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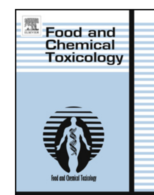

Maternal nicotine exposure leads to higher liver oxidative stress and steatosis in adult rat offspring

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Early nicotine exposure causes future obesity and insulin resistance. We evaluated the long-term effect of the maternal nicotine exposure during lactation in liver oxidative status, insulin sensitivity and morphology in adult offspring. Two days after birth, osmotic minipumps were implanted in the dams: nicotine (N), 6 mg/kg/day for 14 days or saline (C). Offspring were killed at 180 days. Protein content of superoxide dismutase, glutathione peroxidase, catalase, nitrotyrosine, 4HNE, IRS1, Akt1 and PPARs were measured. MDA, bound protein carbonyl content, SOD, GPx and catalase activities were determined in liver and plasma. Hepatic morphology and triglycerides content were evaluated. Albumin and bilirubin were determined. In plasma, N offspring had higher catalase activity, and SOD/GPx ratio, albumin and bilirubin levels but lower MDA content. In liver, they presented higher MDA and 4HNE levels, bound protein carbonyl content, SOD activity but lower GPx activity. N offspring presented an increase of lipid droplet, higher triglyceride content and a trend to lower PPARα in liver despite unchanged insulin signaling pathway. Early nicotine exposure causes oxidative stress in liver at adulthood, while protect against oxidative stress at plasma level. In addition, N offspring develop liver microsteatosis, which is related to oxidative stress but not to insulin resistance.

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1. Introduction

Smoking is a serious health public problem that is associated with diseases such as cancer, pulmonary and cardiovascular diseases where nicotine, the main component of the cigarette, exerts an important effect of dependence (Benowitz et al., 2009). Also tobacco exposure can contribute to obesity development since several epidemiological studies have reported that maternal smoking during pregnancy and/or lactation can be a risk factor for child and teenager obesity and hypertension (Gao et al., 2005; Goldani et al., 2007; Hill et al., 2005; Von Kries et al., 2002; Wideroe et al., 2003). The relationship between environmental, nutritional and hormonal influences at critical windows of plasticity and several chronic adult diseases is named programming (Barker, 2003; de Moura et al., 2008). Therefore, maternal cigarette smoking or nicotine gum and patches during pregnancy and/or lactation are not desired since nicotine can be transferred through placenta and breast milk and can induce future metabolic disturbances, as a programming factor. Indeed, experimental studies of our group have evidenced that nicotine is an imprinting factor during lactation, acting as endocrine disruptor, which programs for overweight, higher visceral adiposity, hyperleptinemia and hypothyroidism in adulthood (Oliveira et al., 2010), as well as peripheral insulin resistance and central leptin resistance (de Oliveira et al., 2010), hypercorticosteronemia and higher catecholamine content in adrenal medulla (Pinheiro et al., 2011).

Several studies have reported the relationship between nicotine and the high pro-oxidant status development, which triggers the oxidative stress. This process represents an unbalance between oxidant radical production and antioxidant molecule availability/activity, resulting in an increase of pro-oxidant status, and consequent cell damage (Halliwell and Gutteridge, 2007). Indeed, the effects of acute nicotine administration upon oxidative stress can be observed in different tissues such as cardiomyocytes (Zhou et al., 2010), 3T3L1 adipocytes (An et al., 2007), aorta (Xiao et al., 2011), pancreas (Bruin et al., 2007, 2008) and liver (Sheng et al., 2001; Halima et al., 2010). The liver is a target for nicotine because its metabolism occurs mainly in this organ through the cytochrome CYP 2A6 (Benowitz et al., 2009), therefore contributing to ROS production (Kirby et al., 2011; Yamazaki et al., 1999).

It has been already demonstrated that smoking contributes to nonalcoholic fatty liver disease (NAFLD), which is the most common form of liver diseases, representing a large spectrum of disorders (Liu et al.,

Abbreviations: CAT, catalase; C, control; CYP 2A6, cytochrome P450 2A6; FAS, fatty acid synthase; GPx, glutathione peroxidase; 4HNE, 4-hydroxy-2-nonenal; IRS1, insulin receptor substrate-1; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; N, nicotine; NAFLD, nonalcoholic fatty liver disease; PPAR, peroxisome proliferator-activated receptor; Akt1, RAC-alpha serine/threonine-protein kinase 1; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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2013). The NAFLD has two stages of establishment; the first is related to metabolic changes, which increases free fatty acids, and *de novo* lipogenesis, leading to steatosis. The second stage is associated with ROS deleterious effect at cell structures, insulin resistance, and release of proinflammatory cytokines, which promotes the progression of steatohepatitis. Thus, the NAFLD is correlated with decrease of antioxidant defenses (Conceição et al., 2013; Rolo et al., 2012; Videla et al., 2004). According to Valenca et al. (2008), oral chronic nicotine exposure in adult rats is associated with impairment of lipid metabolism and microsteatosis. However, these authors did not investigate the oxidative status in this model.

It was already demonstrated that a moderate nicotine exposure via breast milk (administration of 2 mg nicotine/kg maternal body mass per day during lactation) induced lower body weight and liver pro-oxidant status at weaning (21 days old) and pubertal (45 days old) rats. In this experimental model, hepatic morphology was not studied (Halima et al., 2010). Therefore, considering the bare knowledge about the future consequences (at adulthood) of heavy nicotine exposure (6 mg nicotine/kg maternal body mass for 14 days of lactation), since it causes obesity and insulin resistance hypothyroidism (Oliveira et al., 2010), hypothalamic leptin resistance (de Oliveira et al., 2010), higher catecholamine and glucocorticoid levels (Pinheiro et al., 2011), the present study was designed to address the long lasting effects of maternal nicotine exposure upon the oxidative stress markers and antioxidant capacity in plasma and liver, associating with insulin sensitivity and hepatic morphology.

2. Materials and methods

The experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/017/2012), which based its analysis on the principles adopted and promulgated by the Brazilian Law (no. 11.794/2008) that concerns the rearing and use of animals in teaching and research activities in Brazil (Marques et al., 2009). Wistar rats were housed in a light-regulated (12 hour light cycle starting at 7 a.m.) and temperature-controlled room (25 ± 1 °C) over all period. Adult female rats were caged with male at the proportion of 3:1. After mating, each female was placed in an individual cage with water and chow *ad libitum* until delivery.

2.1. Experimental model of postnatal nicotine exposure

Twenty lactating rats were used for this study. To avoid the prenatal litter size influence in the programming effect, only mothers with litter size of 10 ± 2 offspring were studied. At birth (PN 1), the litter size was adjusted to six males for dam to maximize the lactation performance. In the PN 2, the dams were randomly divided into two groups and were anesthetized with i.p. injection of thiopental sodium (40 mg/kg body weight) to insert osmotic minipumps via s.c. (Alzet, 2ML2, Los Angeles, CA, EUA) on their back. For the **nicotine group** (N; n = 10), minipumps were prepared with nicotine free-base (Sigma, St Louis, MO, USA) diluted in isotonic saline (NaCl 0.9%) to release a dose of 6 mg/kg of nicotine throughout 14 days of lactation (Oliveira et al., 2010). For the **control group** (C; n = 10), dams were implanted with osmotic minipumps containing only saline solution. Since weaning until PN 180, offspring's body weight was monitored every 4 days.

2.2. Euthanasia

At PN 180, the animals were fasted for 12 h, killed by decapitation, and their blood was collected from the trunk. The plasma was obtained after centrifugation (1500 × *g* for 20 min at 4 °C) and was frozen (–20 °C) for further analyses. The visceral white adipose tissue (mesenteric, epididymal and retroperitoneal depots) was excised and weighed for evaluation of central adiposity.

The liver was rapidly removed and its hydrostatic weight and volume obtained using the Scherle method. The hydrostatic liver weight was normalized by the right tibia length. The liver was sliced into several minor fragments and stored – frozen or fixed in freshly prepared fixative for 48 h at room temperature (Conceição et al., 2013).

2.3. Preparation of tissue extracts to oxidative status evaluation

The liver extracts were obtained after homogenization with 20% (w/v) of KPE buffer (0.1 M KH2PO4, 0.1 M K_2HPO_4, and 10 mM EDTA, pH 7.5) by homogenizer Potter-Elvehjem (Marconi, São Paulo, Brazil). The homogenates were centrifuged (19.900 × *g* for 5 min at 4 °C), and the supernatants were stored at –80 °C for the enzymatic activity, lipid peroxidation assay and total protein bound carbonyl assays. The protein content was determined using the Bradford assay (1976).

2.4. Determination of the antioxidant enzyme activities

Samples of liver homogenized in KPE buffer were used to superoxide dismutase (SOD) activity evaluation based on inhibition of epinephrine autoxidation in an alkaline medium (pH 10.2) during 3 minutes at 485 nm (Bannister and Calabrese, 1987). The catalase (CAT) activity evaluation was determined through the rate of hydrogen peroxide decomposition during 60 seconds at 240 nm (Aebi, 1984). The glutathione peroxidase (GPx) activity was estimated through oxidation of NADPH to $NADP^+$ indicated by decrease in absorbance at 340 nm during 2 minutes (Flohé and Günzler, 1984). All absorbance were assayed on a spectrophotometer (Hidex Chameleon™, Turku, Finland) and enzyme activities were expressed as U/mg.

2.5. Oxidative damage

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) through thiobarbituric acid reactive substances (TBARS) method (Draper et al., 1993). Liver homogenate in KPE buffer and plasma were mixed with 10% trichloroacetic acid (1:5 v/v) and centrifuged 10 minutes at 8.300 × *g*. The supernatant was incubated with 0.67% thiobarbituric acid (1:1 v/v) for 30 minutes at 95 °C. The absorbance of the pink chromogen was measured at 532 nm (Hidex Chameleon™, Turku, Finland). The total MDA levels are expressed normalized by mg^{-1}protein in 100 µL of sample.

Protein oxidation by ROS, metal and aldehyde ROS-derived was evaluated in liver and plasma by total protein bound carbonyl content reacting with 2,4-dinitrophenylhydrazine (Sigma-Aldrich Co., St. Louis, MO, USA) (Levine et al., 1990). The absorbance were obtained at 380 nm (Hidex Chameleon™, Turku, Finland) and expressed as nMol/mg protein.

2.6. Western blotting analysis

Liver samples were homogenized in lysis buffer (50 mM Hepes, pH 6.4, 1 mM MgCl2, 10 mM EDTA and 1% Triton X-100, plus protease inhibitor cocktail) (Roche Diagnostics GmbH, Mannheim, Germany) using an Ultra-Turrax homogenizer (IKA Werke, Staufen, Germany). After centrifugation (7.500 × *g* for 5 min), homogenates were stored at –20 °C until the SDS-PAGE assay. The total protein content of homogenate was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). The liver protein content of glutathione peroxidase (GPx), Mn and Cu/Zn superoxide dismutase (SOD), catalase, nitrotyrosine-modified proteins, 4-hydroxy-2-nonenal (4-HNE) adducted proteins, insulin receptor substrate-1 (IRS1), phospho-IRS1, RAC-alpha serine/threonine-protein kinase 1 (Akt1), phospho-Akt1, peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ), and β-actin were evaluated using adequate primary antibody incubation (overnight; 1:1.000 antibody from Santa Cruz, CA, USA) followed by proper secondary antibody incubation (1 hour; 1:10.000 antibody from Sigma-Aldrich Co., St. Louis, MO, USA) and streptavidin (1 hour; 1:10.000; Zymed, CA, USA). The protein bands were visualized by chemiluminescence (Kit ECL plus, Amersham Biosciences) followed by exposure to auto radiographic film (Hyperfilm ECL, Amersham Biosciences). Area and density of the bands were quantified by Image J software (Media Cybernetics, Bethesda, MD, USA). The results were normalized by β-actin content and were expressed as relative (%) to the control group.

2.7. Plasma biochemical parameters

The albumin and total bilirubin serum level were measured with colorimetric kit in accordance with manufacturer instructions (Bioclin, MG, Brazil).

2.8. Liver stereology

After fixation and processing, random liver fragments obtained from all lobes were embedded in Paraplast plus (Sigma-Aldrich, St. Louis, MO, EUA), sectioned to 5 µm thick and stained with hematoxylin and eosin (HE) for visualization using light microscopy. The evaluation of hepatic steatosis was performed by point counting methods through a video microscope system and a test system composed of 36 test points (PT). The volume density (Vv) was estimated as: Vv [steatosis, liver] = PP [steatosis]/PT [liver]. Where PP is the number of points counting fat droplets on hepatic tissue (steatosis) and PT is the total test points (Catta-Preta et al., 2011). We highlight that several slices were performed and 10 microscopic fields per animal (n = 5) were analyzed at random (blind analysis).

2.9. Liver triglyceride content

Total triglyceride was extracted from the liver following the adapted Folch and Sloane method (1957). Briefly, 50 mg of liver was homogenized in 1 mL of isopropanol (Vetec Química Fina, RJ, Brazil) and centrifuged at 1.000 × *g* for 10 min, at 4 °C. The triglyceride content was measured by colorimetric assay kit in accordance with manufacturer instructions (Bioclin, MG, Brazil).

2.10. Statistical analysis

Data are presented as mean ± SEM. Differences between groups were analyzed using *Student's t-test*. All statistical analyses were performed with GraphPad Prism

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