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Maternal Triclosan consumption alters the appetite regulatory network on Wistar rat offspring and predispose to metabolic syndrome in the adulthood

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Abstract. The objectives of this study were to evaluate the effects of maternal oral exposure to the antibacterial Triclosan (TCS) during gestation and lactation on the metabolic status of the adult offspring and on the expression of main genes controlling the appetite regulatory network. Pregnant rats were fed ad-libitum with ground food + TCS (1 mg/kg) from day 14 of gestation to day 20 of lactation (n=3) or ground food (n=3). After litter reduction, 12 males and 12 females born from the TCS exposed rats (TCS, n=24) or not (Control, n=24) were used to evaluate monthly body weight, food intake, plasma levels of cholesterol, glucose and triglycerides, and the hypothalamic mRNA expression of agouti-related protein (Agrp), neuropeptide Y (Npy) and propiomelanocortin (Pomc). Body weight for rats in the TCS group was 12.5% heavier for males at 4 months (p<0.001) and 19% heavier for females at 8 months (p=0.01). Food intake was significantly higher for rats in the TCS group at 5 months of age (p<0.01). Cholesterol and glucose levels were significantly higher for rats in the TCS group at 8 months (p<0.05). mRNA expression of Npy and Agrp were significantly increased in hypothalami of rats in the TCS group at 2 months for males or 8 months for females (p<0.05). In conclusion, low doses of oral TCS consumption by the pregnant and lactating dam increase the hypothalamic expression of the orexigenic neuropeptides Npy and Agrp in the offspring and alter their metabolic status during adulthood, resembling development of the metabolic syndrome.

Key words: Endocrine disruptor, Fetal programming, Hypercholesterolemia, Hyperglycemia, Orexigenic neuropeptides

EXPOSURE to several external factors during gestation in critical windows of fetal development can introduce permanent modifications in the expression of certain genes in the offspring, which could alter the offspring's phenotype permanently. These modifications have been associated with the predisposition to present certain diseases in the adult animal [1]. Maternal nutritional status and/or exposure to several chemical compounds are known to induce changes in the expression, localization and action of the hypothalamic neuropeptides playing a central role in the appetite regulatory network, leading to permanent alterations in the energy consumption after birth [2]. The main hypothalamic neuropeptides stimulating the appetite (orexigenic peptides) are the neuropeptide Y (NPY) and the agouti-related protein (AGRP), while the main inhibitor (anorexigenic peptide) is the alpha melanocyte-stimulating hormone (α-MSH), synthesized as part of the propiomelanocortin molecule (POMC) (reviewed in [3]).

Several studies done in rats have shown that maternal undernutrition during gestation result in offspring with hyperphagia and altered metabolic signals controlling the appetite regulatory network. The general output is a strong predisposition to higher fat deposition, overweight and metabolic diseases once the animal reaches the adult age [4, 5]. The effect of chemical compounds that might be endocrine disruptors

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and could be consumed by the pregnant dam is less clear. There are reports about the effect of bisphenol A (BPA) during gestation, which is considered a potential estrogenic disruptor. BPA exposure during pregnancy could induce a similar effect in the fetus than intrauterine restriction: hyperphagia, visceral fat accumulation, higher liver and body weight and development of metabolic syndrome in the adulthood [6, 7].

Another xenobiotic compound with similar chemical structure tan BPA is Triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy)-phenol). TCS is a synthetic antibacterial compound largely utilized in cosmetic industry (hand soap, toothpaste, deodorants, etc) [8]. The antibacterial action of TCS is achieved by inhibition of the bacterial FabI enzyme, necessary for lipid synthesis [9]. As this gene is not expressed in mammals, TCS is considered safe for human health [10]. However, TCS could be a potential endocrine disruptor that has been shown to affect development and reproduction in the fish [8]. TCS can easily diffuse through biological membranes and has been detected in maternal milk, urine and plasma with lower concentrations than those found in personal care products containing TCS [8]. Although TCS could represent a potential threat to the developing fetus, so far few studies have evaluated if TCS exposure during gestation can affect the progeny after they are born. There are reports that maternal TCS intake has an effect on thyroid hormones in the offspring [11, 12]. However, the potential consequences of TCS exposure during gestation on the offspring's appetite regulatory network and metabolic profile during adulthood are not completely elucidated. The objectives of this study were to evaluate the effect of maternal oral TCS intake during gestation and lactation on the metabolic status of the adult offspring and on the expression of the main genes controlling the appetite regulatory network in Wistar rats. We hypothesized that maternal TCS ingestion impairs the normal development of the orexigenic-anorexigenic neuropeptides in the fetuses, and this is translated as altered phenotype in the offspring after they are born.

Materials and Methods

Chemicals

Triclosan was purchased from Sigma Aldrich (Argentina). 1 mg of the drug was dissolved in 100 ml of water at 20ºC. This solution was poured into 1 kg of ground food and mixed properly for homogeneous distribution. Food was stored on a dry environment, avoiding exposure to sunlight. Using this procedure, 5 kg of food were prepared on the same day and stored for the whole experiment.

Animal procedures

All studies were approved by the Institutional Animal Care and Use Committee of the INICSA and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Nulliparous female Wistar rats of similar age (~4 months) and weight (~250 gm) were housed under a 12-h light/dark cycle (lights on at 0700 h) and temperature controlled (22-24ºC) conditions, with food and water ad libitum. At the beginning of this experiment, female rats were bred by placing two females with a male, which also were of similar ages and weight. Every early morning, females were examined for the vaginal plug or presence of sperm in the vaginal smears. If this was observed, the rat was individually housed apart and that day was considered as day 0 of the experiment for that rat. If no signs of mating were detected, the rat was reintroduced in the male cage. The 6 female rats used in this experiment were mated between the second and fourth day after contact with the male.

During the first 14 days, dams were fed with ground food ad-libitum (Rata/Ratón Laboratorio; GEPSA food, Grupo Pilar S. A., Argentina). On day 14 of gestation, 3 rats were fed ad-libitum with ground food + TCS (1 mg/kg of food). This dose resembles the oral intake of TCS by humans through personal care products [13] (*vide infra*). Feeding with TCS continued until day 20 of lactation (maternal TCS rats). This period of TCS exposure was chosen to match the development of the hypothalamic appetite regulatory network in the rat. The NPY/AGRP neurons are present in the arcuate nucleus at 14.5 day of gestation, and the total development of NPY/AGRP neurons projections is completed around 15 days after parturition [14, 15]. Maternal Control rats (n=3) were kept feeding with ground food *ad libitum*.

The first day after parturition, the litter size was reduced to 4 males and 4 females. There were no differences in the average litter size between maternal TCS and Control rats (14 and 13.3 pups, respectively), neither in the proportion of male:female pups born (7:6.3, respectively, for both groups). Thus, 12 males and 12 females born from the maternal TCS

rats (TCS group, n=24) and from the maternal Control rats (Control group, n=24), respectively, composed the experimental groups.

Weaning was done at 30 days after parturition, and two same-gender siblings were housed per cage. Regular food and water were maintained ad libitum during the rest of study.

Body weight and food intake measurements

Dam's body weight was measured at the beginning of the experiment, 4 days after parturition and immediately after weaning. Offspring's body weight was recorded monthly for every rat on each group, starting after weaning (1 month of age) and continuing until 8 months of age. To measure food intake, 4 males and 4 females from TCS and Control groups were randomly chosen and housed in metabolic cages during one week. The same amount of ground food was fed to each rat daily at the same hour, and the amount of food remaining in the feeder was weighted daily before feeding. Food consumption was measured at 2 months and 5 months of age.

Samples collection

For hypothalamic tissue collection, 8 random rats from each group (4 males and 4 females) were euthanized at 2 months of age (after puberty) and 8 months of age (adult age). Rats were immediately decapitated after euthanasia, brains were removed from the skulls and the hypothalami were rapidly dissected and snap frozen in liquid nitrogen. Tissues were stored at -80ºC until processed for RNA extraction.

At 8 months of age, blood samples (~5 ml) were collected from the heart in tubes and centrifuged at 2,500 g for 15 minutes. Serum samples were stored at -20ºC until processed for metabolites concentration determination.

For liver weight determination, 8 random rats from each group (4 males and 4 females) were euthanized at 4 months of age. Liver weight was also measured in rats euthanized at 8 months of age. Liver was completely removed after euthanasia and its weight was determined on a digital scale.

Rats were fasted overnight before euthanasia for sample's collection.

Determination of serum metabolite concentration

Serum concentrations of total cholesterol, triglycerides and glucose were determined using enzymatic assays with reagents from Roche (Buenos Aires, Argentina) and processed with a Cobas 6000 (c501) analyzer. All serum samples were measured in duplicate for each assay.

Quantitative Real-Time (qRT)-PCR

Messenger RNA was extracted from the hypothalami using Trizol reagent (Thermo Scientific) according manufacturer protocol. Isolated RNA concentration was determined with Nanodrop, and RNA was stored at -80ºC until cDNA conversion. An aliquot of the extracted RNA was converted to cDNA using M-MLV Reverse Transcriptase (Promega) following the methodology recommended by the manufacturer. The synthesized cDNA was stored at -20ºC until qRT-PCR was performed.

The following genes were selected for mRNA measurement by qRT-PCR: Agrp (agouti related protein), Npy (neuropeptide Y), Pomc (propiomelanocortin) and Actb (β-actin), as housekeeping gene. Relative expression of selected genes was determined using primers (Invitrogen) and SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed with Primer Express software (Applied Biosystems) from the corresponding *Rattus norvegicus* mRNA. Primers sequences and accession numbers are reported in Table 1.

All primer pairs had efficiencies greater than 95%. All samples were run in triplicate for each gene and for β-actin. There were no differences in β-actin expression among the groups. Relative mRNA expression of each gene was calculated by determining change in threshold cycle (ΔCt) between the mean Ct for

Table 1 Sequences of primers for real-time PCR analysis

Gene	Forward primer	Reverse primer	Accession number
Npy	TGATGCTAGGTAACAAACGAATGG	GCCAGAATGCCCAAACACA	NM_012614
Agrp	GCAGAGGTGCTAGATCCACAGA	GGACTCGTGCAGCCTTACACA	NM_033650
Pomc	TCCTGCTTCAGACCTCCATAGAC	GGATGCAAGCCAGCAGGTT	NM_139326
Actb	TCTGTGTGGATTGGTGGCTCTA	CCTGCTTGCTGATCCACATCT	NM_031144

each gene and the mean Ct for β-actin mRNA from the same sample. The effect of TCS exposure during gestation/lactation on each gene was analyzed by ANOVA using the ΔCt values. Data were graphed as the mean fold change in expression in the TCS group relative to the Control group; fold change in each sample was calculated as 2-ΔΔCt, where ΔΔCt is the difference between ΔCt in each sample and the mean ΔCt in the Control group.

Statistical analysis

Data is expressed as mean ± SD per group and gender. The effect of group (TCS or Control groups) on body and liver weight, food intake and serum metabolites concentration was analyzed by t-test for each gender and age. The GLM procedure of SAS (v. 9.1) was employed to run the analyses.

For all statistical analyses, the criterion for achieving statistical significance was $p<0.05$, while a $p>0.05$ and <0.1 was considered a tendency.

Results

Body weight, food intake and liver weight

Body weight did not differ between dams in the maternal TCS group or maternal Control group at the beginning of the experiment (246.6 ± 15.3 gm versus 251.6 ± 10.4 gm), after parturition (370 ± 60.8 gm versus 356.6 ± 51.3 gm) or after weaning (286.6 ± 20.8 gm versus 290 ± 26.4 gm). For the offspring, body weight was significantly lighter for males in the TCS group compared to the Control group at 2 months of age (312.25 ± 31.1 gm versus 353.75 ± 16.5 gm, $p<0.001$) and for females (247 ± 15.1 gm versus 259.75 ± 14 gm), but this difference was not significant. This situation was reversed, since either males or females rats in the TCS group presented higher body weight from the 3rd month of age until the end of the study compared to males and females in the Control group. However, body weight was significantly heavier for males in the TCS group at 4 months of age only, compared to males in the Control group (515.6 ± 38.3 gm versus 451.25 ± 51.1 gm, $p<0.001$) and for females at 8 months of age only, compared to females in the Control group (378.3 ± 20.2 gm versus 306.6 ± 20.8 gm, $p=0.01$; Table 2).

Food intake at 2 months of age have a tendency to be higher for male rats in the TCS group compared to male rats in the Control group (27.55 ± 2.3 gm/day versus 25.49 ± 1.6 gm/day, $p=0.055$). Food intake at 5 months was significantly higher for male rats in the TCS group compared to male rats in the Control group (52.75 ± 3.8 gm/day versus 41.25 ± 3.3 gm/day, $p<0.01$); and for females in the TCS group compared to females in the Control group (34.6 ± 2.5 gm/day versus 28.15 ± 2.0 gm/day, $p\leq0.01$).

Hyperphagia and overweight are associated with higher liver weight and size. Thus, liver weight was determined twice during adult age. Liver weight was significantly higher for males in the TCS group compared to males in the Control groups at 4 months of age (12.13 ± 1.3 gm versus 8.9 ± 1.16 gm; $p=0.01$) but there was not significant difference for females. Liver weight at 8 months of age was numerically higher for rats in the TCS group compared to rats in the Control group, either for males (16.1 ± 3.7 gm versus 14.2 ± 0.4 gm) or females (10.6 ± 0.75 gm versus 8.6 ± 1.9 gm), but these numerical differences were not statistically significant. Similar results were found for the ratio of liver weight (gm)/body weight (gm). The ratio was significant for males in the TCS group compared to males in the Control groups at 4 months of age (0.022 ± 0.0018 gm versus 0.018 ± 0.0011 gm). The ratio was numerically but not significantly different for rats in the TCS group compared to rats in the Control group at 8 months of age.

Table 2 Maternal Triclosan (TCS) intake increases offspring body weight

Age (months)	Females					Males				
	Control group		TCS group		*p*-value	Control group		TCS group		*p*-value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
2	259.8	14.0	247.0	15.4	0.266	353.8	16.5	312.3	31.1	0.004 *
4	310.0	25.6	328.1	24.5	0.170	451.3	51.1	515.5	38.3	0.012 *
6	362.5	26.3	393.8	7.5	0.062	486.3	20.6	527.5	25.5	0.275
8	305.0	17.3	378.8	16.5	<0.01 *	557.5	20.6	572.5	28.7	0.428

Mean body weight (grams) for male or females rats in the TCS or Control group every two months. Data is shown as mean body weight ± SD. * Significant difference between mean body weight of rats in the TCS group compared to the Control group.

Serum metabolites concentration

Serum concentrations of cholesterol, triglycerides and glucose at 8 months of age are shown as boxplots in Fig. 1. Cholesterol levels were significantly higher in male and female rats of the TCS group compared to male and female rats in the Control group, respectively (176 ± 50.1 mg/dL versus 106.5 ± 5.2 mg/dL, p=0.03 for males and 130.5 ± 8.1 mg/dL versus 92.2 ± 7.3 mg/dL, p<0.01, for females). Similar significant differences between rats in the TCS group and rats in the Control group were found for glucose serum concentrations (192 ± 55.7 mg/dL versus 117.7 ± 17.2 mg/dL, p=0.04, for males and 175 ± 38.8 mg/dL versus 107.7 ± 9.46 mg/dL, p=0.01, for females).

Triglycerides serum concentrations were not different between groups, either for males or females.

Quantitative Real-Time (qRT)-PCR

Differential mRNA expression of key regulator genes of the appetite regulatory network was measured by qRT-PCR. Expression of Npy and Agrp were significantly increased in the hypothalami of male rats in the TCS group compared to males in the Control group at 2 months of age, while this difference was significant in females at 8 months of age (Fig. 2A and B, p<0.05). Expression of Pomc was inhibited in the hypothalami of male and female rats in the TCS group compared to rats in the Control group at 2 months of age. However, this difference was not significant because the high variability between samples. Pomc had a tendency to decrease in the hypothalami of females in the TCS group compared to females in the Control group at 8 months of age (Fig. 2C, p=0.09).

Discussion

The concept of developmental programming involves the exposure of the rapidly growing fetuses to perturbations of the maternal milieu, resulting in programmed changes in organ structure, cellular responses and gene expression that impact metabolism and physiology of the offspring. While some perturbations lead to immediate effects, others are deferred and alterations in the organ function occur at a later age, as results of epigenetic modifications [16]. These epigenetic changes could be associated with the increased use of manmade chemicals that may interact with other factors influencing fetal and postnatal growth and even contribute to the etiology of obesity

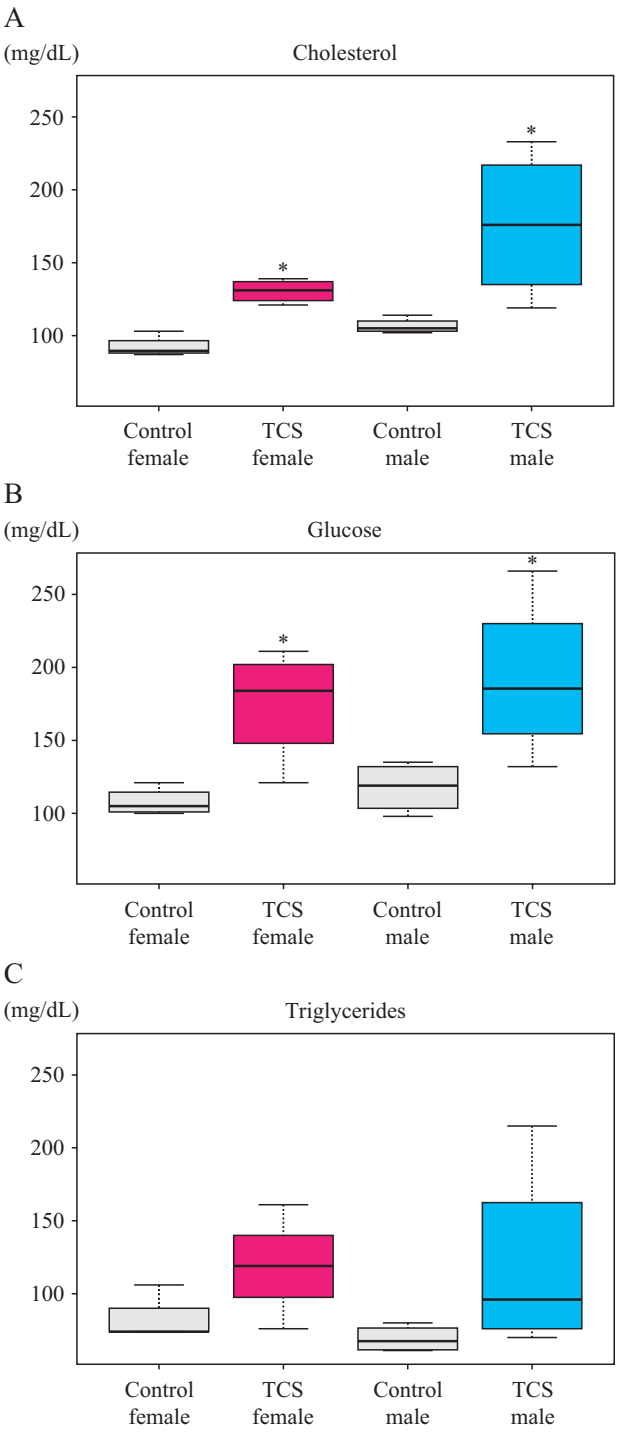

Fig. 1 Maternal Triclosan (TCS) intake increases offspring serum metabolites.
The boxplots show the serum levels (mg/dL) of (**A**) cholesterol, (**B**) glucose and (**C**) triglycerides in males and females rats at 8 months of age in the TCS or Control groups. * Significant differences between metabolite levels in rats in the TCS group compared to the Control group (p<0.05).

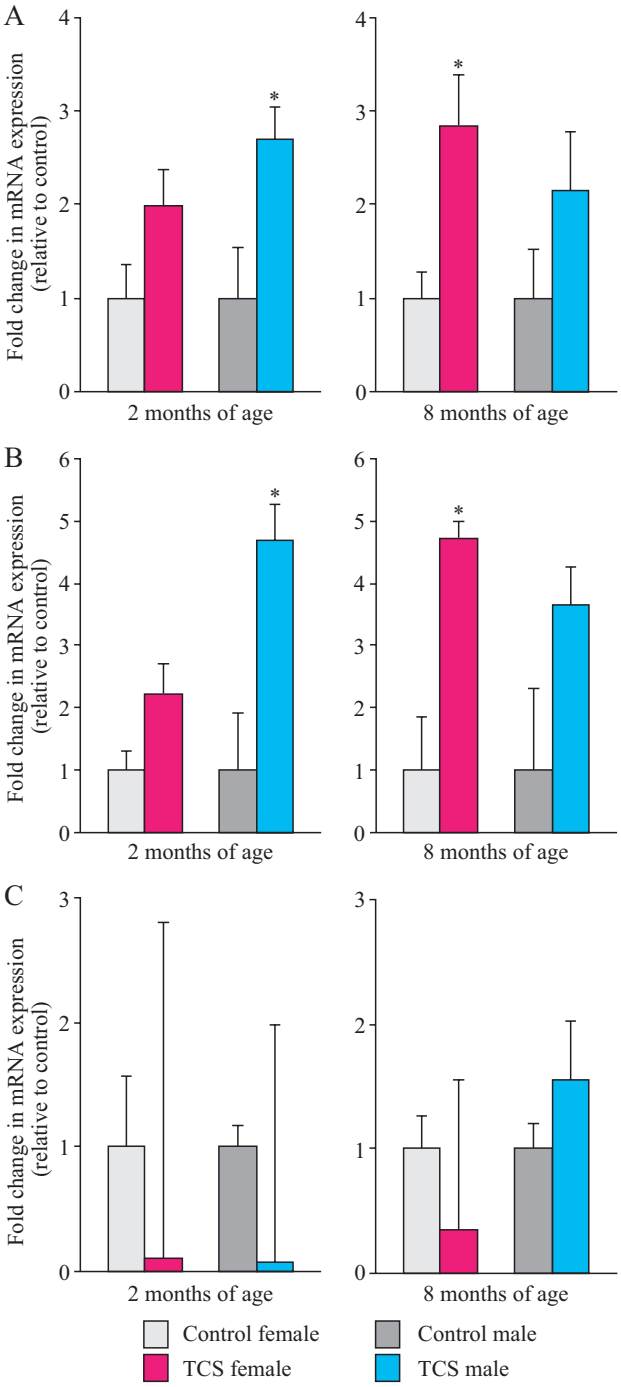

Fig. 2 mRNA expression measured by qRT-PCR in the hypothalami of rats after maternal Triclosan (TCS) intake.
mRNA expression for each group is shown as fold change ± SE with respect to the controls at 2 months and 8 months of age for: **A**) Neuropeptide Y (Npy); **B**) agouti-related protein (Agrp) and **C**) proopiomelanocortin (Pomc). * Statistically significant difference ($p<0.05$) in mRNA expression between TCS and Control group RNA expression.

once the individual reaches the adult age [17]. TCS has been a constituent of personal care products since the late 1960s, including toothpastes, mouthwashes, deodorant and antibacterial soaps, deodorants, cosmetics, and antiseptics; with the primary routes of exposure being oral and dermal [13].

On this study, Wistar rats were orally exposed to TCS during the last stage of gestation and lactation, coincident with the neuronal development of the appetite regulatory network [15]. As rats represent an altricial species, the period of lactation has been correlated with the third trimester of human gestation. We found that low doses of oral TCS consumption by the pregnant and lactating dam alter the metabolic status of the spring during adulthood, on a way that resembles development of metabolic syndrome (hyperphagia, increased body weight, hypercholesterolemia and hyperglycemia).

The observed effects differed in male and female rats: significant increased body and liver weight was measured only in the male offspring in the TCS group compared to males in the Control group at 4-5 months of age, while body weight was significantly increased in females offspring in the TCS group at 8 months of age. However, significant hypercholesterolemia and hyperglycemia was measured in both genders in the TCS group compared to rats in the Control group at 8 months of age. Liver weight was significantly increased in males in the TCS group compared to males in the Control group at 4 months of age and numerically (but not significant) for males and females at 8 months of age. As we can see in Fig. 3, the increased liver weight could be a consequence of lipid infiltration in the organ as well as higher deposition of perivisceral fat than rats in the TCS group. Higher fat deposition could result in hypercholesterolemia and insulin resistance. Although it was not possible on this study to measure insulin levels in the serum samples, the hyperglycemia observed at 8 months of age may reflect insulin resistance. Hypercholesterolemia and hyperinsulinemia/hyperglycemia have been reported in rats that suffered intrauterine growth restriction and developed metabolic syndrome during adulthood [18]. Also, TCS has been shown to inhibit the sulfotransferase enzyme SULT1E1, responsible for the sulfoconjugation of estrogens to estrogen sulfate [19]. Estrone sulfate is a potent antihyperglicemic compound that normalized glucose levels in the obese/diabetic *ob/ob* mouse [20]. Therefore, the hypergly-

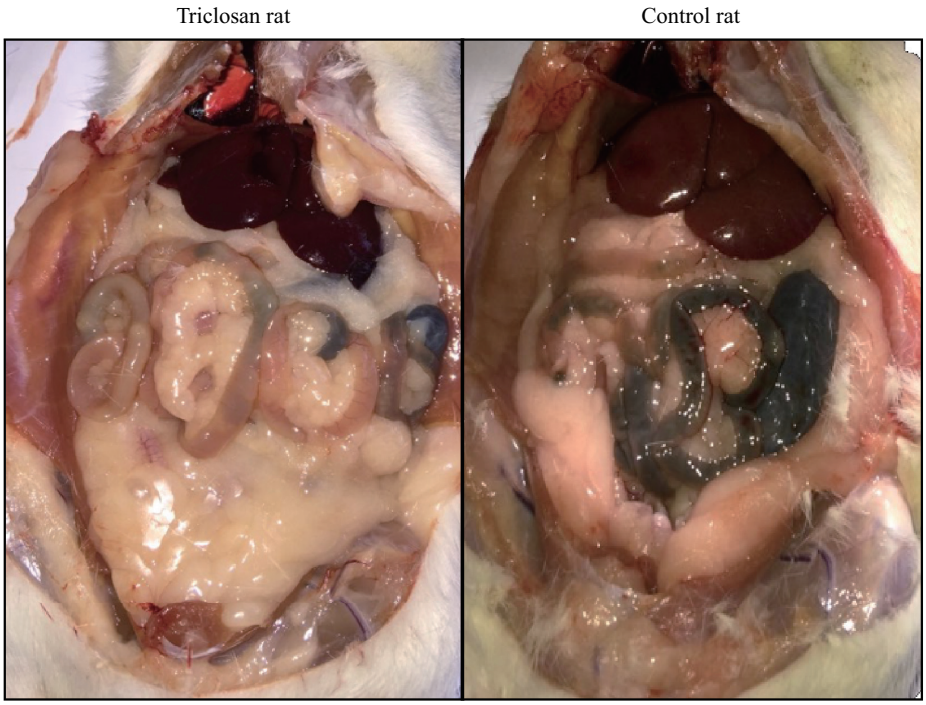

Fig. 3 Triclosan (TCS) increases deposition of perivisceral fat in rats.
The pictures correspond to females at 8 months of age from the TCS or Control group.

cemia observed in rats in the TCS group could be also consequence of the lower estrone sulfate levels indirectly caused by TCS.

Differences in body weight gains between males and females may reflect the expression of genes related with food intake, as TCS also altered the hypothalamic expression of the orexigenic peptides: NPY and AGRP; and of POMC, from which derives the main anorexigenic peptide; α-MSH (reviewed in [3]). The Agrp and Npy genes, encoding for AGRP and NPY, are co-expressed in the arcuate neurons of the hypothalamus [21]. These neurons are adjacent to those that express the Pomc gene [22]. On this study, we showed that mRNA expression of the orexigenic neuropeptides was increased in males' hypothalami in the TCS group at 2 months of age, and in females' hypothalami at 8 months of age.

Several metabolic and hormonal players regulate synthesis and release of the orexigenic neuropeptides. Estradiol is a steroidal hormone that inhibits NPY and AGRP release and so it has anoretic properties [23, 24]. This effect is mediated by the novel Gq-coupled membrane estrogen receptor and by estrogen receptor alpha, presented in the orexigenic neurons [25]. Wistar rats reach puberty around 30-40 days and pubertal brain maturation is necessary for the estrogenic inhibition of eating [26]. Thus, we speculate that food intake around the month of age was inhibited for rats in the TCS group, as TCS affected the expression of the orexigenic neuropeptides, resulting in lower body weight at 2 months of age for rats in the TCS group compared to rats in the Control group. However, food intake tended to increase at 2 months of age for males in the TCS group; corresponding with the overexpression of the orexigenic neuropeptides (Fig. 2A and B) measured at this age. In consequence, male rats in the TCS group were significant heavier than male rats in the Control group at 4 months of age. For female rats in the TCS group, overexpression of the orexigenic neuropeptides was observed later in life compared to rats in the Control group (Fig. 2A and B), resulting in heavier body weight at 8 months of age (Table 2).

Therefore, results found on this study suggest that oral TCS exposure during pregnancy could potentially affect the offspring metabolism, although the mechanism of action is unknown. Other studies have shown that exposure to TCS during pregnancy and lactation is associated with neonatal hypothyroxin-

emia [11, 27]. On this study, the physiology of the hypothalamus-pituitary-thyroid (HPT) axis was not evaluated. However, the observed overexpression of Npy and Agrp might be correlated with hypothyroidism, since both neuropeptides have a central inhibitory role in the HPT axis [28, 29]. Subclinical hypothyroidism is not necessarily associated with development of metabolic syndrome [30, 31] but it might be part of the metabolic dysfunctions programmed by TCS. Also, TCS could be an estrogenic endocrine disrupting chemical with a mode of action similar to BPA: disturbing processes controlled through steroid receptors, particularly estrogen receptor alpha [32]. In fact, it has been reported that mice exposed to a low dose of BPA (10 μg/kg/day) during fetal life were heavier at 4 months relative to controls, and had decreased insulin sensitivity and glucose intolerance [33].

On the present study we used a low dose of TCS intake (1 mg of TCS per kg of ground food). This dose was chosen to mimic human exposure to TCS through personal care products. As mentioned above, oral intake is one of the primary routes of exposure [13]. According the urinary TCS concentrations from the National Health and Nutrition Examination Survey (2.4 to 3,790 μg/L [34]) human daily intake is around 0.2-0.3 μg/kg/day. But, if combined consumer products use is considered, daily intake is estimated to be around 47 to 73 μg/kg/day [35]. So, an average woman of 60 kg of body weight could be daily exposed from low (12 μg) to high (4,300 μg) doses of TCS. Based on our experience, daily food intake for an adult Wistar rat of 300 grams of body weight is about 30 gm [36, 37]. Hence, the pregnant rats on this study consumed at least 30 μg of TCS daily, corresponding to low doses of TCS exposure. A limitation of this study was the inability to measure the real amount of TCS consumed by each dam in the maternal TCS group. Differences in maternal TCS intake could have resulted in the variability of the outcomes measured in the offspring. However, the overall results observed in rats in the TCS group confirmed our hypothesis that TCS exposure during gestation and lactation is indeed impairing the normal development of the orexigenic-anorexigenic neuropeptides in the offspring.

Conclusion

Results found on this study show that maternal oral intake of low doses of TCS, from 14 days of gestation to 20 days after parturition, induced an increase in body weight of the offspring from 3 months of age (significant at 4 months in males and 8 months in females), higher liver weight and food intake, hyperglycemia and hypercholesterolemia during adulthood and increased expression of Npy and Agrp genes. Results differed between males and females, but both genders were affected.

In summary, this study is demonstrating that TCS, similar to BPA, could be an endocrine disruptor affecting the developing fetus and leading to health issues in the adulthood. The chosen dose of TCS exposure could be daily attained by the pregnant woman. Thus, more investigations are needed to understand the potential adverse health effects of TCS exposure in humans and to unravel its mechanisms of action in the exposed fetus.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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