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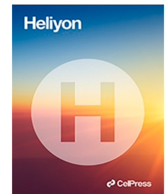

Research article

Periodontitis and diabetes in pregnant rats: Maternal-fetal outcomes

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ABSTRACT

Aim: To evaluate the repercussions of periodontitis and diabetes association on rat pregnancy and newborns.

Methods: Diabetes was induced in female Wistar rats 24 h after birth through the administration of Streptozotocin. The diabetic condition of the rats was further confirmed in adulthood. After mating, the pregnant rats were distributed into four experimental groups (n = 12 rats/group): nondiabetic and diabetic with and without periodontitis. Periodontitis was induced by a ligature inserted into the first molar on day 0 of pregnancy. Body weight, water and feed consumption were evaluated weekly, and an oral glucose tolerance test was performed on day 17 of pregnancy. On day 21 of pregnancy, the animals were anesthetized and killed for organ removal. The hemimandibles were collected to analyze alveolar bone loss. Immunological and biochemical parameters were evaluated in the maternal blood samples, and reproductive performance was analyzed. The newborns were weighed, and anomalies evaluated.

Results: The group with diabetes and periodontitis had a greater degree of alveolar bone loss, along with higher relative pancreatic weight, blood glucose levels, triglyceride and inflammatory cytokine levels, hepatic transaminase activity, and embryonic losses. In addition, these newborns had increased body weight, placental weight, a greater number of ossification centers, and a higher rate of visceral and skeletal anomalies.

Conclusion: The combination of maternal diabetes and periodontitis negatively impacts maternal parameters and fetal development. The findings reinforce the importance of maintaining maternal oral health to ensure the general health of the offspring, especially in cases where diabetes is present.

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1. Introduction

Periodontal disease is considered a public health problem, affecting approximately 65 million (47 %) American adults, and approximately 6 % of adult women [1]. The disease is characterized by the inflammation of the periodontium, caused by products secreted by bacteria, resulting in the disorganization and destruction of supporting dental structures [2]. During pregnancy, studies suggest that maternal exposure to periodontitis can lead to low birth weight and premature birth, causing long-term impacts throughout the life of the offspring [3-5]. Furthermore, the inflammation of the periodontium can cause systemic changes, which would also interfere with the placenta [6].

The correlation between periodontal disease and the risk of adverse health effects including *Diabetes mellitus* (DM) has been studied [7–9]. According to the American Diabetes Association (ADA), periodontitis is the sixth most common clinical sign of diabetes [10]. Depending on the hyperglycemic intensity, there is a greater chance of developing periodontal disease, and its progression can be more severe [11]. Furthermore, diabetic inflammatory responses and tissue repair deficiencies show a significant role in the worsening of periodontitis [12].

Studies using experimental animal models can expand the understanding of the progression and effects of diabetes and periodontal disease. Through the findings of these studies, it is possible to identify the mechanisms involved and the treatments to reduce the complications of these diseases [13]. Laboratory rodents, particularly rats, are used because it is easy to maintain dietary, behavioral, and observational control. Additionally, it is possible to obtain several lineages [14]. Although several studies regarding the impacts of maternal diabetes [15,16] and periodontal disease [7,17] were separately conducted, there are currently no studies that evaluate the impact of these associated factors during pregnancy. Therefore, we hypothesized that periodontal disease and diabetes association intensify the damages induced separately by these diseases both to the mother and to the fetuses. This study aimed to evaluate the effects of diabetes and periodontal disease association during rat pregnancy on dams and their fetuses.

2. Material and methods

2.1. Ethical statement

This study was carried out following the recommendations in the Guide for Care and Use of Experimental Animals and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [18]. The local ethic committee approved all protocols of this study (Protocol 23108.088014/2020-4).

2.2. Animal model

Male and female Wistar rats (210 ± 40 g, 90 days old) were obtained from the Center for Maintenance of Experimental Animals of the Federal University of Mato Grosso and maintained under standard laboratory conditions (22 ± 3°C, humidity 60 ± 10 %, 12-h light/dark cycle), with tap water and pelleted food (Purina® rat chow, Brazil) *ad libitum.*

2.3. Induction, confirmation of diabetes, and experimental groups

After acclimatization period (1 week), the female rats were mated with male rats of same age (ratio 3:1) to obtain female pups for diabetes induction. Half of the pregnant rats were randomly selected, and their female litter formed the diabetic group, which received a dosage of 100 mg/kg of Streptozotocin (STZ, Sigma Chemical Company®, USA), which was diluted in a citrate buffer (0.1 mol/L; pH 4.5). The beta-cytotoxic drug was administered by a subcutaneous injection (dorsal region) 24 h after birth. Female pups from the other half of the pregnant rat group were used in the nondiabetic (control) group, which received an equivalent volume of the citrate buffer [19]. After diabetes was induced, the offspring stayed with their mothers (maximum of eight females = number of functional teats for suckling) until the end of the suckling period (21 days). Subsequently, the female newborns were separated from their mothers.

To inclusion criteria for the experimental groups (diabetic or control), the female rats were submitted to the Oral Glucose Tolerance Test (OGTT) on day 110 of life [10,20]. After 6 h of fasting, blood glucose level was performed by a small puncture in the tail vein (time point 0). Next, the D-glucose solution (Sigma Chemical Company®, USA, 2.0 g/kg body weight) was intragastrically administered by gavage. Later, glycemic levels were determined at 30, 60, and 120 min after D-glucose overload by a conventional glucometer. Only rats with glycemia <140 mg/dL measured at least three times during the OGTT were included in the control group. The animals with glycemia ≥200 mg/dL measured at least once during OGTT were included in the diabetic group [10,21]. The female rats that no presented these parameters in OGTT, according to their experimental groups, were excluded and euthanized.

The effect size was determined considering previous studies in our laboratory on reproductive parameters and the four experimental groups [23,24], using an error type I of 5 % and 90 % power. The effect size showed the minimal n = 12 rats/group. After establishing the exclusion and inclusion criteria, the animals were randomized into the experimental groups: nondiabetic rats without periodontitis (Control); nondiabetic rats with periodontitis (Periodontitis); diabetic rats without periodontitis (Diabetic); and diabetic rats with periodontitis (Diab + Period).

2.4. Mating

At 120 days of life, three female rats were placed in the overnight period with one normoglycemic male of same age, which were purchased for the purpose of mating. In the morning (7–8 a.m.) the male rats were removed, and the female was tested for the sperm presence in the vaginal canal. Gestational day 0 (GD0) was considered when positive sperm was found. This process continued until the number of pregnant rats needed was reached. Non-mated female rats were considered infertile after 15 consecutive days (approximately three-estrous cycles) [22] and discarded from the study.

2.5. Periodontitis induction

Periodontitis was induced immediately after pregnancy was confirmed. Before the procedure, the selected rats were weighed and anesthetized with ketamine (30 mg/kg, intramuscular) and xylazine (10 mg/kg, intramuscular) combination (Syntec®, Brazil). After sedation was confirmed, the females in the Periodontitis and Diab + Perio groups were positioned in a prone position, with the upper incisors supported on a metal arch and the lower on an elastic band to provide adequate mouth opening and access to the animal's lower molars. Periodontal disease was induced using a ligature [13]. Using the active tip of a suture needle and a Mathieu needle holder, the lower molars on the left side were separated to facilitate access to the region. Cotton thread number 4.0 (Coats Correntes®, Brazil) was inserted in the gingival groove between the first and second lower molars on the left side of the mandible, creating a tie that involved the entire cervical region of the dental element. The thread remained for the 21 days of pregnancy and was checked daily.

2.6. Pregnancy period

Body weight, water and feed consumption were evaluated daily until the end of pregnancy. To verify the glucose metabolism, OGTT was performed on day 17 of pregnancy, according to the methodology previously described. The glycemic values on OGTT were used to estimate the total area under the curve using the trapezoidal mathematical method [25,26]. On day 21 of pregnancy, the rats received sodium thiopental (Thiopentax®, Brazil, dose 120 mg/kg intraperitoneal). After confirming that the rats were successfully anesthetized, blood samples were obtained for biochemical and immunological determinations. The rats were then subjected to a laparotomy to collect some organs and uterine horns.

2.7. Analysis of maternal parameters

The relative weights of the heart, liver, spleen, kidneys, and pancreas of each rat were calculated by the ratio of each organ (g) and the difference in the body weight minus the pregnant uterus weight on day 21 of pregnancy. The relative weight of each organ was expressed in grams/100 g of body weight.

The maternal blood samples were collected without anticoagulant and were centrifuged at 1784$\times g$ for 10 min. The serum samples obtained were stored at −20 °C until evaluated by commercial kits. Total proteins (#1770260, LaborLab®, Brazil), cholesterol (#1220114, Wiener®, Argentina), triglyceride (#1780105, Wiener®, Argentina), high-density lipoprotein (HDL-c) concentrations (#1220103, Wiener®, Argentina), aspartate transaminase (AST) (#1752360, Wiener®, Argentina), and alanine transaminase (ALT) (#1762360, Wiener®, Argentina) activities were determined. Very-low density lipoprotein (VLDL-c) level was calculated through the triglyceride concentrations [27].

2.8. Evaluation of immunological profile

Maternal blood was collected (500 μL) and transferred into tubes with anticoagulant (EDTA) for the total and differential leukocyte count. The total leukocyte count was established using a Neubauer's hemocytometer with blood samples diluted 1:20 in Turk's solution. For differential leukocyte cell counting, blood smears were stained with a panoptic solution. According to staining and morphological criteria, differential cell analysis was performed by counting 100 cells, and the percentage of each cell type was calculated [22], using a light microscope.

The serum concentration of tumor necrosis factor-alpha (TNF-α) (#DY510–05, R&D Systems®, USA), interleukin-6 (IL-6) (#DY506–05, R&D Systems®, USA), and interleukin-10 (IL-10) (#DY522–05, R&D Systems®, USA) were measured by the Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) [20]. 50 μL per well of the captured antibody was briefly added in a half-area microplate, followed by overnight incubation at 4 °C. The wells were then washed 3 times using PBS with tween and blocked using 1 % BSA in PBS for 1 h at room temperature. Subsequently, 50 μL of each standard and serum samples were added, followed by another incubation at room temperature for 2 h. Then, the wells were washed 3 times, after which 50 μL of a detection antibody was inserted and incubated at room temperature for 2 h. Next, the wells were washed again 3 times, followed by the addition of 50 μL of streptavidin-horseradish peroxidase (HRP) and incubated at room temperature for 20 min. Lastly, the wells were washed 3 times and then 50 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) was inserted, followed by incubation for 20 min by blocking light. The color reaction was stopped using 50 μL of 2 N H_2SO_4. The absorbance of the samples was determined by an ELISA microplate reader at a wavelength of 450 nm, and the cytokine concentration was calculated.

2.9. Histological analyzes

The histological analysis aimed to measure bone loss related to the inflammatory process resulting from periodontitis. For this, the rats hemimandibles were dissected by removing the muscle tissue, which was fixed in formaldehyde (10 %) for 24 h and decalcified in a 5 % nitric acid solution. After the decalcification process, the hemimandibles were washed in water and neutralized in a sodium bicarbonate solution. To prepare and analyze these samples, the pieces were dehydrated in progressive alcohol concentrations, cleared in xylene, and included in paraffin. Sections of 6 μm width were obtained using a microtome (Thermo Scientific® HM 340E, Brazil), and each piece was stained with hematoxylin and eosin. The photomicrographs were captured using the computerized image system Future Win Joe 1.6, combined with the digital camera image microscope (Mylabor® 10.5 MP, Brazil). The area of bone tissue loss was measured by image analysis software (lmageJ®, National Institutes of Health, USA) with evaluation of linear points from the margin of the alveolar bone to the particular cementoenamel of the buccal and lingual surfaces [28].

2.10. Reproductive outcomes and fetal development

For the evaluation of dead and living fetuses, the amount of reabsorption (embryonic death), along with the number of corpora lutea and implantation sites, was analyzed. Additionally, the gravid uterus was dissected. Salewski method was used to verify the number of undetectable implantation sites [29]. The pre- and postimplantation loss percentage were calculated according to Araujo-Silva et al. [30]. The living fetuses were weighed and classified in small, adequate or large for gestational age according to body weights obtained from the control group [31]. The placentas were also weighed to determine placental efficiency (fetal weight/-placental weight).

The fetuses were analyzed in a microscope concerning the frequency of external anomalies with detailed analysis of the cranial conformation, eyes, ear implantation, mouth, fore and hind limbs, tail, and anal perforation. Subsequently, half of the fetuses were fixed in Bodian's solution and serial sections were prepared [32] for visceral examination. For this, cuts in specific regions of the fetuses were performed to evaluate anomalies in the palate, nose, eyes, brain, spinal cord, thyroid, trachea, esophagus, veins, arteries, heart, lung, diaphragm, liver, kidneys, and genital organs. The other half of the fetuses were processed for bone examinations [33]. Examination of the skeleton allows all bones to be evaluated. Along with the skeletal analyses, the counting of the ossification sites (metacarpals, metatarsals, phalanges, sternebra, and caudal vertebras) was also performed to determine the degree of fetal development [34,35].

2.11. Statistical analysis

Data are showed as mean ± standard deviation (SD). After the use of Shapiro-Wilk normality test, a comparison among groups was performed using the One-way analysis of variance (ANOVA) followed by Tukey's Test. Fisher's Exact test was used to calculated proportions. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Maternal blood glucose measurements

In adulthood, the diabetic animals presented glycemia levels superior to 200 mg/dL at one or more time points on the OGTT according to the inclusion criteria. Additionally, the rats in the control group had glycemia <140 mg/dL at three time points during the

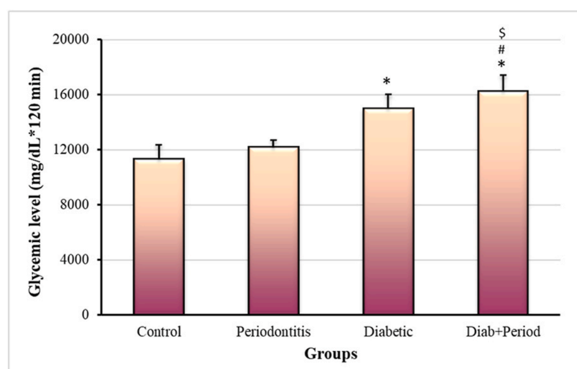

Fig. 1. Area under the curve (AUC) calculated from the blood glucose levels during oral glucose tolerance test (OGTT) on gestational day 17 of nondiabetic (Control) and diabetic rats with or without periodontitis.
N = 12 animals/group. Data are shown as mean ± standard deviation (SD). $p < 0.05$ - *compared to the Control group; #compared to the Periodontitis group; $compared to the Diabetic group (ANOVA followed by Tukey's Multiple Comparisons Test).

OGTT. The Diabetic group presented a larger area under the curve (AUC) compared to nondiabetic animals (mean ± SD: Control: 11332.5 ± 891.9 vs Diabetic: 15936.2 ± 769.8 mg/dL*120 min; $p < 0.0001$), confirming a diabetic status in the STZ-induced animals.

Fig. 1 shows the values obtained in AUC of the OGTT on the 17th day of pregnancy. The diabetic animals presented high glycemic values in relation to the Control group. The Diab + Period group presented a higher AUC than the other groups, demonstrating the high value of circulating blood glucose.

3.2. Degree of alveolar bone loss

Fig. 2A shows the degree analysis of alveolar bone loss. The analysis of the degree of alveolar bone loss in the buccal and lingual regions showed an increase in periodontal degradation in the Periodontitis group (2C) compared to the Control group (2B) in both regions. The Diab + Period (2E) group showed greater bone loss on the buccal surface compared to the other groups, and significant bone loss in the lingual region compared to the Control (2B) and Diabetic (2D) groups.

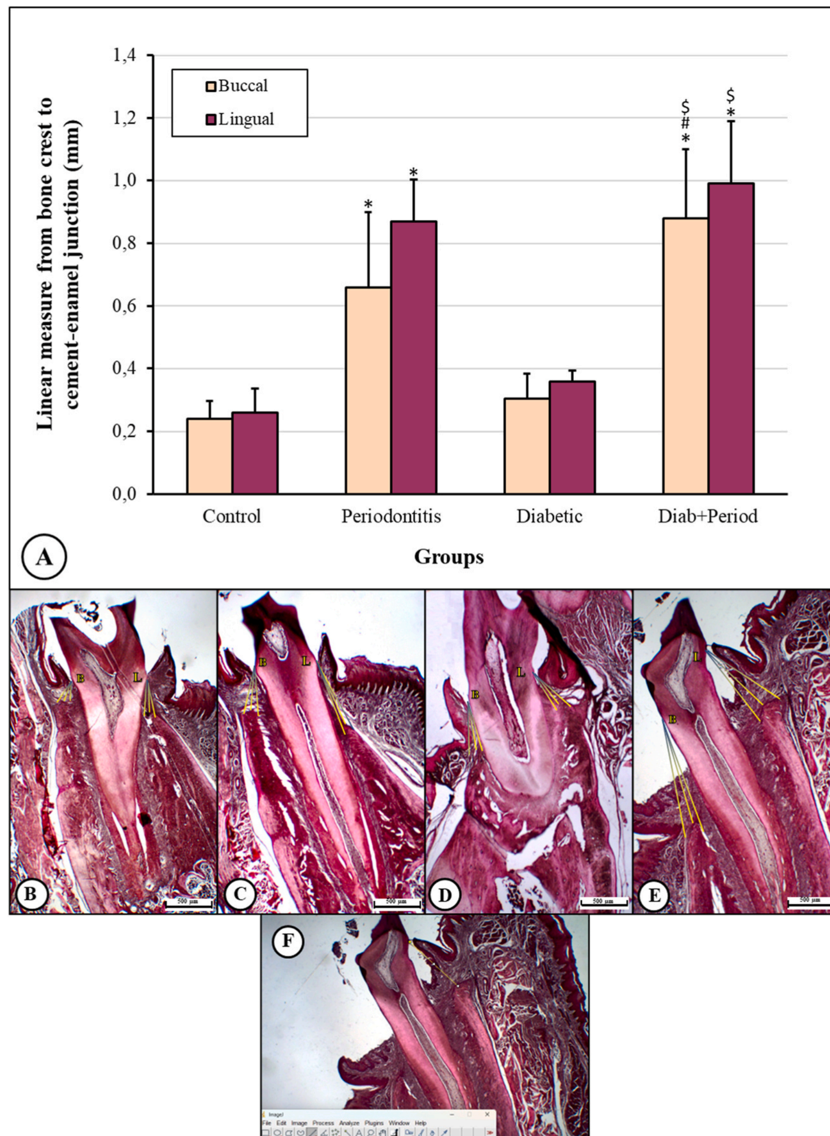

Fig. 2. **A**: Degree analysis of alveolar bone loss (lines) of the buccal (V) and lingual (L) regions in the lower left first molar at term of pregnancy (day 21) from nondiabetic (Control) and diabetic rats with or without periodontitis. **B-E**: Photomicrograph of hematoxylin and eosin (HE) stained of the left first molar of the Control (**B**), Periodontitis (**C**), Diabetic (**D**) and Diab + Period (**E**) groups. (**F**) Representative analysis using ImageJ software. (40× magnification).

N = 12 animals/group. Data are shown as mean ± standard deviation (SD). $p < 0.05$ -*compared to the Control group; #compared to the Periodontitis group; $compared to the Diabetic group (ANOVA followed by Tukey's multiple comparison test).

3.3. Maternal evaluating

During the first week of pregnancy, Diabetic female rats showed less weight gain than the Control group. The Diab + Period dams showed lower values of total maternal weight gain when in relation to the Periodontitis group. Furthermore, there was an increased relative spleen weight in the Diab + Period group than the Control group. Both diabetic groups had higher pancreatic weight in relation to the Control and Periodontitis groups. There was no change in daily water and food intake during pregnancy between the experimental groups (Table 1).

The maternal biochemical and cytokines profiles are presented in Table 2. The Diabetic group showed increased levels of triglycerides and VLDL-c, and a decrease in HDL-c and IL-10 in relation to the Control group. The Diab + Period dams showed higher levels of triglycerides, VLDL-c, and IL-6 compared to the other groups, as well as increased ALT activity and decreased HDL-c than the Control group. Furthermore, the total protein in the Diab + Period group increased significantly, while IL-10 levels decreased compared to the Periodontitis group. There was no difference in the hematological profile between the experimental groups.

3.4. Reproductive outcomes

Reproductive outcomes at term pregnancy are shown in Fig. 3. Diabetic animals with periodontitis presented a greater number of pre-embryonic losses before implantation compared to the other groups, as well as increased embryonic losses after implantation in relation to the Control and Periodontitis group.

3.5. Analysis of fetal development

The Periodontitis group had an increased percentage of small fetuses (SGA) and a decrease in adequate fetuses (AGA) for gestational age when compared to the Control group. Diabetes and periodontitis association increased the number of ossification centers in fetuses in relation to the Control group, along with increasing fetal weight, placental weight, and the rate of fetuses classified as large for pregnancy age (LGA) when compared to the other groups (Table 3).

Fig. 4A shows the percentages of fetal anomalies. The diabetic rat group showed an increase in the percentage of skeletal anomalies and a lower percentage of fetuses without anormal morphology in relation to the Control group. The Diab + Period dams presented a decrease in the rate of fetuses with normal morphology (4B and 4E) and an increase in the rate of fetuses presenting skeletal anomalies, particularly sternebrae alterations (4C and 4D), when compared to the Control and Periodontitis groups, along with a higher rate of fetuses with visceral anomalies (specially dilated trachea - 4F) than the Control group.

4. Discussion

This study was designed to address the effects of the association between maternal diabetes and periodontal disease, two diseases that correlate and can occur concomitantly during pregnancy. The findings show that periodontal disease aggravates maternal and fetal changes when associated with maternal diabetes. To understand the pathophysiological processes implicated in the two conditions (diabetes and periodontitis), we used an experimental model of inducing diabetes and periodontal disease in rats during pregnancy. The use of the beta-cytotoxic drug Streptozotocin (STZ) proved to effectively induce glucose intolerance during the neonatal period and diabetes in adult life [19], as indicated by the increased glycemic value obtained in the area under the curve

Table 1
Maternal physiological parameters of pregnant nondiabetic (Control) and diabetic rats with or without periodontitis.

	Groups			
	Control (n=12)	*Periodontitis (n=12)*	Diabetic (n=12)	*Diab + Period (n=12)*
Weight gain in pregnancy (g)				
1st week	22.9 ± 5.2	20.5 ± 10.5	13.7 ± 7.1*	17.0 ± 4.6
2 nd week	28.5 ± 9.0	37.8 ± 8.1	23.1 ± 5.0	31.8 ± 16.7
3rd week	72.4 ± 10.8	83.6 ± 23.7	72.5 ± 17.1	61.9 ± 26.0
BWG (g)	123.8 ± 17.1	141.6 ± 18.7	109.3 ± 19.3	110.6 ± 27.6#
GUW (g)	77.5 ± 17.0	83.6 ± 25.1	69.5 ± 17.8	60.4 ± 24.4
BWG - GUW (g)	46.3 ± 13.4	57.9 ± 33.7	39.7 ± 12.2	50.4 ± 13.9
Daily food consumption (g)	21.7 ± 3.0	21.5 ± 1.3	20.4 ± 2.8	20.6 ± 2.7
Daily water intake (mL)	45.5 ± 8.5	48.7 ± 5.7	45.6 ± 11.8	45.3 ± 5.5
Relative organ weight (g/100g)				
Heart	0.34 ± 0.06	0.31 ± 0.05	0.31 ± 0.02	0.34 ± 0.05
Liver	4.29 ± 0.49	4.80 ± 0.93	4.36 ± 0.50	4.71 ± 0.37
Spleen	0.21 ± 0.03	0.21 ± 0.04	0.22 ± 0.03	0.25 ± 0.02*
Kidneys	0.62 ± 0.07	0.69 ± 0.10	0.60 ± 0.04	0.67 ± 0.10
Pancreas	0.18 ± 0.03	0.24 ± 0.07	0.30 ± 0.04*	0.33 ± 0.06*#

Legend: BWG - Body weight gain; GUW - Gravid uterus weight.
Data are shown as mean ± standard deviation (SD).
$p < 0.05$ - *compared to the Control group; #compared to the Periodontitis group (ANOVA followed by Tukey's multiple comparison test).

Table 2
Maternal biochemical and immunological parameters on gestational day 21 (at term) of nondiabetic (Control) and diabetic rats with or without periodontitis.

	Groups			
	Control (n=12)	*Periodontitis (n=12)*	*Diabetic (n=12)*	*Diab+Period (n=12)*
Biochemical profile				
Total Protein (g/dL)	5.3 ± 0.7	4.4 ± 0.7	6.0 ± 0.7	6.0 ± 1.6$^{\#}$
Triglycerides (mg/dL)	87.6 ± 15.6	105.6 ± 39.2	156.7 ± 46.8*	284.1 ± 99.3*$^{\#\$}$
Cholesterol (mg/dL)	83.3 ± 4.0	83.8 ± 5.4	81.6 ± 9.8	75.3 ± 5.7
HDL-c (mg/dL)	47.6 ± 6.6	41.8 ± 5.0	36.8 ± 8.0*	35.1 ± 4.3*
VLDL-c (mg/dL)	17.6 ± 3.1	21.1 ± 7.8	31.3 ± 9.3*	57.1 ± 32.1*$^{\#\$}$
ALT (U/l)	40.1 ± 9.3	43.1 ± 9.1	43.9 ± 5.1	54.1 ± 13.8*
AST (U/l)	184.5 ± 11.8	182.7 ± 19.6	184.3 ± 45.4	159.1 ± 21.2
Hematological profile				
Leukocytes (10^3/mm^3)	5.8 ± 0.9	6.6 ± 1.3	6.7 ± 1.4	7.2 ± 1.3
Neutrophils (10^3/mm^3)	2.1 ± 0.5	2.2 ± 0.6	2.6 ± 0.5	2.6 ± 0.7
Lymphocytes (10^3/mm^3)	3.4 ± 0.6	4.2 ± 1.4	3.9 ± 1.2	4.3 ± 1.2
Monocytes (10^3/mm^3)	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Eosinophils (10^3/mm^3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cytokines				
TNF-α (pg/dL)	67.6 ± 20.4	57.3 ± 12.9	83.6 ± 34.5	69.1 ± 41.1
IL-6 (pg/dL)	14.3 ± 2.7	13.6 ± 2.2	15.49 ± 2.71	20.4 ± 5.7*$^{\#\$}$
IL-10 (pg/dL)	7.1 ± 1.1	7.1 ± 0.2	5.4 ± 0.9*	6.1 ± 0.6*$^{\#}$

Legend: HDL-c – High-density lipoprotein; VLDL-c – Very-low-density lipoprotein; AST – aspartate transaminase; ALT – alanine transaminase; TNF-α – Tumor Necrosis Factor-alpha; IL - Interleukin.
Data are shown as mean ± standard deviation (SD).
$p < 0.05$ - *compared to the Control group; #compared to the Periodontitis group; $compared to the Diabetic group (ANOVA followed by Tukey's multiple comparison test).

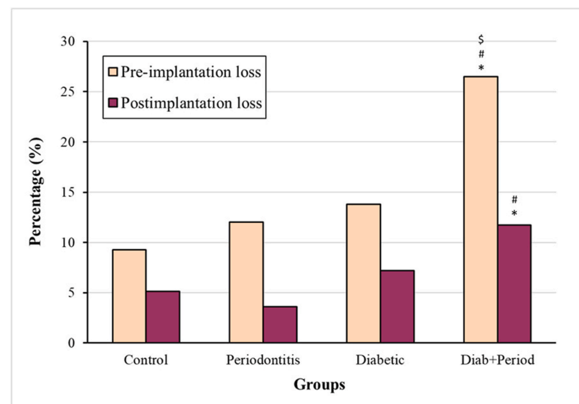

Fig. 3. Percentage of losses of embryos before and after implantation at term pregnancy of nondiabetic (Control) and diabetic rats with or without periodontitis.
N = 12 animals/group. $p < 0.05$ - *compared to the Control group; #compared to the Periodontitis group; $compared to the Diabetic group (Fisher's Exact Test).

during OGTT, without causing excessive loss of animal subjects during diabetes induction. STZ has β-cytotoxic effects in which destruction of the endocrine portion, more specifically of the beta cells of the pancreas, is observed [36]. Through the data analyzed in this study, a glycemic increase in diabetic rats with the concomitant presence of periodontitis during pregnancy using OGTT on day 17 of pregnancy was observed. The presence of periodontal disease alone, however, was insufficient for increasing the glycemic levels of rats during pregnancy. In a periodontal disease experimental model involving a greater number of dental elements or spanning a longer period, it might be possible to increase the animals' blood glucose. Contrastingly, the results of this study establish that the presence of periodontal disease in diabetic rats aggravates glycemic alterations, compared to when these two diseases occur in isolation. The inflammatory process of the periodontium possibly stimulates the production of cytokines, which act as a direct antagonist of insulin receptors. This promotes increased glycemia in diabetic individuals, contributing to the loss of diabetes control [37].

Diabetic animals associated with periodontal disease models through the placement of ligatures or gavage have been described [38–40], with ligation being more reliable, as it presents higher success rates in retaining bacteria [38]. In this study, all animals remained pregnant for 21 days with the thread properly inserted, thus allowing the onset, progression, and monitoring of periodontitis. The use of the lower left first molar was justified by the ease of visualization and monitoring throughout the study compared to the

Table 3
Fetal body weight, placental weight, and efficiency at term of pregnancy (day 21) of nondiabetic (Control) and diabetic rats with or without periodontitis.

	Groups			
	Control (n=125)	*Periodontitis (n=139)*	*Diabetic (n=118)*	*Diab + Period (n=99)*
Fetal body weight (g)[a]	5.47 ± 0.46	5.46 ± 0.72	5.54 ± 0.43	5.81 ± 0.62*#$
SGA Fetuses (%)[b]	3.20	9.35*	3.39	4.04
AGA Fetuses (%)[b]	93.60	82.73*	94.07	75.76*$
LGA Fetuses (%)[b]	3.20	7.92	2.54	20.20*#$
Total ossification sites	24.74 ± 1.77	26.77 ± 3.46	24.89 ± 2.01	27.70 ± 2.67*
Placental weight (g)[a]	0.49 ± 0.09	0.48 ± 0.09	0.48 ± 0.08	0.55 ± 0.12*#$
Placental efficiency[a]	11.34 ± 1.81	11.58 ± 2.19	11.68 ± 1.84	10.96 ± 2.42

Legend: SGA – small for gestational age; AGA – adequate for gestational age; LGA – large for gestational age.
Data are shown as mean ± standard deviation (SD) and proportions (%).
$p < 0.05$- *compared to the Control group; #compared to the Periodontitis group; $compared to the Diabetic group ([a]ANOVA followed by Tukey's Multiple Comparison test; [b]Fisher's Exact Test).

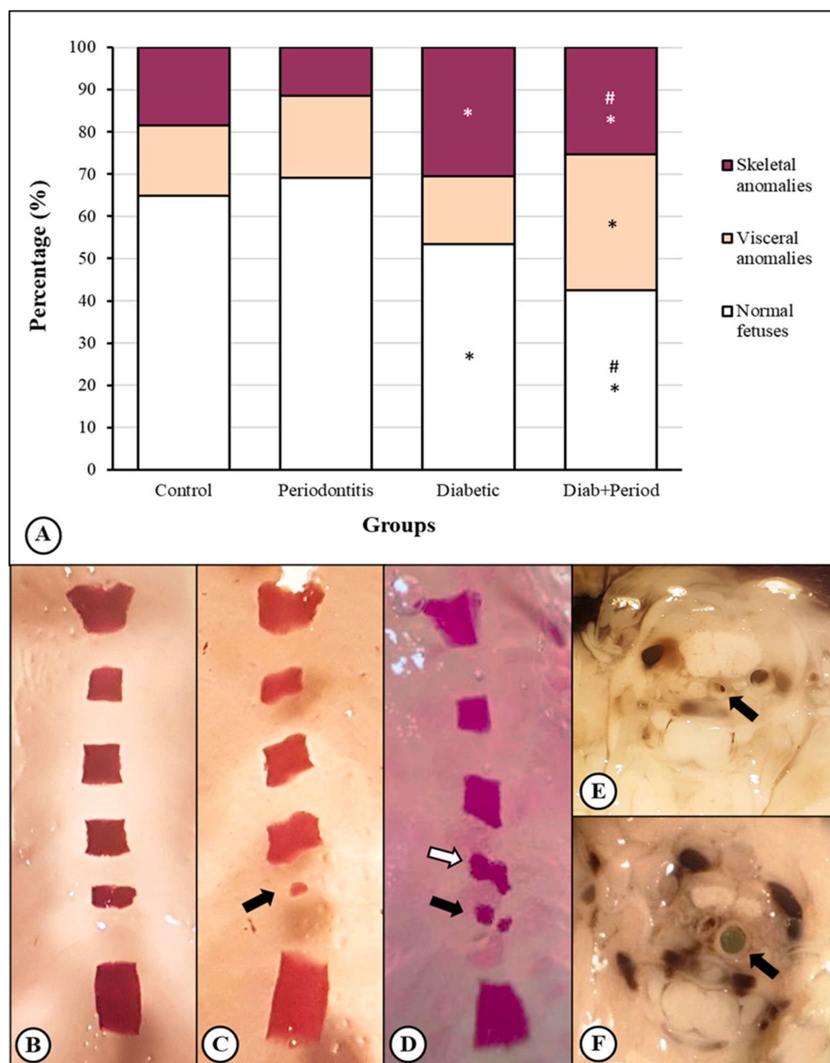

Fig. 4. A: Frequency of fetal anomalies at term of pregnancy (day 21) from nondiabetic (Control) and diabetic rats with or without periodontitis. **B–F:** Representative images of the main anomalies found. Normal sternebra **(B)**, incomplete ossification of sternebra (arrow) **(C)**, abnormally shaped (white arrow) and bipartite (black arrow) sternebra **(D)**, thoracic section with normal trachea (arrow) **(E)** and dilated trachea (arrow) **(F)**.
N = 125 -Control group; 139-Periodontitis; 118-Diabetic; 99-Diab + Period $p < 0.05$ - *compared to the Control group; #compared to the Periodontitis group (Fisher's Exact Test).

other teeth [41]. The presence of cotton thread close to the neck of the tooth and the gingival sulcus allowed bacterial accumulation and consequent inflammatory lesions in the region [42]. The degradation process of the periodontium was observed in teeth that were surrounded by cotton thread, which was notably absent in the periodontal tissue around the molars, where the disease had not been induced. Through histological analysis, it was possible to analyze increased alveolar bone loss in animals with periodontitis induction, demonstrating the effectiveness of the experimental model of ligature periodontitis. The alveolar bone loss observed in this experimental model occurred acutely (on a larger scale) in the first 14 days after ligation [38], and chronically after this period, where the loss was less aggressive. Hyperglycemic environments can lead to microvascular changes and deficiencies in the formation and repair of alveolar bone, accelerating periodontal degradation [43]. Choubaya et al. [37] observed a greater degree of alveolar bone loss in histological analysis in animals with experimental periodontitis associated with diabetes. The data corroborate these findings, showing greater bone loss in the group with diabetes and periodontitis, especially in the vestibular region, in full-term pregnant rats.

Alterations in multiple organs occur in pregnancy. The pancreas, for example, adapts to maternal and fetal demands by increasing the size and/or number of cells [44]. Excessive maternal glucose concentrations, however, can compromise maternal metabolism in the pancreas [45]. The pancreatic hyperplasia observed in this study may be attributed to the response of pancreatic beta (β) cells facing increased insulin demand caused by insulin resistance in diabetes [46], periodontitis [47], and pregnancy itself [48]. Concerning the spleen, there was an increased relative weight of the organ at term pregnancy in rats, which may be related to a response to inflammatory stimuli due to the association between periodontal degradation and hyperglycemia [49], as well as dyslipidemia [9].

The findings showed that diabetes, whether isolated or associated with periodontitis, caused changes in the lipid profile. The Diabetic animals showed decreased HDL-c values, while triglyceride and VLDL-c values were increased, with these biochemical parameters being exacerbated when periodontitis was present. It is common for hypertriglyceridemia to occur in diabetics [50] since insulin resistance is capable of suppressing the action of lipoprotein lipase, which is responsible for lipid oxidation [51]. Furthermore, increased concentrations of triglycerides have already been observed in chronic periodontitis [9]. Additionally, the presence of periodontal disease contributed to a higher concentration of total proteins in the blood. Proteins are vulnerable to hyperglycemia, which causes oxidative stress, mitochondrial dysfunction, and DNA damage [52]. We suggest that changes in insulin action, triggered by inflammatory mediators in periodontitis, lead to the increased protein catabolism and interruption of protein synthesis with the release of large quantities of amino acids into the blood [53]. Similar to another study conducted on rats during pregnancy [21], no change in the transaminases was observed in the diabetic group. The serum concentrations of alanine aminotransferase (ALT) were also increased in rats with diabetes and periodontitis, corroborated by epidemiological and laboratory studies that showed an association between periodontitis, elevated serum ALT activity, and liver diseases [54,55].

An increase in leukocytes, neutrophils, and pro-inflammatory mediators is common during periodontitis or hyperglycemia, representing a fundamental link between the two diseases [56]. Despite the total and differential leukocyte count being reflective parameters of the inflammatory state of the organism, no changes in the leukocyte profile were observed in this study. It is possible that the model of mild-intensity diabetes and periodontal disease did not cause sufficient hyperglycemia to damage the leukocyte profile. However, we observed an increased inflammatory process characterized by higher concentrations of cytokine IL-6 and a decreased anti-inflammatory status by lower concentrations of cytokine IL-10. Periodontal disease can increase the serum concentrations of inflammatory cytokines such as IL-6 [57]. Furthermore, the hormonal changes observed during pregnancy, along with the higher production of IL-6 and the reduced production of IL-10, trigger a high degree of insulin resistance, which impairs the tissue uptake capacity of glucose, leading to a hyperglycemic condition [58].

The IL-6 cytokine family is also involved in early embryonic development and implantation [59]. When analyzing maternal reproductive performance, increased pre-and postimplantation embryonic losses were observed in diabetic rats with associated periodontitis. The observed hyperglycemia or dyslipidemia may have aggravated periodontal tissues, leading to the release of inflammation biomarkers such as IL-6 due to altered cellular immunity and the formation of advanced glycation end products [60–62]. The high levels of IL-6 might contribute to the subsequent production and activation of prostaglandins, which induce uterine contractions, embryo losses, and spontaneous preterm birth [63].

Regarding fetal development and growth, periodontal disease during normoglycemic pregnancy restricted intrauterine growth, confirmed by the higher rate of fetuses classified as small for their pregnancy age. Not only pregnancy can alter the progression of periodontal diseases, but can also periodontitis interfere with pregnancy [64]. The physiological and hormonal alterations induced during pregnancy can modify inflammatory responses, increasing gingival inflammation and the accumulation of bacteria [57]. Increased vascular permeability in the gingival tissues enables the dissemination of pathogenic microorganisms and their products into the bloodstream and the placenta [65], causing changes in the structure of the placental labyrinth. This promotes an insufficient supply to the fetus, which impairs fetal growth and increases the risk of low birth weight [66]. Furthermore, periodontal inflammation interferes with both the fetal insulin production capacity and increased resistance to the hormone, indicating the risk of offspring developing DM2 in the future [67]. However, the inadequate intrauterine environment caused by the presence of diabetes concomitantly with periodontal disease led to macrosomia. The rats from diabetic mothers and those with periodontal disease showed increased body weight of newborns and placenta, along with increased ossification centers. Literature indicates an increased placental weight in the pregnancies of diabetic individuals [68], which can be attributed to an attempt by the maternal organism to stimulate nutrient exchanges between the fetus and the mother during fetal development [69]. The presence of high glucose levels in the placenta can stimulate the secretion of large amounts of insulin by the fetal pancreas, causing macrosomia [70], since greater amounts of insulin would be capable of increasing the production of insulin-like growth factors (IGF-I and IGF-II) [71]. Diabetic animals with periodontal disease had higher glucose levels, which could explain the macrosomia found in newborns. Furthermore, it should be noted that the macrosomia found in this study did not occur due to differences in the food intake of the mother rats, as they all received the same amount and type of food and there were no significant differences in food intake among the groups.

This study demonstrated that diabetes, whether associated with periodontitis or not, reduced the rate of fetuses without congenital alterations. Thus, the diabetic state increased skeletal and visceral anomalies. Hyperglycemia has been associated with increased anomalies in pregnancies complicated by diabetes in humans [72] and in experimental models [30]. The mechanism by which diabetes leads to fetal teratogenicity is not yet fully understood [73]. Most of the anomalies observed in the offspring of diabetic mothers are of cardiac origin and may also occur in bones and the urogenital tract [74].

The key points of the present study are as follows: First, we analyzed a topic that has not yet been explored (the association of diabetes with periodontal disease during pregnancy). Second, we carried out the study on laboratory animals that mimic human blood glucose levels and excluded the possibility of interference from external factors, which allows the standardization of an animal model that can be used in future studies. As a limitation, the model of periodontal disease during pregnancy used in this study only considers the 21 days of ligation; periodontal disease is chronic and can occur for an indefinite period, which would differentiate the experimental model of the disease.

5. Conclusions

The association between periodontal disease and maternal diabetes intensifies the damages induced separately by these diseases, promoting metabolic and biochemical changes that affect embryonic development and fetal growth. Therefore, the findings of this study emphasize the impact of periodontal disease on maternal diabetes and reinforce the importance of maintaining adequate maternal oral health to ensure the general health of the offspring.

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Data availability statement

All of the data analysis results obtained during this study are included in the article. Raw data that support the findings of this study are available from the corresponding author, GTV, upon reasonable request.

CRediT authorship contribution statement

Samuel Santos Souza: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Larissa Lopes Cruz:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Amanda Munnick Alves-Reis:** Investigation, Formal analysis. **Vanessa Queiros Costa:** Investigation, Formal analysis. **Rafaianne Queiroz Moraes-Souza:** Investigation, Formal analysis. **Débora Cristina Damasceno:** Writing – review & editing, Writing – original draft. **Gustavo Tadeu Volpato:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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