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

María Belén Rabaglino¹, ²), María José Moreira-Espinoza³), Juan Pablo Lopez³), Nestor Horacio García³) and Dante Beltramo¹), ⁴)

¹) Centro de Excelencia en Procesos y Productos de Córdoba (CEPROCOR), CONICET, Córdoba, Argentina
²) Dpto. Reproducción Animal, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina
³) Instituto de Investigaciones en Ciencias de la Salud (INICSA), CONICET, Córdoba, Argentina
⁴) Cátedra de Biotecnología, Facultad de Ciencias Químicas, Universidad Católica de Córdoba, Córdoba, Argentina

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

©The Japan Endocrine Society
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 than 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 20°C. 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 time, 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 -80°C 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 -20°C 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 hypothalam using Trizol reagent (Thermo Scientific) according manufacturer protocol. Isolated RNA concentration was determined with Nanodrop, and RNA was stored at -80°C 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 -20°C 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>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npy</td>
<td>TGATGCTAGGTAACAAACGAATGG</td>
<td>GCCAGAAATGCCCCAAACACA</td>
<td>NM_012614</td>
</tr>
<tr>
<td>Agrp</td>
<td>GCAGAGGGTGCTAGATCCACAGA</td>
<td>GGACCTCGTGCAGCCTACACA</td>
<td>NM_033650</td>
</tr>
<tr>
<td>Pomc</td>
<td>TCTCGCTTCAGACCTCCATAGAC</td>
<td>GGATGCAAGCCAGCAGGTT</td>
<td>NM_139326</td>
</tr>
<tr>
<td>Actb</td>
<td>TCTGTGTGGATTGGTGCCCTCTA</td>
<td>CCTGCTTGCTGATCCACATCT</td>
<td>NM_031144</td>
</tr>
</tbody>
</table>
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, \( p ≤ 0.01 \)).

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).

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

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Females</th>
<th></th>
<th></th>
<th>Males</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>TCS group</td>
<td>p-value</td>
<td>Control group</td>
<td>TCS group</td>
<td>p-value</td>
</tr>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>2</td>
<td>259.8</td>
<td>14.0</td>
<td>274.0</td>
<td>15.4</td>
<td>0.266</td>
<td>353.8</td>
</tr>
<tr>
<td>4</td>
<td>310.0</td>
<td>25.6</td>
<td>328.1</td>
<td>24.5</td>
<td>0.170</td>
<td>451.3</td>
</tr>
<tr>
<td>6</td>
<td>362.5</td>
<td>26.3</td>
<td>393.8</td>
<td>7.5</td>
<td>0.062</td>
<td>486.3</td>
</tr>
<tr>
<td>8</td>
<td>305.0</td>
<td>17.3</td>
<td>378.8</td>
<td>16.5</td>
<td>&lt;0.01 *</td>
<td>557.5</td>
</tr>
</tbody>
</table>

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.
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 peri-visceral 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 antihyperglycemic compound that normalized glucose levels in the obese/diabetic ob/ob mouse [20]. Therefore, the hypergly-

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.
Triclosan affects offspring metabolism

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 hypothyroxi-
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. 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.

Acknowledgements

This work was supported by FONCyT grant PICT 2827/13.

Disclosure

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

References

Triclosan affects offspring metabolism


