuals with a clinically health periodontium [15]. One likely explanation for the development of anaerobic microorganisms at sites in individuals with periodontitis may involve microenvironmental factors such as changes in redox potential in areas of periodontal pockets [18] and changes in hormone levels [19,20]. Even with the impossibility of quantifying the bacteria using the conventional PCR method for pathogen detection, the findings presented here corroborate results from real-time PCR for quantifying periodontal pathogens. For example, high prevalence of *Actinobacillus*

actinomycetemcomitans, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Dialister pneumosintes*, *Campylobacter rectus* and *Micromonas micros* were found in subgingival biofilm in 83 patients with periodontitis and 43 healthy volunteers [15]. With regard to the humoral response of the puerperae and their newborns, another important finding was the high serum levels of anti-*Porphyromonas gingivalis* IgG4 in both groups. It is now known that transportation of IgG in humans begins around the twentieth week of pregnancy and that it increases progressively until delivery. Only antibodies in the IgG class cross the placental barrier, and the transmission rate of these immunoglobulins also varies according to the subclass to which they belong. IgG1 and IgG3 seem to cross the placenta slightly more efficiently than IgG4 does. In turn, the latter crosses the mother-fetus interface more efficiently than IgG2

does^{21, 22}. It is possible that, through sensitization due to infection with *Porphyromonas gingivalis*, the puerperae of the present study produced and transmitted IgG4 to their babies, given that the fetus received antibodies against antigens to which their mothers were exposed previously.

On the other hand, the IgG1 and IgG3 levels were low among the puerperae and consequently among their newborns. Although it is known that individuals with periodontitis have higher serum levels of IgG1, while there is no difference in IgG3 between different periodontal conditions²¹. However, out of the 43 puerperae studied in this investigation, only 23.25% presented periodontitis, thus probably explaining the low levels of IgG1 found among this group. As expected, the antibodies IgA and IgG2 were found at higher levels in the serum of the puerperae than in their newborns' serum. This confirms that, despite the high production in the puerperae, these immunoglobulins were not transmitted to the fetus. The evaluation of IgA and IgG2 in the present study was to understand the behavior of the humoral response of mothers which composed the sample.

It is important to note that the choice of Pg extract to assess the humoral response is due to its presence at high levels in periodontitis¹². Furthermore, in puerperae evaluated in this study, the occurrence of this pathogen was high confirming this finding. Since this is a preliminary study, we chose to test only one extract, since future evaluations will be performed with different extracts of pathogens also prevalent. In this manner, and in the light of the initial evidence from the present pilot study, it is not the purpose of this investigation to relate the periodontal pathogens and antibody levels to adverse gestational complications, due to their preliminary results and limitations. But the findings of this study indicate that some IgG subclasses against *Porphyromonas gingivalis* crossed the placental barrier and the puerperae transmitted them to their babies. This can raise two opposing suppositions: in the first, a fetal passive immunization against *Porphyromonas gingivalis* to which the mother was exposed, regardless of having had the disease, can occur. In the second, these immunological changes in the fetoplacental unit, stimulated by the presence of this periodontal pathogen can disrupt the immune profile balance, indicating that the relationship between periodontal disease and possible unfavorable gestational outcomes are likely. Thus, longitudinal studies evaluating the humoral response specific to

Porphyromonas gingivalis in pregnant women and the gestational outcomes are necessary to elucidate this relationship.

CONCLUSION

Continuation of this investigation may contribute important complementary information regarding the biological plausibility of the relationship between periodontal disease and possible unfavorable gestational outcomes.

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ETHICAL COMMITTEE APPROVAL

All the participants asked, agreed to be enrolled in the study. The participants signed a statement of free and informed consent that had been approved by the Research Ethics Committee of Feira de Santana State University (protocols No 020/2002 and 114/2005).

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