

## OBSTETRICS

## No association between periodontitis, preterm birth, or intrauterine growth restriction: experimental study in Wistar rats

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**BACKGROUND:** The biologic plausibility of the possible association between periodontitis and adverse pregnancy outcomes has been assessed with the use of different experimental models. However, most experimental studies did not induce periodontitis in the animals but promoted an acute microbial challenge with selected periodontal pathogens or their products subcutaneous or intravenous or intraamniotic. The present study was then conducted to verify the biologic plausibility of such association by experimentally inducing periodontitis in Wistar rats.

**OBJECTIVE:** An experimental study on an animal model by the induction of periodontitis in 50% of sites and assessment of the presence of cytokines in the gingival tissue, serum, placenta, cord, and amniotic fluid was designed to test the null hypothesis that experimental periodontitis that is induced on rats does not result in adverse pregnancy outcomes.

**STUDY DESIGN:** Forty female Wistar rats were included in 2 groups: a periodontally healthy (without ligatures) and an experimentally induced periodontitis group (test, with ligatures). Forty-five days after the induction, the mating was initiated. Males were placed with females in the ratio of 1:2 for a period of 12 hours. The bodyweight of the female, from then on, was recorded daily. When the pregnancy was confirmed on day 20, laparotomy was performed. The amniotic fluid, placenta, umbilical cord, blood (serum) and maternal and gingival tissue samples were subjected to quantitative analysis for interleukin 1 $\alpha$ , -6, -10, -4, -12p70, and -17a, tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  by multiplex methods. Mean scores, standard

deviations, and standard errors for estimated measures were calculated. For cytokines analyses, the Mann-Whitney test was conducted to compare the concentration of the analytes from control and test groups in the different tissues samples. For comparison of cytokines reduction from gingival tissue to serum and from serum to placenta, the Wilcoxon Test was performed. Spearman's correlation was conducted among cytokines in the 5 different tissues that were evaluated.

**RESULTS:** The induced periodontitis in Wistar rats did not result in adverse outcomes of pregnancy. There were no statistically significant differences between groups in relation to prematurity, fetal, or birth weight. Regarding cytokines, there were no statistically significant differences in concentrations that were measured in each tissue between the groups with periodontitis and controls. Furthermore, all cytokine levels in the placenta, except interleukin-6, were diminished compared with the amniotic fluid or maternal serum, which suggested that the cytokines cannot easily be transferred via this tissue in maternal-fetal or fetomaternal direction. The fertility rate was reduced significantly in the group with periodontitis.

**CONCLUSION:** Periodontitis that is induced in rats is not a risk factor for preterm birth or low birthweight.

**Key words:** animal, experimental periodontitis, preterm birth, low birthweight, Wistar rats

Periodontitis is the outcome of an interrelation of an oral infection that induces an inflammatory response in the host, modified by behavioral elements.<sup>1,2</sup> Because of its chronic, inflammatory, and infectious characteristics,<sup>3,4</sup> periodontitis was proposed as a risk factor for preterm birth (PTB) and low birthweight (LBW).<sup>5</sup>

The biologic plausibility of such association was assessed through

experimental models. One study induced periodontitis in Wistar female rats and evaluated its effect in pregnancy outcomes.<sup>6</sup> The findings suggested that the induction of periodontitis does not significantly alter mortality rates or birthweight of the rats.<sup>6</sup>

However, most experimental studies did not induce periodontitis; however, they promoted an acute microbial challenge with selected periodontal pathogens or their products that were subcutaneous<sup>5,7,8</sup> or intravenous<sup>9-11</sup> or intraamniotic.<sup>12</sup> Those studies showed decreased fetal weight, increased fetal resorption, morbidity and mortality rates<sup>5,7-12</sup>; increased embryoletality<sup>5</sup>; elevated tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-10 serum levels<sup>7</sup>; prematurity<sup>10</sup>; high toxicity to fetuses;

death of some pregnant females by circulatory collapse at the inoculation; uterine inflammation; increased number of leukocytes in amniotic fluid and umbilical arterial blood, and increased white cell infiltration in the lungs in females that survived.<sup>12</sup>

The present study was conducted to verify the biologic plausibility of the possible association between periodontitis and PTB and LBW. An experimental study on an animal model by the induction of periodontitis in 50% of periodontal sites and assessment of the presence of cytokines in the gingival tissue, serum, placenta, cord, and amniotic fluid was designed to test the null hypothesis that experimental periodontitis that is induced on rats does not result in adverse pregnancy outcomes.

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## Materials and Methods

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.<sup>13</sup> The Committee on the Ethics of Animals Experiments of the Federal University of Rio de Janeiro approved the protocol (ODONTO017-05/16).

Forty female Wistar rats, 2-months old, that weighed 200-270 g and 10 male Wistar rats were maintained in the Animal Facility of the Laboratory of Endocrine Physiology at the Sciences Health Center, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Individual cages with open-air tops and wood shavings were used for animal accommodation. Ambient conditions were an inversed light cycle of a 12-hour light/dark photoperiod; temperature was controlled to  $21 \pm 2^\circ\text{C}$ , and relative humidity levels were maintained at 50-70%, with an exhaustion of 15 minutes per hour. Food and water were given ad libitum. Experiments were performed with anesthesia, and efforts were made to minimize suffering.

Female rats were included randomly in 2 groups: a periodontally healthy control group (without ligatures placement) and an induced periodontitis group (test, with ligatures placed around the cervical region of the second maxillary molars and of the first mandible molars). Around every tooth, periodontists usually count 6 periodontal sites, which are 6 different points that periodontitis can affect. In this study, periodontitis was spread in 50% of available sites, which in humans would be a widespread generalized periodontal disease. After the ligatures placement, the rats were observed for 45 days to allow biofilm accumulation and subsequent periodontitis development, with gingival inflammation and alveolar bone resorption. During that period, the ligatures were checked at minimum once a week.

On day 41 after periodontitis induction, each 2 females from the same cage were switched to a cage where the male for mating was housed to be exposed to the male's odor. Female rats housed together for extended periods of time

and that are subsequently paired with a male or exposed to the male's odor demonstrate a high incidence of mating on the third night after pairing with the male.<sup>14,15</sup> The males that were selected for mating had proven fertility previously.

In day 45, in the beginning of the dark photoperiod, the male was put together with the 2 females in each cage from both the control and test groups for the first mating attempt. Twelve hours later, the male was removed from the cage. That half-day period of male-to-female contact was minimal but necessary to estimate more precisely the first gestational day with an imprecision of only 12 hours.

Females were then followed and weighed every other day for 15 days. Those who had a significant increase in weight, abdominal enlargement (usually detectable by day 13 of pregnancy), and mammary gland enlargement (usually detectable by day 14 of pregnancy) were pregnant. A second mating attempt was conducted with the ones that were not pregnant; all of the steps were repeated.

Pregnant rat females usually deliver after gestational day 21. Cesarean surgery was then performed in the end of gestational day 20; if the parturition did not happen spontaneously, PTB was not recorded.

Amniotic fluid, placenta, and umbilical cord were collected aseptically. After 65 days, the animals were killed. After the rats were decapitated, the blood was collected and the gingival tissue samples were taken from the buccal and lingual regions of the first mandible molars. The mandibles were dissected and disarticulated into 2 hemi mandibles. The specimens were prepared adequately for morphometry. Maxillae with teeth and surrounding periodontium were dissected for histopathologic examination.

For morphometry, mandibles were immersed in 2% sodium hypochlorite for 24 hours, brushed, washed in running water, and dried with compressed air. To evidence the cementoenamel junction (CEJ), the mandibles were stained with 1% methylene blue (Hemafarma Comércio Indústria Farmacêutica, São Gonçalo, Brazil), and

its images were captured with the use of a digital camera (Canon Rebel XT350D; Canon, Tokyo, Japan) on a tripod, distant from the specimens at the minimal focal distance, to guarantee standardized images for every mandible. A millimeter ruler was also photographed to show the spatial calibration within the image software used for analyzing the images (Image Pro Plus Software; Media Cybernetics, Inc, Rockville, MD). An observer who was blinded to the groups identification analyzed the images and obtained the measures from the bone crest to the CEJ in 8 different points per hemi mandible, 4 points in buccal surfaces (mesial, buccal, and distal of the first molar and mesial of the second molar), and 4 points in lingual surfaces (mesial, lingual, and distal of the first molar and mesial of the second molar), which is every surface in contact with the ligature. Those measures were obtained from the right and left hemi mandibles of the animals. The CEJ distance that was found in control animals was considered physiologic, and the CEJ distance that was found in test animals was subtracted from the first one to calculate the bone loss.

Placental, gingival, and umbilical cord tissues were homogenized and processed for analysis. Serum that was extracted from blood and amniotic fluid were analyzed also. Measurement of levels of IL-1 $\alpha$ , TNF- $\alpha$ , IFN- $\gamma$ , and IL-12p70, -4, -6, -10, and -17a were conducted, according to the manufacturers' protocols of a multiplex flow cytometric sandwich assay system based on Luminex multi-analyte profiling technology (LabMAP; Luminex Corp, Austin, TX). The methods were provided in detail.<sup>16</sup>

## Pilot study

Before the main study, 5 female Wistar rats with an average weight of 232 g were mated according to the same protocol described earlier. No intervention, periodontitis induction, or cesarean surgery was conducted. In the first mating attempt, all of them got pregnant and had a spontaneous delivery date at 22 days (SD, 1) with an average of 10 pups per litter (SD = 1-51) and an average birthweight of 3,67 g (SD = 0-5). Those

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