Effects of tobacco smoke exposure during lactation on nutritional and hormonal profiles in mothers and offspring

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Abstract

Exposure to tobacco smoke is related to changes in energy balance regulation and several endocrine dysfunctions. Previously, we showed that maternal nicotine (the main addictive compound of tobacco) exposure exclusively during lactation affects biochemical profiles in mothers, milk, and pups. As the possible consequences for mothers and offspring of maternal smoking during lactation are still unknown, we evaluated the effects of tobacco smoke exposure on nutritional, biochemical, and hormonal parameters in dams and pups at weaning. After 72 h from birth, lactating rats were divided into two groups: smoke-exposed (S) in a cigarette-smoking machine, 4×1 h per day throughout the lactation period without pups; control (C), rats were treated the same as the experimental group but exposed to filtered air. Dams and pups were killed at weaning (21 days of lactation). Body weight and food intake were evaluated. Milk, blood, visceral fat, adrenal,

and carcass were collected. S dams showed hyperprolactinemia (+50%), hypoinsulinemia (−40%), hypoleptinemia (−46%), as well as lower triglycerides (−53%) and very low-density lipoprotein cholesterol (−50%). Milk of S dams had higher lactose (+52%) and triglycerides (+78%). S pups presented higher body protein (+17%), lower total (−24%) and subcutaneous fat contents (−25%), hypoglycemia (−11%), hyperinsulinemia (+28%), hypocorticotesteronemia (−40%), lower adrenal catecholamine content (−40%), hypertriglyceridemia (+34%), higher high-density lipoprotein cholesterol (+16%), and lower low-density lipoprotein cholesterol (−45%). In conclusion, tobacco smoke exposure leads to changes in nutritional, biochemical, and hormonal parameters in dams and, passively through the milk, may promote several important metabolic disorders in the progeny.

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Introduction

Globally, it is estimated that one-third of adults are regularly exposed to environmental tobacco smoke (ETS), and among them, 17% are women (WHO 2009). Smoking during pregnancy or lactation is a risk factor for the child's health since it is often associated with adverse effects such as ectopic pregnancy, spontaneous abortion, neurological abnormalities, fetal growth restriction, preterm delivery, and lower nutritional supply in the milk (Einarson & Riordan 2009). Maternal exposure to ETS that contains a variety of toxic compounds is associated with low birth weight (Hegaard *et al.* 2006) and reduction of breastfeeding (Chou *et al.* 2007).

The progeny from smoking mothers show higher gastroenteritis episodes and more hospital admissions due to infections (Ladomenou *et al.* 2009) in childhood. Newborns from smoking mothers have lower leptin concentration in the cord blood (Mantzoros *et al.* 1997). Among compounds of tobacco smoke, nicotine is the most studied and the most

addictive. Rats prenatally exposed to nicotine are heavier when compared to controls (Williams & Kanagasabai 1984, Newman *et al.* 1999), suggesting that fetal nicotine exposure results in increased adiposity in the offspring.

Lactation is a critical period of life: important cognitive and neurological developments occur in this period. Mother's milk represents the primary source of nutrition in this period (Golding *et al.* 1997). Nevertheless, there are few studies approaching maternal smoking during lactation. Zanardo *et al.* (2005) showed that maternal smoking decreased pro-inflammatory cytokine IL1 α in breast milk. In an experimental model of maternal nicotine exposure during lactation, our group evidenced higher milk lactose levels and energy content (Oliveira *et al.* 2010). These previous findings suggest that smoke or its isolate components can alter breast milk and influence nutritional and immunologic status in pups. Nicotine-treated lactating dams presented hyperprolactinemia at the end of nicotine exposure, associated with lower food intake and hyperleptinemia (Oliveira *et al.* 2010). This was

associated with some alterations in lipid profile of mothers and pups, such as higher high-density lipoprotein cholesterol (HDL-C). At weaning, offspring whose mothers were exposed to nicotine had type 2 diabetes (Bruin *et al.* 2007) and increased adipose tissue weight (Soom *et al.* 2008). When maternal nicotine exposure occurred only during lactation, the 15-day-old pups showed higher adiposity, hyperleptinemia, hypothyroidism, hypercorticosteronemia, and higher adrenal catecholamine content (Oliveira *et al.* 2009, 2010, Santos-Silva *et al.* 2010).

Smoking mothers are exposed to thousands of components that are present in tobacco smoke. Despite the fact that there have been an increasing number of animal studies on the basic neurobiology of nicotine exposure during development, tobacco compounds other than nicotine are likely to have their own effects or to have combined effects, including interactions with nicotine, resulting in altered nutritional and hormonal profiles of the offspring. Some compounds of cigarette smoke such as perchlorate and thiocyanate are excreted in urine and milk of breastfeeding women (Steinmaus *et al.* 2007). Breastfeeding infants from smoking mothers presented high urinary cotinine levels (Becker *et al.* 1999), confirming previous studies that showed that nicotine is transferred through the breast milk (Luck & Nau 1987). Since nicotine alone has several effects during lactation both in mothers and in pups, we hypothesized that cigarette smoke as a whole may have the same or even more deleterious effects on maternal and pup physiology. The present study was thus designed to determine the effects of active smoking on maternal metabolism and the short-term endocrine and metabolic consequences for offspring passively exposed via milk, using an experimental model of postnatal cigarette smoke exposure.

Materials and Methods

Wistar rats were kept in a room with controlled temperature ($25 \pm 1^\circ\text{C}$) and with artificial darkness–light cycles (lights on from 0700 to 1900 h). Three-month-old, virgin female rats were caged with male rats at a proportion of 2:1. After mating, each female was placed in an individual cage with free access to water and food until delivery. The use of animals was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/016/2009) which based its analysis on the principles adopted and promulgated by Brazilian Law 11.794/2008, concerning the rearing and use of animals in teaching and research activities in Brazil (Marques *et al.* 2009).

Model of maternal exposure to cigarette smoke during lactation

Three days after birth, 16 lactating rats were randomly assigned to either the smoke (S) or control (C) group until 21 days of lactation. In the S group, dams ($n=8$) were separated from pups and placed into a smoking chamber, 4×1 h per day

throughout the lactation period. This chamber received the smoke generated from an automatic cigarette-smoking machine (Model TE-10, Teague Enterprises, Davis, CA, USA) that burned one research cigarette type 2R1F at a time (1.7 mg nicotine/cigarette; Tobacco Research Institute, University of Kentucky, Lexington, KY, USA). A smoke mixture containing 89% sidestream smoke (smoke released from the burning end of a cigarette) and 11% mainstream smoke (smoke inhaled by an active smoker) as a surrogate for active smoking (Slotkin *et al.* 2001, Abreu-Villaça *et al.* 2010) was generated by the smoking machine in a staggered manner at the rate of a single 35 ml puff of 2 s duration each min. During exposure, the total suspended particulate was measured by weighing teflon-coated fiber filters (TX40H120-WW, Pallflex Products Co., Putnam, CT, USA) before and after a 5 min period, when air was collected from the chamber. There were 12 periods of collection, of which each generated levels of 38.4 ± 3.9 mg/m³ (mean $\pm$ S.E.M.). In the C group, dams ($n=8$) were separated from pups and placed into the chamber as described for the experimental group but exposed to filtered air.

At birth, litter adjustment was performed, with six male pups kept per S or C dam to maximize lactation performance. While dams were kept inside the chamber, pups were kept in their cages in a room with controlled temperature. At weaning (21 days of lactation), dams and pups were killed by rapid decapitation with no prior anesthesia because anesthesia affects hormone and lipid metabolism. We used two offspring from each mother (S pup, $n=16$ and C pup, $n=16$). Blood samples were collected and frozen at -20°C for further analysis.

Nutritional evaluation

During lactation, mother's body weight (BW) and food intake were daily monitored as well as pup's BW. Body length of the pups was measured every 3 days.

Milk collection

Milk samples were collected at 21 days of lactation. For this, mothers were separated from litters for 2 h before milking (Bonomo *et al.* 2005). After i.p. injection of oxytocin (5 UI/ml s.c. – Eurofarm, São Paulo, SP, Brazil) under pentobarbital anesthesia (30 mg/kg BW), milk was manually collected from all teats. We obtained 0.5–1.0 ml from each lactating rat, and samples were frozen at -20°C for further analysis.

Milk biochemistry analysis

Total milk protein was measured according to the Peterson method (1977), with BSA as a standard. Protein concentration was determined based on the Stauffer formula (1975), and results were expressed in mg/ml. Total lipids were measured in milk samples diluted in distilled water (1:25) by colorimetric assay using a Bioclin commercial kit, and results

were expressed in mg/dl. Milk lactose was measured by a colorimetric method using picric acid (Khramov *et al.* 2008), using commercial lactose as a standard (Sigma), and results were expressed in mg/ml. Milk energy was estimated using caloric value of each macronutrient (carbohydrate, lipids, and protein), and expressed in kcal/ml.

Body composition evaluation

Visceral fat mass (VFM) was excised and weighed for evaluation of the central adiposity – mesenteric, epididymal, and retro-peritoneal (Toste *et al.* 2006). The carcasses of C and S pups were weighed, autoclaved for 1 h, and homogenized on distilled water (1:1). Homogenate was stored at 4 °C for analysis (Fagundes *et al.* 2007). Three grams of homogenate were used to determine fat content gravimetrically. Samples were hydrolyzed on a shaking water bath at 70 °C for 2 h with 30% KOH and ethanol. Total fatty acids and free cholesterol were removed with three successive washing with petroleum ether. After drying overnight in vacuum, tubes were weighed, and data were expressed as grams of fat by 100 g of carcass. One gram of homogenate was used for protein content determination by Lowry method (Lowry *et al.* 1951). Data were expressed as grams of protein per 100 g of carcass. The estimate of the subcutaneous fat was calculated by subtracting the visceral fat from the total fat.

Morphological evaluation of maternal lung

Samples of the right lung from S and C mothers were fixed in 4% paraformaldehyde for 72 h; after that, all tissues were dehydrated, cleared, and then paraffin-embedded. Five micrometer-thick sections at the same level were obtained and stained with hematoxylin/eosin to assess morphology. To obtain uniform and proportional lung samples, 16 fields of each slide were observed at a magnification of 200×, using a video microscope (Olympus BX40 linked to an Optronics CCD video camera system; Olympus, Center Valley, PA, USA). All evaluations were carried out by experimenters who did not know the group of dam or pup from which they received lung samples (numbered 1–32).

Detection of cotinine (nicotine metabolite)

Serum and milk cotinine levels were determined using a kit from Orasure Technologies (Bethlehem, PA, USA). At weaning, dams were separated from their litters and, 2 h later, milk was collected and stored at –20 °C until assayed (Bonomo *et al.* 2005). After that, S and C dams and pups were killed, and blood was collected. Blood was centrifuged (2000 g, 20 min), and supernatant was stored (–20 °C) until assaying.

Serum biochemical parameters

Glycemia was determined in the blood sample from the tail vein of fasting rats using a glucosimeter (ACCU-CHEK Advantage, Roche Diagnostics).

Total cholesterol (TC), triglycerides, and HDL-C were analyzed using Biosystem commercial test kits with an automated A15 spectrophotometer (Biosystems S.A., Barcelona, Spain). Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated according to the Friedewald equation (Friedewald & Levy 1972):

$$\text{VLDL-C} = \text{triglycerides}/5$$

$$\text{LDL-C} = (\text{TC} - \text{HDL-C} - \text{triglycerides})/5.$$

Serum hormone quantification by RIA

Blood samples were centrifuged (1500 g/20 min per 4 °C) to obtain sera, which were kept at –20 °C until the assay. All measurements were performed in one assay. Insulin was determined by commercial RIA kit (ImmuChem TM 125 I, coated tube, ICN Biomedicals, Inc., Orangeburg, NY, USA). Assay sensitivity was 1 µUI/ml, and intra-assay variation was 8.9%. Leptin was measured by a specific RIA kit (Linco Research, Inc., St Louis, MO, USA) that measures both rat and mouse leptin (range of detection, 0.5–50 ng/ml; intra-assay variation, 2.9%). Adiponectin was determined by a commercial RIA kit (Millipore MADP, Billerica, MA, USA), with an assay sensitivity of 1 ng/ml and intra-assay variation of 3.7%. Total corticosterone was measured using a commercial RIA kit (ICN Biomedicals, Inc., Aurora, OH, USA) with an assay sensitivity of 50 ng/ml and an intra assay

Figure 1 Photomicrographs of lung from tobacco smoke-exposed mothers during lactation (B and D). Tissue sections were stained with hematoxylin–eosin. (A and C) Control mothers. B, bronchioles; V, vessel; A, alveolus; and AS, airspace. *n*=8 dams per group. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-10-0410>.

variation coefficient of 7%. Prolactin (PRL) was determined by specific RIA using reagents supplied by the National Institute of Health (Maryland, MD, USA). Data were reported in ng/ml from the reference preparation RP-3 with an assay sensitivity of 0.3 ng/ml and an intra-assay coefficient of 7.5%. Total triiodothyronine (TT₃) and free thyroxine (FT₄) were measured by RIA (ICN Pharmaceuticals, Inc., Los Angeles, CA, USA). Sensitivity limits were 25 ng/dl for TT₃ and 0.3 ng/dl for FT₄, and intra-assay variations were 3.5 and 6.5% respectively.

Insulin sensitivity evaluation

Insulin sensitivity was analyzed according to two formulae (Matthews *et al.* 1985):

The homeostasis model assessment of insulin resistance (HOMA-IR): (Insulin (μIU/ml) × serum glucose (mmol/l))/22.5; HOMA-β: (Insulin (μIU/ml) × 20)/serum glucose (mmol/l) – 3.5.

Figure 2 Body weight (A) and food intake (B) evolutions and visceral fat mass at weaning from tobacco smoke-exposed mothers during lactation (black) or control mothers (gray). Values represent mean and S.E.M. of eight dams per group.

Table 1 Glucose homeostasis and hormonal profile of tobacco smoke-exposed mothers during lactation. Values represent mean ± S.E.M. of eight lactating rats per group

	Control group	Smoke group
Glycemia (mg/dl)	77.0 ± 5.6	69.3 ± 3.9
Serum insulin (μIU/ml)	43.7 ± 3.9	26.6 ± 3.5*
HOMA-IR	9.4 ± 1.2	4.4 ± 0.8*
HOMA-β	234.3 ± 32.2	160.0 ± 29.4
Serum leptin (ng/ml)	1.6 ± 0.2	0.9 ± 0.1*
Serum adiponectin (ng/ml)	2.0 ± 0.2	1.9 ± 0.2
Serum PRL (ng/ml)	3.9 ± 0.6	7.8 ± 1.5*
Adrenal catecholamine content (μM/g)	0.40 ± 0.23	0.69 ± 0.21
Serum corticosterone (ng/ml)	364.0 ± 61.2	255.3 ± 59.5
Serum TT ₃ (ng/dl)	48.7 ± 4.5	45.3 ± 3.1
Serum FT ₄ (ng/dl)	0.51 ± 0.11	0.50 ± 0.09

*Significant differences between groups ($P < 0.05$).

Adrenal catecholamine content measurement

For catecholamine content, left adrenal glands were homogenized in 500 μl of 10% acetic acid and centrifuged (10 000 *g* for 1 min). To assay, 50 μl of supernatant/epinephrine standards were mixed with 250 μl of buffer phosphate 0.5 M, pH 7.0 and 25 μl of potassium ferricyanate 0.5%, followed by incubation (20 min; ice bath). Reaction was stopped with 500 μl of ascorbic acid/NaOH 10 M (1:19 proportion). Parameters used in the fluorometer (Victor2, PerkinElmer, Waltham, MA, USA) were 420 nm excitation and 510 nm emission. Results were obtained by plotting the values into a linear regression of the standard epinephrine curve. Data were expressed by μM catecholamines/mg gland (Trevanzoli *et al.* 2007).

Statistical analysis

Results were reported as mean ± S.E.M. GraphPad Prism 5 was used for statistical analyses and graphics (GraphPad Software, Inc., La Jolla, CA, USA). Changes in BW and food intake were analyzed by two-way ANOVA and Newman-Keuls multiple comparison tests. The other experimental data were analyzed by unpaired Student's *t*-test with significance level set at $P < 0.05$. We studied two offspring from each mother (16 pups per group). However, for the analyses, litter was used as the experimental unit so that we considered the average of values from animals of the same litter instead of using individual animal values.

Results

Tobacco smoke exposure affected cotinine milk and serum levels. In S dams, milk and serum cotinine concentrations were similar (milk, 107.6 ± 6.6 ng/ml; serum, 105.1 ± 9.6 ng/ml). S pups at 21 days old had serum cotinine levels of 17.9 ± 4.2 ng/ml. Control dams and pups had cotinine levels below the detection limit of the technique (< 8 ng/ml).

Table 2 Lipid profile of tobacco smoke-exposed mothers during lactation. Values represent mean $\pm$ S.E.M. of eight lactating rats per group

	Control group	Smoke group
Total cholesterol (mg/dl)	70.4 $\pm$ 3.6	67.6 $\pm$ 4.5
Triglycerides (mg/dl)	116.3 $\pm$ 5.7	55.4 $\pm$ 5.8*
HDL-C (mg/dl)	51.4 $\pm$ 2.0	52.4 $\pm$ 2.4
LDL-C (mg/dl)	8.0 $\pm$ 0.9	7.3 $\pm$ 1.3
VLDL-C (mg/dl)	23.3 $\pm$ 1.1	11.1 $\pm$ 1.2*

*Significant differences between groups ($P < 0.05$).

The pulmonary morphology (Fig. 1) showed histological abnormalities (as goblet cell depletion) in mothers who were exposed to cigarette smoke, such as alteration of bronchiolar structure, blood vessel, and alveolus. The bronchioles showed alteration in the epithelium as well as an increase of vessel thickness with hypertrophy, where tunica adventitia and media were not differentiated. Alveolus showed septa destruction with disruption and decrease of functional airspace, inflammation with macrophages, and neutrophils.

At weaning, S dams exhibited no change in BW, food intake, and VFM compared to control group as observed in Fig. 2. Hormonal profile is shown in Table 1. At the end of lactation, S dams presented hypoinsulinemia (-40% , $P < 0.05$), hypoleptinemia (-46% , $P < 0.05$), and hyperprolactinemia ($+50\%$, $P < 0.05$), while glycemia, adiponectinemia, serum thyroid hormones, corticosteronemia, and adrenal catecholamine were unchanged. Concerning insulin sensitivity (Table 1), S dams presented lower HOMA-IR (-53% versus C, $P < 0.05$) but no significant change of HOMA- β . As shown in Table 2, S dams showed lower serum triglycerides (-53% , $P < 0.05$) and VLDL-C (-50% , $P < 0.05$), while cholesterol evaluations were normal. In milk (Table 3), tobacco smoke exposure caused higher lactose, triglycerides content ($+52$ and $+78\%$, $P < 0.05$ respectively), and calories ($+19\%$), while protein, cholesterol, and total lipids were normal.

Pups whose mothers were smoke-exposed during lactation were comparable to control pups concerning BW gain and body length (Fig. 3). Concerning body composition evaluation (Fig. 4), S pups showed lower total and subcutaneous fat (-24% Fig. 4B and -25% Fig. 4C, $P < 0.05$ respectively) and no alteration of the VFM with higher body protein content (Fig. 4D, $+17\%$, $P < 0.05$). Hormone levels of pups whose mothers were smoke-exposed during lactation are depicted in Table 4. Weaned S pups showed higher insulinemia ($+28\%$, $P < 0.05$), lower glycemia (-11% , $P < 0.05$), lower corticosteronemia (-40% , $P < 0.05$), and lower adrenal catecholamine content (-40% , $P < 0.05$) compared to C pups; however, adiponectin, PRL, and thyroid hormone levels were unchanged. This group also showed higher HOMA- β ($+47\%$, $P < 0.05$, Table 4). The lipid profile of S pups is described in Table 5. At 21 days old,

S pups presented higher serum triglycerides ($+34\%$, $P < 0.05$), lower LDL-C, and higher HDL-C levels (-45 and $+16\%$, $P < 0.05$ respectively).

Discussion

There are a large number of compounds found in tobacco smoke, at least 4000 bioactive compounds were already reported, most of them with toxicological effects such as nicotine, formaldehyde, cadmium, and urethane (Balharry *et al.* 2008). Our data using cigarettes with a high nicotine dose (1.7 mg/cigarette; the main addictive compound of cigarettes) showed similar cotinine concentrations in serum and milk. This fact confirms the transfer of nicotine to the milk and shows the effectiveness of our model of passive exposure of offspring to smoke. The short half-life of cotinine (5.2 h) in rats (Kyerematen & Vesell 1991) and the interval between the last exposure of mothers to smoke and the killing (2 h) explain the low cotinine levels of S pups. Thus, offspring were exposed to low nicotine levels and only through the milk, similar to what occurs with infants whose mothers smoked between the breastfeeding periods, away from their babies.

We showed pathological changes in the lung of S dams, which were also found in other models of tobacco smoke exposure (Chen *et al.* 2008, Zhang *et al.* 2008a,b). Dams of S group presented alterations in bronchioles, vessels, and alveoli. These alterations lead to an alveolar disturbance, cellular hyperplasia, and inflammatory infiltration. Clearly, this suggests that smoke has potent inflammatory agents that activate inflammatory proteins such as cytokines, growth factors, and adhesion molecules, which causes changes in lung function and structure. It is known that quinones and hydroquinones detected in tobacco smoke are responsible for the generation of reactive oxygen species (Hecht 1999, Yu *et al.* 2002). This oxidative stress may cause a local inflammatory response, with higher IL12, tumour necrosis factor- α , neutrophils, macrophages and lymphocytes that may be related to the morphological changes observed (Zheng *et al.* 2009, Braber *et al.* 2010). Taken together, the morphological changes of maternal lung and high cotinine levels in serum and milk confirm the effectiveness of smoke

Table 3 Milk biochemical composition of tobacco smoke-exposed mothers during lactation. Values represent mean $\pm$ S.E.M. of eight lactating rats per group

	Control group	Smoke group
Lactose (mg/ml)	24.4 $\pm$ 3.1	37.2 $\pm$ 3.1*
Protein (mg/ml)	109.7 $\pm$ 18.4	96.9 $\pm$ 7.5
Cholesterol (mg/dl)	530.3 $\pm$ 54.7	530.6 $\pm$ 31.3
Triglycerides (mg/dl)	18 140 $\pm$ 3155	32 320 $\pm$ 2474*
Total lipids (ng/ml)	3717 $\pm$ 861	4125 $\pm$ 581
Calories (kcal/ml)	26.0 $\pm$ 3.7	31.0 $\pm$ 4.7*

*Significant differences between groups ($P < 0.05$).

Figure 3 Body weight (A) and body length (B) of pups whose mothers were tobacco smoke-exposed (black) or not (gray) during lactation. Values represent mean and s.e.m. of eight pups per group.

exposure in our experimental model of active maternal smoking during lactation.

Despite the data showing the reduction of BW and food intake in animals under tobacco smoke or nicotine exposure (Arai *et al.* 2001, Chen *et al.* 2005, 2006, 2008), we observed that S dams did not present changes of these parameters throughout the lactation period. A simplistic explanation for this fact could be the relatively short period of smoke exposure (18 days of lactation). However, this short period was already enough to produce important lung morphological changes. Further, we cannot discard the importance of maternal hormonal changes observed in the present study, such as hypoleptinemia and hyperprolactinemia, also observed by Oliveira *et al.* (2010) in nicotine-treated mothers during lactation, to produce a counterbalance of nicotine effects upon BW and food intake control. Leptin is an anorexigenic hormone produced by adipocytes (Ahima *et al.* 1996), and PRL, which allows the initiation and maintenance of breastfeeding (Hill *et al.* 1999), is responsible for maternal hyperphagia during this period (Grattan 2001). Thus, lower leptin and higher PRL could avoid the anorexigenic effect of smoking, promoting the unchanged food intake and BW of dams, perhaps in order to ensure the maintenance of energy homeostasis in this critical period.

Regarding glucose homeostasis, S mothers showed normoglycemia and hypoinsulinemia. Normoglycemia was described in rats exposed to tobacco smoke during pregnancy (Sinzato *et al.* 2008) associated with lower liver glycogen content and glucose intolerance. In addition, some studies have associated smoking in adult humans with the development of insulin resistance (Chiolero *et al.* 2008). In contrast, in the present study, S mothers presented lower HOMA-IR suggesting higher insulin sensitivity. This hypoinsulinemia can be due to lower insulin secretion from pancreatic β cells that in a long term could lead to the development of glycemic intolerance. It is known that PRL stimulates and leptin inhibits insulin secretion in pancreatic β cells (Arumugan *et al.* 2010, Park *et al.* 2010). However, both hypoleptinemia and hyperprolactinemia of S mothers were not sufficient to maintain insulin levels. Thus, the physiological period of life, especially lactation, or the duration of smoke exposure can determine whether the organism will be more sensitive or resistant to insulin in response to smoking.

Smoking affects the thyroid gland (Christensen *et al.* 1984, Fisher *et al.* 1997, Utiger 1998), and thiocyanate is associated with hypothyroidism (Fukayama *et al.* 1992, Muller *et al.* 1995). During lactation, smoking mothers transfer less iodine through milk (Lauberg *et al.* 2004). In our study, S dams did not show serum thyroid hormone alterations. Probably, the short-term smoke exposure was not enough for changing the maternal thyroid function.

Milk from S mothers presented high triglycerides and lactose levels that contribute to an offer of a hypercaloric milk to pups. Lower triglyceridemia of S mothers can be due to a higher transfer of this macronutrient to the pups through the milk and confirms previous studies (Ng *et al.* 2009). Among cigarette substances, nicotine can be responsible for this change because a similar effect was found in the model of maternal nicotine exposure during lactation (Oliveira *et al.* 2010). Accordingly, these biochemical

Figure 4 Visceral fat mass (A), body fat (B), subcutaneous fat (C), and body total protein (D) of pups whose mothers were tobacco smoke-exposed (black) or not (gray) during lactation. Values represent mean and s.e.m. of eight pups per group. * $P < 0.05$.

Table 4 Glucose homeostasis and hormonal profile of pups whose mothers were tobacco smoke-exposed during lactation. Values represent mean $\pm$ S.E.M. of eight pups per group

	Control group	Smoke group
Glycemia (mg/dl)	109.8 $\pm$ 2.5	98.2 $\pm$ 2.4*
Serum insulin (μ U/ml)	27.5 $\pm$ 2.9	35.1 $\pm$ 2.6*
HOMA-IR	7.1 $\pm$ 0.8	9.0 $\pm$ 1.2
HOMA- β	82.1 $\pm$ 7.4	121.2 $\pm$ 12.5*
Serum leptin (ng/ml)	2.0 $\pm$ 0.3	2.4 $\pm$ 0.3
Serum adiponectin (ng/ml)	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1
Serum PRL (ng/ml)	4.43 $\pm$ 0.85	4.45 $\pm$ 1.05
Adrenal catecholamine content (μ M/g)	0.53 $\pm$ 0.07	0.32 $\pm$ 0.05*
Serum corticosterone (ng/ml)	155.7 $\pm$ 27.0	94.5 $\pm$ 13.4*
Serum TT ₃ (ng/dl)	148.6 $\pm$ 5.9	146.4 $\pm$ 4.8
Serum FT ₄ (ng/dl)	0.92 $\pm$ 0.09	0.91 $\pm$ 0.12

*Significant differences between groups ($P < 0.05$).

alterations in milk can be a protective mechanism, providing more energy to the pups. In accordance with this suggestion, previous studies show that smoke exposure is associated with reduced oxygen partial pressure in dams signaling a deficient nutritional offer to the mother.

Smoke in pregnancy is related to being overweight in childhood (Von Kries *et al.* 2002, Wideroe *et al.* 2003), and higher total body fat mass was described in suckling pups whose mothers were nicotine-treated during lactation (Oliveira *et al.* 2009). However, in the present model, S pups showed no change in BW at weaning, despite higher body protein and lower body fat mass. Higher body protein can be due to hyperinsulinemia of S pups that can increase amino acids uptake in the muscle. Since visceral adiposity was not altered in S pups, lower body fat was caused exclusively by lower subcutaneous fat content. Although the subcutaneous adipocyte is the main site of leptin production, its reduction did not affect serum leptin levels in S pups. This kind of dissociation between fat mass and leptinemia was already reported in other models (Toste *et al.* 2006, Rodrigues *et al.* 2009). Although we cannot draw a direct comparison between the models of nicotine exposure and ETS exposure, Oliveira *et al.* (2009) observed higher adiposity and hyperleptinemia in pups from nicotine-treated dams on lactation, while in the present study, we found lower body fat in S pups, which can be associated with other cigarette components or substances conveyed through the milk, since S offspring had no direct contact with cigarette smoke.

Concerning the lipid profile of S pups, we detected higher serum triglycerides, resulting from the higher transfer through maternal milk. Hypertriglyceridemia can still be indicative of fatty liver development that is related to smoking (Chiang *et al.* 2009, Mallat & Lotersztajn 2010). Tobacco smoke is related to decreased Apo AI and other cardiometabolic alterations in children exposed to ETS (Nagel *et al.* 2009); however, in our model, S pups had higher HDL-C levels suggesting an adaptive defense mechanism against tobacco smoke effects. Perhaps,

nicotine is responsible for higher serum HDL-C because recently we evidenced this effect in suckling pups whose mothers were treated with nicotine during lactation (Oliveira *et al.* 2010). In addition, according to Ng *et al.* (2009), female pups whose mothers were exposed to tobacco in pregnancy showed higher HDL-C.

As already mentioned, smoking exposure and nicotine exposure are related to the development of insulin resistance and type 2 diabetes (Chiolo *et al.* 2008, Sommi *et al.* 2009). Despite lower glycemia (only 11% less than controls) and no change of adiponectin level, S pups showed hyperinsulinemia and higher HOMA- β , suggesting a risk for the future development of insulin resistance.

Detection of lower serum corticosterone and adrenal catecholamine content in S offspring under basal conditions may be related to lower response to stress and suggest that some components of smoke have a direct inhibitory effect in the adrenal gland of the pups. On the contrary, exclusive exposure to nicotine (Oliveira *et al.* 2010) leads to higher corticosteronemia and adrenal catecholamine content in suckling pups. This fact shows that exposure to nicotine alone has opposite effects when associated with other cigarette components. Thus, other smoke substances may have contrary and more marked effects than nicotine over adrenal function. Since it is well known that catecholamine decreases insulin secretion, and both catecholamines and corticosterone are hyperglycemic hormones, the lower levels of these two hormones of S offspring could help to explain the hyperinsulinism combined with lower blood glucose levels.

Maternal smoking influences the thyroid function of the infants (Meberg & Marstein 1986, Chanoine *et al.* 1991). In the present study, S pups did not display changes of thyroid hormone levels. Previously, we demonstrated that neonate pups from-nicotine treated dams presented primary hypothyroidism (Oliveira *et al.* 2009). It is interesting to note that in the two models, the serum levels of cotinine detected in the progeny, although low, are similar as well as the period of exposure. Thus, again there was a dissociation between the effects of isolated nicotine exposure and those of other smoke components.

Previously, we evidenced that maternal nicotine exposure during the lactation period leads to metabolic alterations in both dams and pups (Oliveira *et al.* 2009, 2010). In the present

Table 5 Lipid profile of pups whose mothers were tobacco smoke-exposed during lactation. Values represent mean $\pm$ S.E.M. of eight pups per group

	Control group	Smoke group
Total cholesterol (mg/dl)	157.3 $\pm$ 10.5	137.7 $\pm$ 5.0
Triglyceride (mg/dl)	155.6 $\pm$ 22.9	208.2 $\pm$ 17.0*
HDL-C (mg/dl)	58.9 $\pm$ 2.0	68.5 $\pm$ 1.7*
LDL-C (mg/dl)	68.0 $\pm$ 3.2	37.6 $\pm$ 2.9*
VLDL-C (mg/dl)	35.2 $\pm$ 5.8	41.6 $\pm$ 3.4

*Significant differences between groups ($P < 0.05$).

model of maternal exposure to smoke in postnatal life, we observed that some nutritional, hormonal, and metabolic alterations were similar (i.e. high milk lactose and maternal hyperprolactinemia), while others were different (i.e. hypothyroidism, adrenal hormonal changes, hyperleptinemia, and higher adiposity in suckling pups) when compared to maternal exposure to nicotine during lactation (Oliveira *et al.* 2009, 2010), which suggests different effects of nicotine-isolated exposure when compared to effects elicited by tobacco smoke components. However, we must be careful in making direct comparisons between the two models, due to differences in the route of administration, time, and frequency of exposure, which hinders a clear interpretation. It is the first time that the effects of smoking exposure during lactation on this dataset were shown.

In conclusion, our model of maternal exposure to cigarette smoke exclusively during lactation highlights the deleterious effects on nutritional, biochemical, and hormonal profiles of both mothers and neonates. Therefore, the implication from these results is that women who are breastfeeding should avoid exposure to tobacco smoke, even in the absence of the child.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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