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Maternal periodontitis decreases plasma membrane GLUT4 content in skeletal muscle of adult offspring

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ABSTRACT

Aims: The fetal programming hypothesis suggests that intrauterine stimuli can induce metabolic changes in offspring, increasing the disease risk in adulthood. Periodontal disease may enhance serum cytokine levels. Cytokines such as tumor necrosis factor-alpha (TNF-α) have been associated with reduced glucose transporter type 4 (GLUT4) expression, decreased protein kinase B (Akt) phosphorylation, and insulin resistance. This study aimed to evaluate GLUT4 content, and Akt serine phosphorylation status in the gastrocnemius skeletal muscle (GSM), glycemia, insulinemia and change in body weight in offspring of rats with periodontal disease.
Main methods: Female Wistar rats were distributed into a control group (CN) and an experimental periodontal disease group (PD), in which a ligature was placed around the mandibular first molars. Seven days after ligature placement, both groups were mated with normal male rats. The ligatures remained throughout pregnancy until weaning, after which the male offspring were distributed into groups: CN-o, control rat offspring; and PD-o, periodontal disease rat offspring. The body weight from 0 to 75 days of age was measured. At 75 days, the glycemia, insulinemia, TNF-α levels, Akt serine phosphorylation, and GLUT4 content in the GSM were measured in the offspring.
Key findings: The PD-o group showed a low birth weight (LBW), unchanged glycemia, increased insulinemia, insulin resistance, increased TNF-α levels, decreased Akt serine phosphorylation status, and reduced GLUT4 content in the plasma membrane and translocation index after insulin stimulation.
Significance: Maternal periodontal disease causes LBW, insulin resistance, and alterations in the final stage of insulin signaling in the GSM of adult offspring.

1. Introduction

Periodontal disease is a local inflammatory process characterized by the release of toxic products, such as lipopolysaccharides, by pathogens and the host immune response comprising neutrophil, macrophage, and lymphocyte infiltration, and the release of cytokines such as tumor necrosis factor-alpha (TNF-α) [1].

During pregnancy, periodontal pathogens in the oral cavity can reach the amniotic cavity and cause adverse outcomes such as low birth weight (LBW) [2]. There is a correlation between LBW and an increased predisposition to hypertension, coronary heart disease, and diabetes in adulthood [3–5]. This is explained by the fetal programming mechanism, in which environmental stimuli within the uterus affect fetal growth and health, not only during gestation, but also during adult life [4,6,7].

Pregnant women with periodontitis showed increased serum TNF-α and interleukin (IL)-6 concentrations [8]. Excessive TNF-α concentration causes insulin resistance by modifying the insulin signal transduction [9]. Insulin binds the α-subunit of its receptor, which triggers autophosphorylation of the β-subunit followed by stimulation of tyrosine kinase activity, tyrosine phosphorylation of the insulin receptor substrate (IRS), and recruitment of phosphatidylinositol 3-kinase (PI3K). Ultimately, this pathway triggers activation of several serine/threonine kinases, most notably protein kinase B (PKB/Akt), which

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enables glucose uptake into the cells by translocating the glucose transporter type 4 (GLUT4) from the intracellular compartment to the plasma membrane [10,11].

Shirakashi et al. [12] showed that maternal periodontal disease altered the initial steps of the insulin-signaling pathway, as indicated by a reduction in pp185 tyrosine phosphorylation in the gastrocnemius skeletal muscle (GSM) and white adipose tissue.

Therefore, this study evaluated the relationship between maternal periodontal disease, LBW, and alterations in the final stage of insulin signaling in the GSM of their offspring, as abnormal insulin activity in muscle represents an early marker for an increased risk of diabetes.

2. Materials and methods

2.1. Animals

This study followed the Ethical Principles and Guidelines for Animal Experimentation, and was approved by the local ethics committee (protocol number 00863-2013). The Wistar rats were housed with a 12-h light/dark cycle (lights on at 07:00), ambient temperature of 23 ± 2 °C, and food (Presence®, Paulínia, São Paulo, Brazil) and water available *ad libitum*. The diet (3.6 kcal/g-digestible energy) contained an average macronutrient composition as follows: 23% from protein, 4% fat and 58% from carbohydrate (Presence®, Paulínia, São Paulo, Brazil).

2.2. Experimental design

Sixteen Wistar female rats (approximately 200 g each) were randomly divided into two groups: CN, control group ($n = 8$); and PD, experimental periodontal disease group ($n = 8$). In the PD group, periodontal disease was induced by installing a ligature with 4-0 silk (Seda-Silk, Johnson & Johnson, São José dos Campos, Brazil) thread onto the cervical region of the mandibular first molars bilaterally, as described by [13]. Seven days later, the rats in both groups were mated with normal male rats. Vaginal smears were taken daily to verify the presence of sperm. After confirming pregnancy, the female rats were housed individually. The food intake and body weight of the mother rats were evaluated weekly during the periods of pregnancy and lactation. The ligature was maintained throughout pregnancy until weaning the offspring.

After birth, the numbers of offspring in the CN and PD groups were matched so that each mother had the same number of offspring. After weaning, the adult female rats were euthanized (thiopental overdose, 150 mg/kg), and the male offspring were studied to avoid any effect from hormonal oscillation during the estrous cycle in the juvenile female rats. The male offspring were distributed into two groups: CN-o, which were the offspring of the control rats ($n = 16$); and PD-o, which were the offspring of the rats with experimental periodontal disease ($n = 16$). The body weights of the offspring were measured once weekly from birth until 75 days of age, and experiments were performed once the offspring were 75 days old. Food intake of the CN-o and PD-o groups was evaluated weekly from weaning until the end of the experiment.

Animals were fasted for 14 h prior to experiments and anesthetized with sodium thiopental (Thiopentax®, Cristália, Itapira, Brazil) (3%, 50 mg/kg, intraperitoneal). Experiments were performed 10–15 min later.

A median laparotomy was performed in rats from the CN-o ($n = 8$) and PD-o ($n = 8$) groups, and blood samples (4 mL) were taken from the inferior vena cava. The blood samples were then transferred into tubes containing heparin (Liquemine, Hoffmann-La Roche, Basle, Switzerland). Plasma was prepared by centrifuging the blood at 3000 $\times g$ for 15 min at 4 °C, and stored in aliquots at −70 °C until glucose, insulin and TNF-α quantification.

The periepididymal white adipose tissue and left GSM were collected, and the absolute weight (g) and relative weight (per 100 g of body weight) of these tissues ($n = 8$) were measured. The left GSM ($n = 6$) was used to measure the GLUT4 protein content. After this procedure, the animals were euthanized. Finally, the remaining animals from the CN-o ($n = 8$) and PD-o ($n = 8$) groups were used to evaluate the Akt serine phosphorylation status in the GSM following insulin (Novolin ®, Novo Nordisk, Bagsvaerd, Dinamarca) administration (1.5 U, intravenously into the portal vein).

2.3. Radiographic and histological analysis of maternal periodontal tissues

The periodontal health (CN group) or effectiveness of periodontal disease induction (PD group) was confirmed using radiographic and histological analysis of the maternal jaws.

The adult female rats were euthanized after weaning the offspring, and the right and left hemi-mandibles were dissected and fixed for 24 h in 4% formaldehyde. The mandibles were radiographed at a 70 kvp, 10 mA, and 0.10 s exposure. The source-to-film distance was always set at 40 cm. The digital image was obtained directly with an optical digital plate (Digora, Soredex, Orion Corporation, Helsinki, Finland), and the optical plates were read in sensitized laser scanner equipment (Windows Digora 1.51, Soredex, Orion Corporation, Helsinki, Finland). The images were analyzed to verify the presence of alveolar bone loss in the first molars.

After obtaining the radiographic images, the hemi-mandibles were demineralized in 10% EDTA (UltraPureTM EDTA, Invitrogen, Scotland, UK) (pH 7.4) for 3 months. The specimens were processed conventionally and embedded in paraffin. Semi-serial sections (5 μm) were obtained in the distal-mesial direction and stained with hematoxylin and eosin (H&E). Histological analysis was performed as described by Garcia et al. [14]. A certified histologist (EE) evaluated to the following parameters: nature and degree of inflammation; extent of the inflammatory process; presence and extent of tissue necrosis; presence, extent, and nature of bone, cementum, and dentin resorption; state of the vasculature; extracellular matrix structure of the periodontal tissues; and cellularity pattern of the periodontal tissues.

2.4. Glycemia, insulinemia, HOMA-IR index and plasma concentration of TNF-α in offspring

Using the 8 plasma samples in each group (CN-o and PD-o), the glucose concentrations were measured by the glucose oxidase method (Enzymatic glucose, ANALISA Diagnóstica, Belo Horizonte, MG, Brazil), and insulinemia was measured by radioimmunoassay (Sensitive Rat Insulin, SRI-13K, Millipore, St Charles, MO, EUA). Insulin resistance was evaluated by the HOMA-IR index (homeostasis model assessment of insulin resistance), which was calculated according to the following formula: HOMA-IR = fasting glycemia (mmol/L) × fasting insulinemia (μIU/mL)/22.5 [15,16]. Plasma concentrations of TNF-α ($n = 8$) were quantified using an enzyme-linked immune sorbent assay (ELISA) kit (Invitrogen Corporation, Camarilio, CA, USA).

2.5. Evaluation of Akt serine phosphorylation status in skeletal muscle of offspring

The GSM was collected from eight animals in each group (CN-o and PD-o) before and 90 s after administering 1.5 U of regular insulin (intravenously through the portal vein). The tissue samples were prepared as described by Carvalho et al. [17] and the Akt serine phosphorylation status following insulin stimulus was quantified using Western blotting and antiphosphoserine AKTSer473 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

The total Akt was quantified using Western blotting and antitotal Akt antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) served as the loading control. Immunoreactive bands were detected by autoradiography using a chemiluminescent substrate system (GE Healthcare,