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REVIEW ARTICLE

Critical analysis of endocrine disruptive activity of triclosan and its relevance to human exposure through the use of personal care products

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Abstract

This review examines the mammalian and human literature pertaining to the potential endocrine disruptive effects of triclosan (TCS). Dietary exposure to TCS consistently produces a dose-dependent decrease in serum thyroxine (T4) in rats without any consistent change in TSH or triiodothyronine (T3). Human studies reveal no evidence that the TCS exposure through personal care product use affects the thyroid system. TCS binds to both androgen and estrogen receptors in vitro with low affinity and evokes diverse responses (e.g., agonist, antagonist, or none) in steroid receptor transfected cell-based reporter assays. Two of three studies in rats have failed to show that TCS exposure suppresses male reproductive function in vivo. Three of four studies have failed to show that TCS possesses estrogenic (or uterotrophic) activity in rats. However, two studies reported that, while TCS lacks estrogenic activity, it can amplify the action of estrogen in vivo. The in vitro, in vivo, and epidemiologic studies reviewed herein show little evidence that TCS adversely affects gestation or postpartum development of offspring. Furthermore, previously reported toxicity testing in a variety of mammalian species shows little evidence that TCS adversely affects thyroid function, male and female reproductive function, gestation, or postpartum development of offspring. Finally, doses of TCS reported to produce hypothyroxinemia, and occasional effects on male and female reproduction, gestation, and offspring in animal studies are several orders of magnitude greater than the estimated exposure levels of TCS in humans. Overall, little evidence exists that TCS exposure through personal care product use presents a risk of endocrine disruptive adverse health effects in humans.

Keywords

androgen receptor, estradiol, estrogen receptor, female reproduction, gestation, Leydig cells, male reproduction, postpartum development, testosterone, thyroid, thyroxine, triiodothyronine, TSH, uterotrophic effects

History

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Introduction

Triclosan (TCS, 2,4.4′-trichloro-2′-hydroxy-diphenyl ether) is a biphenyl compound linked together by an oxygen as an ether. One benzene ring possesses two chlorines positioned at meta to one another, whereas the other benzene ring possesses a single chlorine at the meta position to an hydroxyl group (Figure 1). By virtue of its antimicrobial (antibacterial and/or antifungal) properties, TCS has been a constituent of personal care products since the late 1960s. Among these products are toothpastes, mouthwashes, deodorant and antibacterial soaps, antiperspirants and deodorants, cosmetics, and antiseptics with the primary routes of exposure being oral and dermal. TCS is also present in a number of other consumer products such trash bags, clothes, bedding, toys, and kitchen utensils (Rodricks

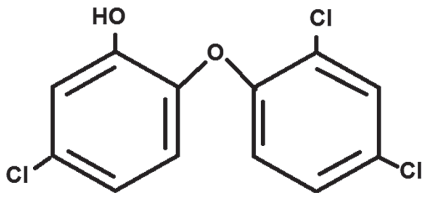

Figure 1. Chemical structure of triclosan.

et al. 2010, Witorsch and Thomas 2010). As a result of its wide exposure through consumer products, TCS is detectible in nanomolar (nM) concentrations in human body fluids, such as urine, serum (1–55 nM) (Rodricks et al. 2010, Allmyr et al. 2008, Honkisz et al. 2012), and breast milk (1–120 nM) (Allmyr et al. 2006a, Dayan 2007). Estimates of daily human intakes of TCS are divergent depending upon the model employed. Based upon the urinary TCS concentrations from the National Health and Nutrition Examination Survey (NHANES) survey of 2003 and 2004 (range: 2.4–3,790 μg/L) (Calafat et al. 2008), mean daily intakes have been estimated to be 0.2–0.3 μg/kg/day, whereas daily intakes based upon combined consumer product use is estimated to be about two orders of magnitude higher (47–73 μg/kg/day) (Rodricks et al. 2010). The most recent NHANES survey of 2011–2012 suggests a similar range of urinary TCS concentrations (1.63–3,830 μg/L) (CDC 2013).

As will be discussed in subsequent sections of this review, recent laboratory studies have addressed the issue of whether TCS affects the endocrine system particularly with regard to thyroid function, male and female reproduction, and postpartum development. Since the early 1990s, the field of endocrine disruption, that dealing with the interference of the endocrine system by environmental chemicals, has been gaining considerable attention (Witorsch and Thomas 2010). Because the endocrine system represents a major mode of communication within the body via hormones, environmental chemicals can potentially interfere with reproduction and normal development, as well as physiological functions, among these neural function, metabolism, growth, fluid balance, and cardiovascular function. Environmental chemicals can interfere with the endocrine system in a number of ways, such as interacting with a target cell via hormone receptors, affecting hormone secretion or clearance, and interfering with feedback relationships that exist in the thyroid, gonadal, and adrenocortical systems. The aspect of endocrine disruption that has received the most attention has been the interaction between a xenobiotic and the estrogen receptor (ER). The promiscuity of the ER is well known, as it can interact with a diverse array of chemicals (e.g., polychlorobiphenyls or PCBs, bisphenol A, flavonoids, alkylphenols, DDT derivatives, and kepone) and evoke an agonistic and/or antagonistic response in vitro and in vivo. With rare exception, the interaction between xenoestrogens and binding pocket of the ER is imperfect, which compromises the binding affinity of the ligand. As a result, high exposures to xenoestrogens are usually required to evoke an endocrine disruptive effect through this pathway (Witorsch 2002). This promiscuity is shared by receptors for other steroid hormones, as well (Eick et al. 2012).

Several incidents have sparked interest in endocrine disruption as a public health issue. Among these is the phenomenon of "clover disease", impaired fertility in livestock linked to the consumption of grasses containing flavonoids. Other incidents of endocrine disruption have been the observation of reproductive abnormalities in wildlife that reside in or near bodies of water polluted with such chemicals as DDT derivatives, PCBs, and dioxins, and the existence of reproductive abnormalities in human infants associated with in utero exposure to the synthetic estrogen, diethylstilbestrol (Witorsch 2002). These and other episodes, laboratory reports, and public pressure led to incorporation of endocrine disruption into the Food Quality Protection Act and Safe Drinking Water Act in 1996. As a consequence of this legislation, the Environmental Protection Agency has initiated a two-tiered program, the Endocrine Disruptor Screening Program (EDSP) to identify and characterize environmental chemicals as endocrine disruptors. Tier 1 is a battery of 11 in vitro and in vivo assays currently in use designed to identify chemicals with estrogen, androgen, and thyroid hormone agonist/antagonist activities. Tier 2, which is currently under development, will be a battery of assays intended to establish a dose-range for adverse effects of chemicals identified by Tier 1 (Witorsch 2002, Endocrine Disruptor Screening Program 2013).

Another resource for gaining insight regarding endocrine disruption is already available in the literature. The purpose of the current review is to provide an objective and critical analysis of the laboratory data in mammals and humans, as well as any published epidemiologic data that deals with the relationships between the TCS exposure and endocrine-related endpoints, in the current case the thyroid system, male and female reproduction, gestation, and postpartum development. The ultimate goal of this review is to determine whether the exposure to TCS through the use of personal care products poses an adverse health risk to humans through endocrine disruption. Some controversy has existed in the appropriate definition of the term "endocrine disruptor." In particular, the controversy pertains to the broadness of this definition, whether the term applies to substances that interact with the endocrine system, but do not necessarily produce adverse effects or is restricted to those substances that produce *adverse effects* as result of their endocrine interaction (EFSA 2013). Most national agencies comply with the more restrictive definition proposed by the World Health Organization (WHO 2002) in which an endocrine disruptor is defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or sub(populations)" (EFSA 2013). In the current assessment of the literature, both the WHO definition and the existence of endocrine activity per se will be taken into consideration. In order to arrive at a conclusion as to whether the TCS exposure through personal care product use poses a health risk to humans through endocrine disruption, the Hill criteria of causation served as a guideline for evaluating the available evidence. Among the most important criteria considered were strength and consistency of association, dose-dependency, temporality, plausibilty, and coherence (Hill 1965).

Studies considered in this review were those dealing with the relationship between the exposure to TCS and endocrine-related endpoints in animals and humans, both in vitro and in vivo. Scholarly articles evaluated in this review were identified from a variety of sources. A search was conducted in the PubMed database using the term "triclosan" cross-referenced

with the a variety of other search terms, namely "endocrine", "endocrine disruptor", "thyroid", "male reproduction", "testes", "female reproduction", "ovary", and "gestation". The search was conducted from January 2009 through April 2013. Citations were also provided from the sponsor of this study, Colgate Palmolive Company. Relevant literature was also available from the work done on a previously published review article on endocrine disruption and personal care product constituents (Witorsch and Thomas 2010). Additional citations were identified as a result of examination of individual studies as well as from other assessments of the literature (SCCP 2009, SCCS 2011, Health Canada 2012, Rodricks et al. 2010).

Effects of TCS on thyroid function

In Vitro studies of TCS effects on thyroid function

Three recent in vitro studies have explored the effects of TCS on the thyroid system. Butt et al. (2011) examined the effect of TCS on deiodinase (DI) activity in a cell-free system (human liver microsomes) using thyroxine (T4) as a substrate. A dose-dependent inhibition of DI activity was observed with an IC_{50} of over 300 μM. Hinther et al. (2011) examined the effect of TCS in the presence or absence of triiodothyronine (T3), on the expression of thyroid hormone responsive genes using a mammalian culture system (rat pituitary tumor GH3). TCS (1–1000 nM) failed to affect gene expression in this system (with or without T3). Methyl TCS, a major bacterial metabolite of TCS, inhibited T3-induced expression in rat pituitary GH3 cells but only at higher concentrations (1000 nM). Sankoda et al. (2011) examined thyroid hormone activity of TCS and its oxidative products in yeast cells transfected with thyroid hormone receptors. Oxidative products of TCS were generated by exposure of an aqueous solution of TCS to UV light. TCS and its oxidative products were tested for thyroid hormone activity with this yeast-based assay in the presence and absence of metabolic activation by S9 (rat liver preparation). In the absence of S9, TCS failed to exhibit activity, whereas in the presence of S9, TCS and its oxidative products, tetraclosan and 2,4 dichlorophenol, exhibited weak thyroid hormone activity (0.05% or less relative to T3).

In view of the fact that the assay systems employed in the above studies (i.e., cell free, neoplastic cells, or yeast based) differ from the in vivo situation and, at best, a weak endocrine-related activity is observed (i.e., requiring high concentrations of TCS), these observations are of limited value in determining whether TCS is an endocrine disruptor in vivo. Furthermore, as will be evident from the discussion in next section, these observations have little or no relevance to effects of TCS on thyroid function observed in vivo in rats.

In Vivo studies: TCS-induced decrease in serum T4 in rats

Based upon the fact that TCS and thyroid hormone are both halogenated biphenyl ethers, and the existence of some data suggesting that the former might promote hepatic catabolism of the latter, Crofton et al. (2007) initiated a series of studies examining the effect of TCS on thyroid function in rats. These investigators administered TCS to weanling Long Evans female rats (27–29 days old) through oral gavage at varying doses (0, 10, 30, 100, 300, and 1000 mg/kg/day; n = 8–16 per dose) for 4 days. Rats were sacrificed the following day by decapitation. TCS produced a dose-dependent decrease (28–58%) in serum total T4 between 100 and 1000 mg/kg/day. With the aid of USEPA dose software, Crofton et al. (2007) estimated the benchmark dose (BMD) and lower confidence limit (BMDL) for changes in serum T4 according to the Hill Model fit of the data. The BMD is a prediction of the dose of TCS that produces a 20% decrement in serum T4, while the BMDL predicts the 95% lower confidence interval of this effect. The BMD and BMDL were estimated at 69.7 mg/kg/day and 35.6 mg/kg/day, respectively. No other indices of thyroid function (e.g., serum TSH or T3) were measured (Table 1).

A subsequent study from the same research group included animals from the preceding study, and eight additional animals per group (Paul et al. 2010a). This study confirmed a TCS-induced dose-dependent decline in serum total T4 (26–57%) between TCS doses of 100 and 1000 mg/kg/day (BMD and BMDL: 99.4 and 65.6 mg/kg/day, respectively). In addition, a decrease was observed in serum T3 levels of 11 and 25% at 300 and 1000 mg/kg/day of TCS, respectively, and no change in serum TSH was observed associated with this decrease in serum thyroid hormone levels (Table 1).

The effect of TCS exposure for periods longer than 4 days on thyroid function has also been examined. Zorrilla et al. (2009) administered TCS through oral gavage (0, 3, 30, 100, 200, and 300 mg/kg/day) to weanling Wistar males rats for 31 days, from postnatal day (PND) 23 to 53. Serum T4 decreased dose dependently from 30 to 300 mg/kg/day of TCS, while no effect was observed at 3 mg/kg/day. Decrements in T4 were 45–50% at 30 and 100 mg/kg/day and about 80% at 200 and 300 mg/kg/day (BMD and BMDL were estimated to be 14.5 and 7.2 mg/kg/day, respectively). Serum T3 exhibited an erratic pattern, being significantly increased at 3 mg/kg/day and significantly decreased at 200 mg/kg/day. In both cases the deviations from the control level were no more than ± 25%. This apparent erratic pattern in serum T3 may have been attributed to a design artifact in the experiment, because TCS was administered to two different blocks of animals. One block involved controls, and doses of 3, 30, and 300 mg/kg/day of TCS, whereas the second block involved controls and doses of 100 and 200 mg/kg/day. As in the preceding study, no significant changes in serum TSH were observed at any level of TCS exposure. Histological examination of the thyroid gland revealed minimal, if any, effects. No changes were observed in thyroid epithelial cell height at any TCS dose level, while it was noted that there was a "significant" decrease in thyroid colloid (described as "depletion") at the 300 mg/kg/day-dose level, although no data or images were presented (Table 1).

Stoker et al. (2010) employed two basic EDSP protocols to explore the effects of TCS on thyroid function in weanling Wistar female rats, the pubertal assay (21-day treatment) and the uterotrophic assay (3-day treatment). In the pubertal design, rats received TCS through oral gavage (0, 9.375, 37.5, 75, or 150 mg/kg/day) from PND 22 to 42. Total serum T4 decreased dose dependently (by about 40–70%) from doses ranging from 37.5 to 150 mg/kg/day. Free T4 (fT4), the bioavailable component, was decreased at 75 and 150 mg/kg/day by 70–80%. Serum T3 was not measured in this study. No effect of TCS was observed on serum TSH. With the uterotrophic design, females were treated through oral gavage with varying doses of TCS (0, 1.18, 2.34, 4.69, 9.375, 18.75, 37.5, and 75 mg/kg/day) from

Table 1. Effects of TCS on thyroid system hormone levels and related endpoints in rats.

Study	Experimental design	Results
Crofton et al. (2007)	Long Evans (LE) females, PND 27 to PND 29, TCS (0, 10, 30, 100, 300, 1000 mg/kg/day) × 4 days, oral gavage.	**Serum T4** decreased 28 to 58% between 100 and 1000 mg/kg/day BMD = 69.7 mg/kg/day BMDL = 35.6 mg/kg/day
Paul et al. (2010a)	LE females, PND 27 to PND 29, TCS (0, 10, 30, 100, 300, 1000 mg/kg/day) × 4 days, oral gavage	**Serum T4** decreased 26 to 57% between 100 and 1000 mg/kg/day BMD = 99.4 mg/kg/day BMDL = 65.6 mg/kg/day **Serum T3** decreased 11 and 25% at 300 and 1000 mg/kg/day, respectively **Serum TSH** no change (NC)
Zorrilla et al. (2009)	Wistar (W) males, TCS (0, 3, 30, 100, 200, 300 mg/kg/day) PND 23 to PND 53, oral gavage	**Serum T4** decreased 45 to 80% between 30 and 300 mg/kg/day BMD = 14.5 mg/kg/day BMDL = 7.2 mg/kg/day **Serum T3** no consistent pattern **Serum TSH** NC **Thyroid histology** minimal effects
Stoker et al. (2010)	W females, TCS (0, 9.375, 37.5, 75, 150 mg/kg/day) PND 22 to PND 42, oral gavage (pubertal design)	**Serum T4** decreased 40 to 70% between 37.5 and 150 mg/kg/day **Serum fT4** decreased 70 to 80% between 75 and 150 mg/kg/day **Serum TSH** NC
	W females, TCS (0, 1.18, 2.34, 4.69, 9.375, 18.75, 37.5, 75 mg/kg/day) PND 19 to PND 21, oral gavage (uterotrophic design)	**Serum T4 and fT4** decreased 20 to 40% between 18.75 and 75 mg/kg/day
Paul et al. (2010b)	LE adult females, TCS (0, 30, 100, 300 mg/kg/day) GD 6 to PND 21, oral gavage	**Dams:** *PND 22*, **serum T4** decreased 30% at 300 mg/kg/day BMD = 229 mg/kg/day BMDL = 104 mg/kg/day **Pups:** *PND 4*, **serum T4** decreased 27% at 300 mg/kg/day BMD = 113 mg/kg/day BMDL = 58 mg/kg/day *PND 14 and PND 21,* **serum T4** NC
Paul et al. (2012)	LE adult females TCS (0, 10, 30, 100, 300 mg/kg/day) GD 6 to PND 21, oral gavage	**Dams:** *GD 20*, **serum T4** decreased 30% at 300 mg/kg/day BMD = 124 mg/kg/day BMDL = 25.2 mg/kg/day **Serum T3 and Serum TSH** NC *PND 22,***serum T4** decreased 15 and 30% at 100 and 300 mg/kg/day, respectively BMD = 115 mg/kg/day BMDL = 62.1 mg/kg/day **Serum T3** and **Serum TSH** NC **Pups:** *GD 20*, **serum T4** decreased 23 and 28% at 100 and 300 mg/kg/day, respectively BMD - 95.4 mg/kg/day. BMDL = 33 mg/kg/day. *PND 4*, **serum T4** decreased 26% at 300 mg/kg/day BMD = 150 mg/kg/day BMDL = 61.8 mg/kg/day **Serum TSH** NC *PND 14, 21*, **serum T4, T3, and TSH** NC
Axelstad et al. (2013)	W adult females, TCS (0, 75, 150, 300 mg/kg/day) GD 7 to PND 16, oral gavage	**Dams:** *GD 15*, **serum T4** decreased 59 to 72% between 75 and 300 mg/kg/day *PND 16*, **serum T4** decreased 38 to 72% between 75 and 300 mg/kg/day, **thyroid weight or histology** no effect **Pups:** *PND16*, **serum T4** NC, **thyroid weight or histology** no effect.
	W pups (not treated with TCS in utero), TCS (0, 50, 150 mg/kg/day) from PND 3 to PND 16, oral administration	*PND 16*, **serum T4** decreased dose-dependently (16 and 37%)
Rodriguez and Sanchez (2010)	W adult females, TCS (0, 1, 10, 50 mg/kg/day) 8 days pre-gestation to PND 21 in drinking water	**Dams:** *GD 5 to GD 20, PND 5 to PND 20*, **serum T4** decreased 20 and 30% at 10 and 50 mg/kg/day, respectively **Serum T3** no consistent pattern, some modest suppression

PND 19 to PND 21 and were sacrificed 6 h after the final dose. Serum total T4 and fT4 were decreased by about 20–40% at TCS doses ranging from 18.75 to 75 mg/kg/day (Table 1).

In addition to the preceding studies of TCS effects on thyroid function of pre-pubertal and developing male and female rats, several studies have examined the effects of TCS exposure during gestation and post-partum on thyroid function of adult female rats and their offspring. Paul et al. (2010b) gave varying doses of TCS (0, 30, 100, and 300 mg/kg/day) through oral gavage to Long Evans adult female rats from gestational day (GD) 6 to PND 21. Serum was obtained after decapitation for the measure-

ment of total T4 in dams at PND 22 and in offspring on PND 4, 14, and 21. Total T4 decreased by about 30% in PND 22 dams fed with 300 mg/kg/day TCS. No significant decrease in total T4 was observed in dams at other dose levels (BMD and BMDL estimated at 229 and 104 mg/kg/day, respectively). In pups, the effect on serum T4 was transient and only observed at the 300 mg/kg/day dose level. On PND 4, serum T4 was decreased by 27% (BMD and BMDL were 113 and 58 mg/kg/day, respectively) with no change in serum T4 in pups at PND 14 and PND 21 (Table 1). Paul et al. (2010b) suggest that the transient nature of the T4 response in offspring (decrease at PND 4 with recovery at later postnatal ages) is unique when it comes to perinatal maternal exposure to xenobiotics that disrupt the thyroid system. Maternal exposure of offspring to other substances, such as polybrominated diphenyl ethers, polychlorinated biphenyls, and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), during gestation and lactation produce effects on the thyroid system that persist throughout the exposure period (Paul et al. 2010b). They further suggest that transiency of the T4 response to TCS in offspring is due to toxicokinetic and/or toxicodynamic factors, such as a limitation of maternal transfer of TCS to the pup through breast milk, a transient residual in utero effect of transplacental exposure to TCS, and/or the development by the neonate of resistance to TCS effects by the thyroid system (Paul et al. 2010b).

Paul et al. (2012) extended the preceding study of TCS on thyroid function in dams and offspring to obtain additional data, among these the additional time points as well as levels of serum T3 and TSH. Long Evans adult female rats were given varying doses of TCS (0, 10, 30, 100, and 300 mg/kg/day) through oral gavage from GD 6 to PND 21. Serum and livers were collected from dams at GD 20 and PND 22 and from offspring at GD 20, PND 4, PND 14, and PND 21. In TCS-exposed GD 20 dams, serum T4 decreased 30% at 300 mg/kg/day (BMD and BMDL estimated at 124 and 25.2 mg/kg/day, respectively). A 15% and 30% decrease in serum T4 was also observed in PND 22 dams at 100 and 300 mg/kg/day, respectively (BMD and BMDL estimated at 115 and 62.1 mg/kg/day, respectively). In GD 20 fetuses, TCS exposure reduced serum T4 by 23 and 28% at 100 and 300 mg/kg/day, respectively. The BMD and BMDL for TCS-induced decrease in serum T4 were estimated to be 95.4 and 33 mg/kg/day, respectively. In PND 4 pups serum T4 decreased 26% at a dose of TCS of 300 mg/kg/day with a BMD and BMDL of 150 and 61.8 mg/kg/day, respectively. No TCS-induced change in serum T4 was observed in pups at PND 14 and PND 21. Overall these data on serum T4 are consistent with the data of the preceding study (Paul et al. 2010b), with a decline in serum T4 in dams as well as a transient decline in serum T4 in offspring after exposure to high doses of TCS. No effect of TCS was observed on serum T3 in dams at GD 20 and PND 22 or pups at PND 14 or PND 21 (T3 was not measured in GD 20 and PND 4 offspring due to limited volumes of serum). No effect of TCS exposure was observed on serum TSH levels in GD 20 and PND 22 dams and pups at PND 4, PND 14, and PND 21 (GD 20 fetuses were not examined due to the limited amount of serum) (Table 1).

Paul et al. (2012) also measured total TCS (parent and conjugated) concentrations in serum and liver from control and TCS-exposed animals. At a dose of 300 mg/kg/day, total serum TCS concentrations were similar in magnitude (20–30 μg/ml) for GD 20 dams, PND 22 dams, GD 20 fetuses, and PND 4 pups, but declined markedly in PND 14 and PND 21 pups (50% and 95%, respectively). Total TCS content in the liver of these animals exhibited a pattern among dams and offspring similar to that of total serum TCS, with relatively high levels in dams and early offspring that declined in older pups (GD 20 and PND 22 dams $>$ GD 20 fetus and PND 4 pups $>$ PND 14 pups $>>$ PND 22 pups). Paul et al. (2012) suggest that the pattern of total serum and hepatic levels of TCS in dams and offspring reflect the route of exposure to TCS and, as a consequence, determine the pattern of T4 in dams and offspring. Relatively high concentrations of total TCS in serum and liver in dams at GD 20 and PND 22 and GD 20 fetus and PND 4 pups are largely due to TCS received through gavage to the dams or delivered systemically to the fetus through the placenta, whereas the progressive decline of these levels in PND 14 and PND 21 offspring are the result of reduced and indirect TCS exposure during lactation. The TCS concentrations in serum and liver, as internal indices of TCS exposure, exhibit an inverse relationship with serum T4 concentrations in TCS-treated dams and offspring. Paul et al. (2012) also report that in TCS-treated dams (300 mg/kg/day) and offspring most of the TCS were conjugated (90%), the only exception being in PND 4 neonates where parent TCS was about 40%, suggestive of a window of retarded TCS catabolism in pups in the early postnatal period (PND 4). Whether this altered conjugation of TCS at PND 4 contributes to the transient decrease in serum T4 post-partum remains to be determined.

The effects of TCS in dams and their offspring were also examined by Axelstad et al. (2013). Wistar dams received varying doses of TCS (0, 75, 150, and 300 mg/kg/day) through gavage from GD 7 to PND 16. Total T4 in serum was measured in dams at GD 15 and PND 16 and in offspring at PND 16. Serum T4 was decreased in dams dose dependently by TCS (75–300 mg/kg/day) at both GD 15 (59–72%) and PND 16 (38–58%). The TCS-induced hypothyroxinemia in dams observed herein are consistent with previous reports of TCS-induced hypothyroxinemia in dams (Paul et al. 2010b, 2012), although the response appeared more robust in the current study. In contrast to these dose-dependent effects of TCS in dams, no significant effect of TCS was observed on serum T4 in offspring at PND 16 consistent with observations of Paul et al. (2010b, 2012). Thyroid weight in PND 16 dams and offspring were unaffected by TCS treatment and no histopathological effects in thyroid were observed in PND 16 offspring (Table 1).

In a follow-up experiment, dams were not treated with TCS while post-partum their offspring received TCS orally (0, 50, and 150 mg/kg/day) from PND 3 to PND 16 at which time offspring were sacrificed for measurement of total serum T4 (Axelstad et al. 2013). In PND 16 offspring direct oral TCS exposure produced a dose-dependent decrement in serum T4 (16 to 37%) (Table 1). Taken together, absence of effect in offspring exposed to TCS indirectly via nursing compared with the presence of an effect of TCS via direct oral exposure

supports the concept that TCS exposure through lactation is inadequate to disrupt the thyroid system in offspring. On the other hand, the authors acknowledge the possibility of artifact in the direct exposure experiment since all of the controls came from a single litter (Axelstad et al. 2013).

Rodríguez and Sanchez (2010) examined the effect of varying doses of oral TCS on thyroid function in adult female rats prior to, during, and after pregnancy. Adult female Wistar rats received varying doses of TCS (0, 1, 10, or 50 mg/kg/day) from 8 days prior to mating, through gestation, to PND 21. TCS was administered via drinking water in order to minimize the stress that might occur through forced feeding. Blood was obtained from the tail vein at GD 5, GD 10, GD 15, GD 20, and days 5, 10, 15, and 20 of lactation (PND). While offspring were also exposed to TCS, only dams were examined for thyroid function. TCS exposure decreased serum T4 at 10 and 50 mg/kg/day (20 and 30%, respectively at all time points examined, namely GD 5, GD 10, GD 15, GD 20, PND 5, PND 10, PND 15, and PND 20. Effects on serum T3 seem uncertain (Table 1). In fact, there is an apparent misstatement in the text of the paper regarding effects of TCS on serum T3. The narrative states that "Triclosan significantly increased serum T3 concentrations (Figure 3A)." However, the figure referred to shows that there are increases in serum T3 in control dams between GD 15 and PND 5, and TCS-treated rats exhibited a similar pattern. As revealed in the figure, TCS produced a modest suppression in T3 (about 10% below controls) at GD 10 (10 and 50 mg/kg/day), GD 15 (1–50 mg/kg/day), GD 20 (1–50 mg/kg/day), PND 5 (1–50), PND 10 (1–50) without effects on PND 15 or PND 20. These data suggest that the effect of TCS on serum T4 were more robust and persistent than they were for serum T3.

In all of the above studies in the rat the consistent finding is a dose-dependent TCS-induced decrease in serum T4 (hypothyroxinemia) without any change in serum TSH. When examined, no consistent effect of TCS was observed on serum T3.

Effects of TCS on hepatic clearance of T4 in rats

Based upon previous studies, among those of Hanioka et al. (1996) and Jinno et al. (1997), Crofton et al. (2007) speculated that the decreases in serum T4 associated with TCS treatment in rats result from increased clearance of the hormone via activation of Phase I (cytochrome P450) and Phase II (glucuronidation) enzymes in the liver. The link between decreased serum T4 and increased hormone clearance was tested by Zorrilla et al. (2009). As noted previously, Zorrilla et al. (2009) observed TCS-induced dose-related decreases in serum T4 (45–80%) in Wistar male rats gavaged with TCS (30–300 mg/kg/day) for 31 days (PND 23–53) (Table 1). In this study, Zorrilla et al. (2009) also measured hepatic enzyme activities in animals receiving varying doses of TCS (0, 3, 30, and 300 mg/kg/day). The enzyme activities of interest were ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD), markers for phase I enzymes (CYP1A1 and CYP2B, respectively), and uridine diphosphoglucuronosyl transferase (UGT), the phase II enzyme involved in glucuronidation. Zorrilla et al. (2009) observed the following: (1) a 4-fold increase in PROD at 300 mg/kg/day with no change at other doses of TCS tested (3 and 30 mg/kg/day); (2) a decrease in EROD activity by 40–50% at all doses of TCS; and (3) no statistically significant change in UGT activity (although mean UGT activity appeared elevated 2-fold at the 300 mg/kg/day dose) (Table 2). The authors note that the decrease in EROD activity is consistent with other reports suggesting that TCS is a competitive inhibitor of the enzyme, while the absence of EROD activation suggests that TCS is devoid of dioxin contamination [being incapable of interacting with the aryl hydrocarbon receptor (AhR)]. Finally, since the changes in hepatic enzyme activities were not correlated with dose-related changes in serum T4, the authors could not conclude that there exists evidence of a causal relationship between TCS-induced hepatic clearance and hypothyroxinemia (Zorrilla et al. 2009).

In the extension of the study by Crofton et al. (2007), Paul et al. (2010a) also explored the relationship between hypothyroxinemia and increased hepatic clearance of T4. As shown in Table 1, Paul et al. (2010a) observed a dose-dependent decrease in serum total T4 (26–57%) between TCS doses of 100 and 1000 mg/kg/day in Long Evans weanling female rats gavaged with varying doses of TCS (0, 10, 30,100, 300, and 1000 mg/kg/day) for 4 days. The activities and gene expression of Phases I and II enzymes and Phase III transporters were examined in hepatic microsomes from these animals. A non-dose–dependent decrease (26–32%) in EROD activity was observed at TCS doses of 30 mg/kg/day TCS and above. PROD activity was elevated dose-dependently, 2.5-, 8-, and 9.4-fold at TCS doses of 100, 300, and 1000 mg/kg/day, respectively, although the increase at 100 mg/kg/day dose was not statistically significant. T4-glucuronidation (UGT activity) was increased by 82% at a TCS dose of 1000 mg/kg/day but was not statistically significantly elevated at any of the lower doses. Nevertheless, the main effect on T4-UGT activity was statistically significantly related to dose of TCS (Paul et al. 2010a). The effects of TCS on cytochrome P450 (Cyp), glucuronidation (Ugt), and sulfonation (Sult) mRNA expression appeared to be isoform specific. Dose-related increases in mRNA expression were observed for Cyp2b1/2 (2-fold at 300 mg/kg/day), Cyp3a1/23 (2.5- and 4-fold at 100 and 300 mg/kg/day, respectively), Ugt1a1 (1.7- and 2.3-fold, 100 and 300 mg/kg/day, respectively), and Sult1c1 (2.5-fold at 100 and 300 mg/kg/day). On the other hand, no change was observed for Cyp1a1, Ugt1a6, Ugt 2b5, and Sult1b1. Furthermore, no changes were observed for hepatic transporter mRNA expression, namely Oatp1a1 (Oatp1), Oatp1a4. (Oatp2), Mrp2, or Mdr1b (Paul et al. 2010a; Table 2).

Primarily on the basis of the TCS effects on T4-UGT activity and the expression of specific Cyp, Ugt, and Sult isoforms, Paul et al. (2010a) conclude that TCS-induced hypothyroxinemia was the result of increased hepatic catabolism of T4 due to the upregulation of Phases I and II enzymes, but not transporters for iodothyronines. The particular mRNA isoforms of Cyp (e.g., 2b1/2 and 3a1/23) and Ugt (Ugt1a1) stimulated by TCS exposure also suggested to Paul et al. (2010a) the involvement of hepatic constitutive androstane receptor (CAR) and pregnane X receptor (PXR) xenobiotic nuclear receptor pathways. According to Paul et al. (2010a) the lack of stimulation of EROD and Cyp1a1 suggested that TCS is not acting through the AhR pathway, usually associated with

Table 2. Effects of TCS on activities and gene expression of phase I and phase II enzymes and phase III transporters and pathway.

Study	Experimental design	Results
Zorrilla et al. (2009)	W males, TCS (0, 30, 100, 200, 300 mg/kg/day) PND 23 to PND 53, oral gavage	***Phase I:*** EROD decreased 40 to 50% at all doses PROD increased 4-fold at 300 mg/kg/day ***Phase II:*** UGT, no significant elevation at any dose
Paul et al. (2010a)	LE females, PND 27 to PND 29, TCS (0, 10, 30, 100, 300, 1000 mg/kg/day) $\times$ 4 days, oral gavage	***Phase I:*** EROD decreased 26 to 32% at 30 mg/kg/day and above (no dose-response) PROD increased 2.5 to 9.4-fold at 100 to 1000 mg/kg/day Cyp2b1/2 mRNA increased 2-fold at 300 mg/kg/day Cyp3a1/23 mRNA increased 2.5 and 4-fold at 100 and 300 mg/kg/day Cyp1a1 mRNA, NC (isoform specific) ***Phase II:*** UGT increased 82% at 1000 mg/kg/day, NS at lower doses Ugt1a1 mRNA increased 1.7-fold and 2.3-fold at 100 and 300 mg/kg/day Ugt1a6 and Ugt2b5 mRNA, NC (isoform specific) Sult1c1 mRNA increased 2.5-fold at 100 and 300 mg/kg/day Sult1b1 mRNA, NC (isoform specific) ***Phase III:*** Oatp1a1, Oatp1a4, Mrp2, or Mdr1b mRNA, NC
Paul et al. (2012)	LE adult females, TCS (0, 10, 30, 100, 300 mg/kg/day) GD 6 to PND 21, oral gavage	***Phase I:*** **EROD activity** *GD 20 dams* decreased 40 to 50% (100 to 300 mg/kg/day) *PND 22 dams* decreased 30 to 50% (30 to 300 mg/kg/day) *PND 4 pups*, NC *PND 14 pups* decreased 40 to 50% (100 to 300 mg/kg/day) *PND 21 pups*, NC **PROD activity** *GD 20 dams*, NC *PND 22 dams*, increased 3-fold at 300 mg/kg/day *PND 4 pups* increased 2-fold at 300 mg/kg/day *PND 14 and PND 21 pups*, NC **CYP mRNA** Cyp1a1, Cyp2b2, Cyp3a1/23, Cyp3a9, Cyp4a2, few and only modest effects not correlated with decrease in serum T4 ***Phase II:*** **UGT activity** *GD 20 dams*, NC *PND 22 dams*, increased 50% at 300 mg/kg/day *PND 4 pups,* NC **mRNA** No consistent treatment related effects for Ugt gene expression (Ugt1a1, Ugt1a6) or Sult gene expression (Sult 1b1, Sult 1c3) ***Phase III:*** No consistent treatment related effects of gene expression of Mct8, Mrp2, and Oatp1a4
Paul et al. (2013)	Cell-based nuclear receptor reporter assays transfected with rat (r), mouse (m), and human (h) PXR and CAR	TCS failed to activate rPXR, mPXR, but activated hPXR at TCS concentrations that produced cytotoxicity (6 to 25 μM) TCS produced mixed (agonist/antagonist) effects for m, r, and hCAR (μM TCS)

dioxins or coplanar PCB congeners. The authors acknowledge that the inverse relationship between T4 decrease and increased T4 metabolism is not robust and suggest the contribution of other, as yet unexamined, causes of TCS-induced hypothyroxinemia, such as impaired iodide uptake by the thyroid, elevated DI activity, or competitive binding to thyroid hormone serum proteins (Paul et al. 2010a).

As shown in Table 1 and previously discussed, Paul et al. (2012) reported TCS-induced effects on serum T4 in dams and offspring in which the dams received TCS by gavage (0, 10, 30, 100, and 300 mg/kg/day) from GD 6 to PND 21. It will be recalled that decreases in serum T4 ($<30\%$) were observed at the higher doses of TCS (100 and 300 mg/kg/day) in dams at GD 20 and PND 22 as well as GD 20 fetuses and PND 4 pups. In older pups (PNDs 14 and 21) serum T4 was not decreased. Hepatic enzyme activities and gene expression were also examined by Paul et al. (2012) to explore the relationship between TCS-induced hypothyroxinemia and the involvement of Phases I and II enzymes, and Phase III transporters. TCS-induced decreases (30–50%) in EROD activity were observed both in GD 20 dams (100 and 300 mg/kg/day) and PND 22 dams (30–300 mg/kg/day) and PND 14 pups (100–300 mg/kg/day TCS) while no changes in EROD activity were observed in PND 4 and PND 21 pups indicating some lack of positive correlation between TCS-effects on EROD activity and changes in serum T4. TCS (at 300 mg/kg/day) was associated with an increase in PROD activity in PND 22 dams (3-fold), PND 4 pups (2-fold), whereas no change was observed in GD 20 dams, PND 14 pups and PND 21 pups. The only lack of an inverse correlation between serum T4 and this endpoint occurred in GD 20 dams. Five isoforms of Cyp mRNA were examined (Cyp1a1, Cyp2b2, Cyp3a1/23, Cyp3a9, and Cyp4a2). TCS exposure was associated with "few and only modest significant effects at any time point in the study" (Table 2).

No consistent correlation was observed for hepatic phase II enzymes and TCS-induced changes in serum T4. Hepatic microsomal UGT-T4 activity (glucuronidation) increased about 50% in PND 22 dams at 300 mg/kg/day with no effect observed in GD 20 dams or PND 4 pups. No consistent treatment-related effects were observed for UGT gene expression (Ugt1a1 and Ugt1a6) or sulfotransferase gene expression (Sult1b1 and Sult1c3; Paul et al. 2012; Table 2). The absence of TCS-induced changes in the expression of Ugt1a1 contradicts previously published increases of this isoform following 4-day exposure to TCS in weanling female rats (Paul et al. 2010a). Finally, no significant dose-dependent effects of TCS exposure were observed for transporter gene expression (Mct8, Mrp2, and Oatp1a4; Table 2).

In spite of several inconsistencies between TCS-induced effects on Phases I and II markers and serum T4 alluded to above, Paul et al. (2012) conclude that their data are consistent with the hypothesis that the TCS-induced hypothyroxinemia (observed in GD 20 dams and fetuses, PND 4 pups and PND 22 dams) results from increased clearance of thyroid hormone due to TCS-induced activation of Phase I (Cyp450) and Phase II (glucuronyltransferase and sulfonyltransferase) hepatic enzymes mediated by PXR and CAR pathways. In order to accommodate inconsistencies in their data, Paul et al. (2012) suggest that "TCS is a low-potency and low-efficacy thyroid hormone disruptor" in the rat. The authors also indicate that the weak association between T4 and Phase I and Phase II markers was not due to methodology since positive controls (e.g., hepatic microsomes from rats treated with AhR agonists, such as TCDD and Aroclor 1254) behaved appropriately. In addition, Paul et al. (2012) suggest, as they did previously (Paul et al. 2010a), that other, as yet, unspecified modes of action contribute to the TCS-induced hypothyroxinemia.

Finally, Paul et al. (2013) extended their studies to determine whether TCS activation of PXR and CAR pathways, previously hypothesized to occur in the rat (Paul et al. 2010a, 2012), also occurs in humans. To approach this question they employed cell-based nuclear receptor (NR) reporter assays in which the dose-related effects of TCS (0.39–100 μM) were examined in cells transfected with rat (r), mouse (m), and human (h) PXR and CAR cDNA. In the case of human CAR, three isoforms were examined, hCAR1, hCAR2, and hCAR3. TCS failed to activate rPXR and mPXR but activated hPXR at a TCS concentration range of 6–25 μM. The response of hPXR ranged from 30 to 65% with a potency for TCS of 3–5% relative to the positive control, rifampicin. The dose–response curve for hPXR activation had to be truncated since cells exposed to 50 μM TCS exhibited "overwhelming cytotoxicity". TCS produced a dose-related diminution of hCAR1, rCAR, and mCAR activity suggestive of a weak antagonistic effect (referred to as an "inverse agonist"), as well as a weak agonist effect for hCAR3 generally in the dose range of 10–30 μM TCS. No effect of TCS was evident with cells transfected with hCAR2 (Table 2).

Based upon their data, Paul et al. (2013) suggest that TCS activates human PXR and produces a mixed response (agonist and antagonist) among the various isoforms of human CAR. They further suggest that activation of these NRs could initiate upregulation of hepatic catabolism pathways (Phases I and II enzymes) leading to decreases in serum T4, as postulated in previous studies of TCS effects in rats (Paul et al. 2010a, 2012). Several limitations with regard to the current in vitro data and their interpretation are worth noting. The concentrations of TCS producing effects in the cell-based assays, be they activation of hPXR or the inverse effects for rCAR and mCAR and mixed responses for hCAR isoforms, are quite high and cytotoxic. In fact, Paul et al. (2013) acknowledge that on the basis of doses required to activate hPXR and hCAR, human exposure to TCS would be unlikely to activate these pathways leading to hypothyroxinema. Despite the suggestion by Paul et al. (2013) of cross-species similarities with regard to TCS activation of PXR and CAR and their relationship to hypothyroxinema, the data suggest the contrary. In addition to the differences between TCS effects on hPXR and rPXR, the data do not support the hypothesis suggested previously (Crofton et al. 2007, Paul et al. 2010a, 2012) that TCS upregulates thyroid catabolism in the rat via PXR/CAR signaling. Paul et al. (2013) acknowledge these contradictions, with the following statement: "Failure to measure activation of rPXR in vitro may accurately reflect the in vivo biological response, or may have been inherent to the model used or to the concentration range tested, i.e. activation of rPXR may require a higher concentration of TCS than hPXR activation." In any event, this study offers little support to the hypothesis that the TCS-induced hypothyroxinemia in rats is caused by activation of Phases I and II enzymes.

Effects of TCS on thyroid function in humans

Three studies have examined whether TCS exposure adversely affects thyroid function in humans. Allmyr et al. (2009) determined whether toothpaste use (Colgate Total, containing 0.3% TCS, 2 cm strip twice a day) for 2 weeks affected serum levels of 4-β OH cholesterol (a marker for CYP 3A4) and selected thyroid function endpoints (fT4, free T3 (fT3) or TSH in plasma) in 12 human subjects (5 men and 7 women). According to the authors, upregulation of CYP3A4, which plays a major role in the biotransformation of endogenous hormones and prescription drugs, is associated with activation of the hPXR xenobiotic nuclear receptor signaling pathway. No changes in 4-β OH cholesterol or indices of thyroid function (fT4, fT3, or TSH) were observed in this study after toothpaste use. According to the authors, normal use of toothpaste containing TCS is unlikely to alter CYP3A4-mediated metabolism or adversely affect thyroid function. It is also noteworthy that the levels of plasma TCS attained in humans after toothpaste use are slight relative to those associated with hypothyroxinemia in animal studies. In the current study, median plasma TCS (parent and conjugated) increased after 14 days of toothpaste use, from trace levels (under 0.08 ng/ml) to a median of 54 ng/ml. By contrast, the lowest serum concentration of TCS that associated with a statistically significant decline in serum T4 (of 15%) in PND 22 dam rats was 5120 ng/ml (Paul et al. 2012). This represents about two orders of magnitude difference in exposure between the current study in humans and those in adult female rats. The dose of TCS producing that 15% decrease in T4 in rats was 100 mg/kg/day (Paul et al. 2012). By contrast, Allmyr et al. (2009) estimated that the TCS uptake through the use of toothpaste is 0.01 mg/kg/day representing four orders of magnitude difference between the rat and human. These authors

suggest that either the amount or duration of TCS exposure in humans was too low to affect thyroid function or the response observed in rats was species-specific.

Cullinan et al. (2012) conducted a double-blind randomized clinical trial involving subjects with heart disease over a five-year period comparing the effects of a toothpaste containing 0.3% TCS (n = 64) with placebo toothpaste (n = 68). Thyroid function was evaluated at Years 1 and 5, the endpoints of interest being serum TSH, fT4, fT3, antithyroglobulin antibody, and antithyroid peroxidase antibody. No differences in these endpoints were observed between TCS and placebo toothpaste groups in Year 1. In Year 5 the only statistically significant difference observed was a small decrease in serum-free T4 (about 6%) in the placebo group compared to that of TCS group. Cullinan et al. (2012) conclude that there is no evidence that continuous use of toothpaste containing TCS over this extended period affected thyroid function and that toothpaste containing 0.3% TCS is "safe and free of significant thyroid adverse effects."

Koeppe et al. (2013) conducted a cross-sectional study involving 1831 subjects above the age of 12 years from the 2007 NHANES which examined the relationship between urinary TCS and serum endpoints of the thyroid system, namely free and total T3 and T4, thyroglobulin, and TSH. TCS (both parent and conjugated) was determined from spot urine samples. Multivariate regression analysis in which urinary TCS concentrations were organized as quartiles revealed a positive statistically significant association between TCS and total T3 in adolescents aged 12–19 years (n = 352). No such association was observed when adolescents were stratified according to gender. No other association between urinary TCS and thyroid function endpoints was observed for adolescents or adults (20 years of age and older). The positive association observed was modest, representing an increase in serum total T3 of 3.8% (95% CI: 0.1, 7.5%) relative to the median serum total T3 indicating that the levels of total T3 were well within the normal range. While they consider the observation worthy of further investigation, Koeppe et al. (2013) acknowledge that the positive statistically significant relationship between TCS and total T3 in adolescents could be due to residual confounding (regression models were adjusted for age, sex, BMI, and urinary creatinine) or chance. They also acknowledge that the cross-sectional design of their study has limitations and cannot establish causality. This modest relationship between urinary TCS and serum total T3 in adolescents, devoid of changes in any other indicator of thyroid function, is not consistent with animal data nor does it have a plausible physiological explanation. In view of the many statistical comparisons made in this study (including associations between several urinary parabens and thyroid system endpoints) it appears very likely that it is a chance statistical variation of little or no toxicological significance.

Analysis and conclusions regarding TCS effects on thyroid function

Six of the eight studies conducted in rats examining the effects of TCS on the thyroid system are from the same research group (Crofton et al. 2007, Zorrilla et al. 2009, Stoker et al. 2010, Paul et al. 2010a, 2010b, 2012). Numerous variations exist in the protocols employed in these eight studies. One variation is the route of TCS administration where the six from the same group plus the study of Axelstad et al. (2013) administered TCS dissolved in corn oil via gavage, whereas Rodríguez and Sanchez (2010) administered TCS ad libitum through drinking water. Another variation is strain of rat employed, either Long Evans (Crofton et al. 2007, Paul et al. 2010a, 2010b; 2012) or Wistar (Zorrilla et al. 2009, Stoker et al. 2010, Rodríguez and Sanchez 2010, Axelstad et al. 2013). Other variations are doses of TCS employed (ranging from 1 to 1000 mg/kg/day), durations of exposure (from 3 to 50 days), age windows, gender and/or physiological status when exposure occurred. Among the various windows of exposure employed were prepubertal females exposed for 3 or 4 days, male and females exposed from prepuberty to early adulthood for durations of 21–31 days, adult females exposed from early gestation to mid to late lactation (up to 36 days), or from 8 days prior to gestation through lactation (50 days). Offspring were exposed to TCS in utero as well as post-partum through lactation (Table 1).

Despite these variations in experimental design, a consistent finding in all of the studies is a TCS-induced dose-dependent decline in serum T4 ranging between 15 and 80% (Table 1). A decrease in serum T3 appeared less consistent (Paul et al. 2010a, 2012, Zorrilla et al. 2009, Rodríguez and Sanchez 2010), whereas no change was observed in serum TSH (Paul et al. 2010a, 2012, Zorrilla et al. 2009, Stoker et al. 2010) (Table 1). One study reported minimal, if any, effects of TCS on thyroid histology (Stoker et al. 2010) and another observed no effect of TCS on thyroid weight (Axelstad et al. 2013). The effective doses of TCS producing a decrement in serum T4 (ranging from 10 to 1000 mg/kg/day) as well as magnitude of effect appear to have been influenced by variations in the experimental conditions. Due to the number of factors involved, the influential ones may be difficult to identify. One such identifiable factor appears to be rat strain, since Wistar rats appear more sensitive to the effects of TCS on serum T4 than Long Evans rats. For example, prepubertal Wistar rats receiving TCS by oral gavage exhibited lower BMDs or LOELs (18.75–40 mg/kg/day) (Zorrilla et al. 2009, Stoker et al. 2010) for durations ranging from 3 to 31 days compared to those of prepubertal Long Evans rats receiving TCS by the same route for 4 days (70–100 mg/kg/day) (Crofton et al. 2007, Paul et al. 2010a). Rat strain may also explain the difference in TCS doses required to lower serum T4 in adult pregnant/lactating Long Evans rats (100–300 mg/kg/day) (Paul et al. 2010b, 2012) compared to that required to lower serum T4 in adult pregnant/lactating Wistar rats (10–50 mg/kg/day) (Rodriguez and Sanchez 2010). Wistar dams also exhibited a more robust suppression of T4 in response to TCS than Long-Evans dams (Paul et al. 2010b, 2012, Axelstad et al. 2013) (Table 1).

Based upon the BMDs for TCS-induced decrements in serum T4 in Long Evans rats, it is uncertain whether age or physiological status (e.g., weanling, pregnant/lactating female, fetus, and neonate) can affect the sensitivity of the thyroid system to TCS since there is overlap in some of these estimates across physiological states. However, it is certain that neither pregnant/lactating adults nor GD 20 fetuses are more sensitive than weanlings to the effects of TCS (Crofton et al. 2007, Paul et al. 2010a, 2010b, 2012). The transient nature of serum T4 in offspring (suppression at GD 20 and PND 4 but not at PND 14

and PND 21) and the postpartum decline in total serum TCS (parent and conjugated) at PND 14 and PND 21 suggests that the in utero route is a more effective system for the delivery of TCS than the lactational route. In fact, it appears that TCS exposure via nursing is inadequate for evoking a depression in serum T4 in pups (Paul et al. 2010b, 2012, Axelstad et al. 2013) (Table 1).

As discussed previously, a mode of action has been proposed to explain the TCS-induced hypothyroxinemia. This mode of action involves the activation of PXR and CAR NR pathways leading to increased activity of Phases I and II enzymes and ultimately increased clearance of T4 from the body (Crofton et al. 2007, Zorrilla et al. 2009, Paul et al. 2010a, 2012). While not entirely ruled out, the evidence in support of this mode of action is not very compelling. As discussed previously and listed in Table 2, there is a lack of consistency between specific hepatic Phases I and II markers and TCS-induced decrements in T4 as well as a lack of confirmation of particular associations from one study to the next. This lack of consistency in a relationship between TCS-induced hypothyroxinemia and TCS-induced changes in Phases I and II markers is evident for the following endpoints: EROD activity, UGT-T4 activity, and gene expression of specific isoforms of Cyp, Ugt, or Sult (Table 2; Zorrilla et al. 2009, Paul et al. 2010a, 2012). Also countering the proposed mode of action of TCS are the results of the in vitro study employing the cell-based reporter assays involving transfection with species-specific forms of PXR and CAR. This study provided no evidence that TCS activates rPXR, while activation of hPXR and inverse effects or mixed responses for rCAR, and hCAR isoforms involved doses of TCS that were cytotoxic and are thus, unlikely to be meaningful in terms of human exposure (Paul et al. 2013). At best, the mechanism by which TCS induces decreases in serum T4 levels in the rat is, as yet, unexplained.

The observation of a TCS-induced decrease in serum T4 devoid of any change in serum TSH, as reported in this series of studies, appears to be an atypical physiological adjustment. The typical physiological response accompanying a decrease in serum T4 (hypothyroxinemia) should be a change in serum TSH. Depending upon the mechanism involved, this change in TSH can either be negative or positive. If hypothyroxinemia results from a defect at the hypothalamic-pituitary level (either TRH or TSH) a decrease in serum TSH would occur and, in fact, would be the cause of the drop in serum T4. Over a period of time a prolonged decrease in serum TSH would produce thyroid atrophy. On the other hand, if hypothyroxinemia involves a defect at the thyroid level (as produced by antithyroid drugs) elevated serum TSH would accompany a decreased serum T4. This is due to a disruption of the negative feedback effect of T4 on the pituitary secretion of TSH. The prolonged hypersecretion of TSH would promote thyroid hyperplasia and ultimately thyroid tumors (García et al. 1976, Lechan and Kakucska 1992, Hood et al. 1999a, DeVito et al. 1999). Hypothyroxinemia resulting from increased catabolism of T4, as postulated by Crofton et al. (2007) and Paul et al. (2010a, 2012) would also likely be associated with increased TSH and ultimately thyroid hyperplasia and tumors in rats consistent with numerous compounds that act via this pathway like phenobarbital (PB), dioxins, dioxin like compounds, and pregnenolone-16a-carbonitrile (PCN) (Hood et al. 1999a, Hood and Klaassen 2000). This is clearly not the case with TCS since hypothyroxinemia produced under these circumstances is not associated with elevations in serum TSH (Paul et al. 2010a, 2012, Zorrilla et al. 2009, Stoker et al. 2010) nor is there evidence of thyroid gland stimulation or thyroid hyperplasia in rats treated with this compound (Stoker et al. 2010, Rodricks et al. 2010, Axelstad et al. 2013).

While physiologically atypical, there are other observations of induced hypothyroxinemia without an effect on serum TSH. Microsomal enzyme inducers (MEIs), substances that affect the clearance of thyroid hormone from the body, decrease serum T4, but produce divergent effects on serum TSH. Studies with four such MEIs, namely PB, pregnenolone-16α-carbonitrile (PCN), 3-methylcholanthrene (3-MC), and Aroclor 1254 (a mixture of co-planar and non-coplanar PCB congeners) on the thyroid system of Sprague–Dawley (SD) rats revealed two distinct patterns of response. PB and PCN decrease serum T4 levels and elevate TSH in typical fashion ultimately leading to thyroid hyperplasia and thyroid tumors. On the other hand, 3-MC and Aroclor 1254 produce decreased serum T4 but fail to elevate TSH and do not stimulate the thyroid enlargement or tumor formation (Hood et al. 1999b, Hood and Klaassen 2000, Klaassen and Hood 2001). As yet, the mechanistic basis for this difference in the TSH response is unknown.

Since there is no evidence of TSH hypersecretion, it is likely that TCS-induced decreases in serum T4 represent a mild perturbation of the thyroid system in rats. Accordingly, TCS-exposure is unlikely to be associated with thyroid hyperplasia and thyroid tumors in rats. This is not only apparent from the histological data of Zorrilla et al. (2009) and gravitometric data of Axelstad et al. (2013) described previously (Table 1) but also from numerous subchronic and chronic studies in various mammalian species (mice, rats, dogs, hamsters, and baboons) exhibiting no evidence of thyroid enlargement or thyroid hyperplasia at doses of TCS as high as 900 mg/kg/day as discussed by Rodricks, et al. (2010). Since thyroid hormone deficiency in utero or in the early postnatal period is linked to developmental abnormalities (DeVito et al. 1999, Choksi et al. 2003) concern has been expressed regarding an indirect effect of TCS-induced hypothyroxinemia during pregnancy and on offspring (SCCP 2009, Health Canada 2012). As discussed in detail in a later section, the prevailing evidence suggests that TCS exposure during this window does not adversely affect the processes of gestation or postpartum development in the rat.

While the basic physiological relationship and servomechansims between the elements of the hypothalamo-pituitary-thyroid system are conserved among mammalian species, distinct differences exist between rats and humans that have major physiological and toxicological implications. A principal difference exists in the plasma proteins that bind T4 and T3 and determine the amount of free or bioavailable thyroid hormone. fT3 and fT4 are responsible for biological effects in target cells and are inactivated and cleared from the body. In rats the principal binding protein is transthyretin (TTR) whereas in humans this function is mediated by thyroid-binding globulin (TBG). The affinity of TBG for binding T4 and T3 is significantly greater than that of TTR. This difference in binding means that the levels of fT4 and fT3 are much greater in the rat than in the human and that

the thyroid system of the former is significantly more active than that of the latter (Choksi et al. 2003, USEPA 1998, DeVito et al. 1999, Goodman et al. 2010). For example, the biological half-life for T4 in the rat (0.5–1 day) is markedly shorter than the biological half-life of T4 in humans (5 days or more). The required daily replacement dose of T4 per unit body weight (μg/kg) is about 10-fold greater in the rat than it is in the human. This is consistent with species differences in rats and humans of T4 production by the thyroid gland. TSH levels are higher in the rat relative to the human as reflected in thyroid follicular cell morphology where such cells are cuboidal in rat and low cuboidal in humans (Choksi et al. 2003). Because the thyroid system of the rat operates at a much more dynamic pace than that of humans, the former is more prone to disruption by xenobiotics. In the rat numerous diverse compounds (such as dioxins, phenobarbital, and statins) are known to lower serum T4, elevate serum TSH, and produce thyroid hyperplasia and tumors with little evidence that they produce such effects in humans (Wu and Farrelly 2006, Goodman et al. 2010). In fact, as a result of this enhanced sensitivity of the thyroid system of the rat compared to that of the human, the utility of the rat as a model for the identification of thyroid disruptors has been questioned as it might "confound the toxicity profile explored in preclinical toxicity testing" (Wu and Farrelly 2006). On the basis of specific differences between rats and humans, both the Scientific Committee on Consumer Products (SCCP 2009, SCCS 2011) and Health Canada Preliminary Assessment on TCS (Health Canada 2012) have concluded that the effects of TCS on the thyroid in rats is of little relevance to human health.

The relevance of hypothyroxinemic effects of TCS in rats to humans is further in doubt when one considers the marked difference in exposure to TCS occurring in animals to that observed in humans. As noted earlier, the plasma TCS concentration in PND 22 dams exhibiting a 15% decrement in T4 is almost two orders of magnitude greater than the median concentration in humans consuming toothpaste containing TCS under experimental conditions (Paul et al. 2012, Allmyr et al. 2009). Furthermore, exposure levels are three orders of magnitude different when one compares the estimated exposure of humans consuming toothpaste (0.01 mg/kg/day; Allmyr et al. 2009) to the lowest dose of TCS (10 mg/kg/day) associated with a decrease in serum T4 in Wistar rats of about 20% (Rodriguez and Sanchez 2010). Along similar lines, Rodricks et al. (2010) conducted a safety assessment of TCS exposure for human adult males, adult females, and children relative to toxic exposure to TCS in rodents. In this assessment the Margin of Safety (MOS) was calculated for exposures estimated in humans relative to a BMDL in rodents of 47 mg/kg/day (based upon kidney effects of TCS in hamsters). When human exposures were estimated on the basis of biomonitoring (creatinine normalized urinary TCS), where such exposures were estimated to be 0.2 to 0.3 μg/kg/day, the MOS ranged from 5,222 to 11,750. When based upon human exposures estimated from combined product use where such exposures were estimated be 47–73 μg/kg/day, the MOS ranged from 634 to 1000. As indicated in Table 1 of this review, BMDLs associated with hypothyroxinemia were comparable to that used by Rodricks et al. (2010) in their calculations of MOS. Accordingly, MOS based on effects of TCS on thyroid function appear to be of comparable magnitude. Finally, of the three studies that have examined the relationship between TCS exposure in humans to effects on the thyroid system (Allmyr et al. 2009, Cullinan et al. 2012, Koeppe et al. 2013), none have reported plausible effects.

In summary, studies in rats indicate that TCS at doses in the mg/kg/day range produce a decrease in serum T4 without any other consistent change in T3 or any noted change in TSH. While it has been postulated that this hypothyroxinemia is a result of increased clearance of T4 from the body, the data supporting this hypothesis are not compelling and require a better understanding of the complexity of thyroid hormone metabolism, excretion, and transport in the rat. In any event, the decrease in serum T4 associated with TCS exposure in the rat appears relatively mild and, as discussed later, is unlikely to produce any adverse effects in developing and adult animals. Thyroid-altering effects of TCS in rats appear to have little relevance to the human thyroid system. First, the thyroid system of the rat exhibits a much more active level of function than does the human. These species differences, attributable largely to a lower degree of plasma binding of thyroid hormone in the rat, account for the increased sensitivity of the rat to thyrotoxic agents relative to that of humans. In addition, the levels of exposure to TCS associated with hypothyroxinemia in rats are several orders of magnitude higher than TCS levels reported in humans. Finally, studies in humans have failed to reveal any adverse effect of TCS on thyroid function.

Effects of TCS on male reproduction

In Vitro studies

Gee et al. (2008) reported that a 1,000 and 10,000 molar excess of TCS inhibited the binding of [^{3}H] testosterone to recombinant androgen receptor (AR) protein by about 49% and 77%, respectively, suggesting that TCS binds specifically with low affinity to the AR. However, several AR reporter cell-based assays, such as transfected human embryonic kidney cells, transfected mouse mammary tumor cells, and yeast-based bioluminescent screens have failed to demonstrate agonistic activity of TCS (Chen et al. 2007, Gee et al. 2008, Svobodová et al. 2009) although anti-androgenic activity of TCS was observed (Chen et al. 2007, Gee et al. 2008). On the other hand, Christen et al. (2010) observed in MDA-kb2, human breast cancer cells stably expressing an AR-response construct that TCS produced partial androgenic activity between 10 nM and 1 μM, achieving about 50% of that attained by 0.5 nM dihydrotestosterone (DHT). Instead of exhibiting anti-androgenic activity as reported in other cell-based studies cited above, TCS was shown to amplify the response to 0.5 nM DHT up to 80% in this concentration range. This amplification was regarded by the authors as a novel mode of action (Christen et al. 2010).

Two in vitro studies have explored the effects of TCS on steroidogenesis in Leydig cells. Kumar et al. (2008) examined the effect of TCS on testosterone (T) production in purified rat Leydig cells. TCS inhibited LH-induced T synthesis dose dependently (13–40%) at concentrations ranging from 0.01 to 10 μM without affecting cell viability. In addition, TCS also reduced adenyl cyclase activity and cAMP generation while T production in TCS-exposed cells was restored by the addition of forskolin, an adenyl cyclase activator. TCS also inhibited the

transcription and translation of major steroidogenic enzymes, namely, P450scc, 3β HSD, 17β HSD, and StAR. The effective dose range for TCS actions on transcriptional and translational endpoints correlated with effects on steroidogenesis. This concentration range overlapped with those observed in human body fluids (< 1 to 50 nM) (Allmyr et al. 2006a, 2006b, 2008, James et al. 2010). Kumar et al. (2008) conclude from their observation that the major site of TCS-induced inhibition of T production is at the activation of adenyl cyclase. Forgacs et al. (2012) examined the effects on TCS on basal and hCG-induced steroidogenesis in BLTK1 murine Leydig cells. TCS (30 μM) inhibited hCG-induced T production by about 25%, but had no significant effect on basal T production.

In Vivo studies

As a follow-up to their study of TCS effects on the testes in vitro (Kumar et al. 2008). Kumar et al. (2009) examined the effects of TCS treatment on testicular function in vivo. Adult male Wistar rats received varying doses of TCS by oral gavage (0, 5, 10, and 20 mg/kg/day) for 60 days.[1] Dose-dependent decreases in the weights of the testes, and sex accessory organs (e.g., ventral prostate, seminal vesicle, epididymis, and vas deferens) ranging between 20 and 50% were observed at doses of 10 and 20 mg/kg/day without any effect on body weight. Significant decreases were observed in the mRNA levels of testicular steroidogenic enzymes (StAR, P450scc, P450C17, 3βHSD, and 17βHSD) and ARs ranging between 33% and 58% in rats treated with 20 mg/kg/day of TCS. This dose of TCS was also associated with reductions in protein expression of StAR and AR (both decreases about 40%). The enzyme activities of two testicular steroidogenic enzymes, 3βHSD, and 17βHSD, were decreased dose dependently (ranging from 27 to 47% at 10 and 20 mg/kg/day of TCS. At the dose of 20 mg/kg/day TCS produced significant decreases in serum LH (39%), FSH (17%), cholesterol, pregnenolone (31%), and testosterone (41%) (Table 3). Immunohistochemistry revealed a diminution in StAR protein expression in Leydig cells of rats treated with 20 mg/kg/day of TCS. Daily sperm production as estimated from testicular homogenates was reported to decrease by 34% at 20 mg/kg/day of TCS (Table 3). Based upon histopathogical examination of sex accessory organs, Kumar et al. (2009) report decreased sperm density in the tubules of cauda epididymis, and various malformations or indices of hypofunction or degeneration in the vas deferens and prostate, without any effects in the seminal vesicle in rats exposed to the high dose of TCS. The absence of any changes in the seminal vesicle is unexpected in view of the 35% decline in the weight of this organ at the high dose of TCS.

If, as suggested from their preceding in vitro study (Kumar et al. 2008), the testes is the primary site of TCS action, the decreases in serum LH and FSH observed in the current study (Kumar et al. 2009) are unexpected and not consistent with the usual gonadotrophic response following effects at the testicular level. A primary decline in testosterone of 40% would be expected to elevate, not lower, gonadotrophic hormones (LH and FSH) as a consequence of the negative feedback relationship between testosterone and the gonadotrophic hormones. Alternatively, the simultaneous decline in testosterone and gonadotrophic hormones could occur if TCS acts in a multistage fashion at both the testicular and hypothalamic-pituitary levels.

Zorrilla et al. (2009) also examined the male reproductive system in vivo in the same study discussed earlier with regard to thyroid function. Weanling male Wistar rats received TCS by oral gavage (0, 3, 30, 100, 200, or 300 mg/kg/day) for 31 days from PNDs 23–53. No effect of TCS was found on growth or the onset of puberty, as indicated by the age of preputial separation . No consistent effects of TCS were observed on male reproductive development including puberty, sex organ weights (testes, ventral prostate, epididymis, levator ani-bulbocavernosus muscle, seminal vesicle, and coagulating gland), serum and pituitary levels of LH and prolactin (PRL), and serum androstenedione. Serum testosterone was significantly decreased (by about 60%) at a TCS dose of 200 mg/kg but not at 100 or 300 mg/kg/day (Table 3). While "nonmonotonic" dose–response patterns have been hypothesized at low doses of xenobiotics (Vandenberg et al. 2012), this seems very unlikely and toxicologically implausible in the 100–300 mg/kg/day dose range of TCS. Such a dose–response pattern in serum testosterone is not supported by changes in the weight of androgen sensitive sex accessory organs (such as the ventral prostate and seminal vesicle) or the time of onset of puberty. Furthermore, the mean levels of testosterone and its precursor androstenedione appear quite variable from one dose of TCS to the next. This erratic pattern seems more likely to be a chance variation or an artifact of the experimental design. It will be recalled from the thyroid section, that this particular study was conducted using two different blocks of animals, one in which TCS was administered at 3, 30, and 300 mg/kg/day and the other where 100 and 200 mg/kg/day was administered. In fact, the authors of this study suggest that the significant decrease in serum testosterone at the 200 mg/kg/day dose of TCS could reflect "block variability". Finally, histological examination of the testes and epididymis revealed no treatment-related effects.

In addition, the recent findings of Axelstad et al. (2013), a study previously discussed in relation to TCS effects on thyroid, appear more consistent with the observation of Zorrilla et al. (2009) than that of Kumar et al. (2009). These investigators report in PND 16 offspring of Wistar dams gavaged with TCS (0, 75, 150, and 300 mg/kg/day) from GD 7 to PND 16 that no effects were observed on anogenital distances, nipple retention, or on prostate weights and histopathology (Table 3). This suggested to Axelstad et al. (2013) the absence of antiandrogenic activity for TCS administered to offspring via in utero and lactational routes.

Analysis and conclusions regarding TCS effects on male reproduction

The fact that TCS appears to be a ligand for the AR is not unexpected since steroid receptors exhibit some degree of promiscuity toward other endogenous hormones, as well as xenobiotics. In fact, the concept of steroid receptor promiscuity, which is a function of the stereochemical relationship between the

[1]In this study TCS is dissolved in phosphate buffered saline. This is in contrast to other studies in which TCS was administered through oral gavage administration where corn oil was the vehicle (Zorrilla et al. 2009; Paul et al. 2010a, 2010b, 2012; Stoker et al. 2010, Axelstad et al. 2013).

Table 3. Effects of TCS on male reproduction in rats in vivo.

Study	Experimental design	Results
Kumar et al. (2009)	Adult W males, TCS (0, 5, 10, 20 mg/kg/day) × 60 days, oral gavage	Dose-dependent decreases (20 to 50%) in weights of testes, ventral prostate, seminal vesicle, epididymis, vas deferens at 10 to 20 mg/kg/day Significant decreases in mRNA of steroidogenic enzymes (StAR, P450scc, P450C17, 3βHSD, 17βHSD, and AR (33 to 58%) and StAR and AR protein (40%) at 20 mg/kg/day Significant decreases in serum LH (39%), FSH (17%), and testosterone (41%) at 20 mg/kg/day TCS DSP decreases (34%) at 20 mg/kg/day
Zorrilla et al. (2009)	W males, TCS (0, 3, 30, 100 mg/kg/day) PND 23 to 53, oral gavage.	No effect of TCS on growth or onset of puberty, weights of testes, ventral prostate, epididymis, levator-ani bulbocavernosus muscle, seminal vesicle, coagulating gland No effect on serum or pituitary levels of LH, prolactin, and serum androstenedione Serum testosterone decreased (about 60%) at 200 mg/kg/day, not at other doses
Axelstad et al. (2013)	W adult females, TCS (0, 75, 150, 300 mg/kg/day) GD 7 to PND 16, oral gavage	PND 16 pups: No effects on anogenital distances, nipple retention, or weights or histology of prostates (i.e., no anti-androgenic effect)

ligand and the binding pocket of the receptor, serves as a basic concept in hormonal activity of xenobiotics. While xenobiotics may exhibit weak affinity for a hormone receptor, high enough concentrations of such compounds can interact with the hormone receptor binding pocket and evoke a response (Witorsch 2002, Eick et al. 2012). The fact that cell-based assay systems provide a diversity of effects (such as agonist and/or antagonist or no response at all) is also not surprising. The nature of a response evoked by ligands interacting with a member of the NR family is complex and depends on the particular ligand, the conformational changes in the receptor induced by a ligand, as well tissue-specific factors, such as co-activators, co-repressors, and genomic response elements (Witorsch 2000). While such complexities of NR signaling underscore limitations in cell-based screening assays, they also explain why different tissues exhibit unique responses in vivo (Witorsch 2002). The fact that TCS can amplify the action of DHT in human breast cancer cells in vitro (Christen et al. 2010) is provocative. However, it is uncertain from the data presented whether this amplification represents a positive interaction or an additive effect. Furthermore, AR receptor-mediated agonistic or interactive effects of TCS have yet to be reported in vivo.

As noted above, two studies employing Leydig cell-based assays report that TCS suppresses testosterone production in vitro. Kumar et al. (2008) report a dose-dependent suppression in LH-induced testosterone synthesis as well as the inhibition of key steroidogenic enzymes in rat Leydig cells. The effective dose range is broad, but lower levels of this range (10–100 nM) overlap with serum levels in exposed humans (Allmyr et al. 2006a, 2006b, 2008, James et al. 2010). The site where TCS suppresses testosterone production appears to be adenyl cyclase. On the other hand, caution should be exercised in relating observations in vitro to health effects in humans, in view of the limitations that exist in vitro models. Furthermore, as will be discussed, there is concern about the source of rat Leydig cells from this particular laboratory. The other in vitro observation (Forgacs et al. 2012) showing TCS-induced suppression of hCG-induced T production in a murine Leydig cell line, while consistent with the observation of Kumar et al. (2008), should be viewed with caution since the response was evoked with a very high concentration of TCS.

The in vivo studies of Kumar et al. (2009) and Zorrilla et al. (2009) exhibited conflicting results for every male reproductive endpoint examined, namely testicular weight, sex accessory organ weight, serum testosterone levels, and LH, despite the fact both groups employed the same strain of rat. Differences exist in the designs of each study, namely age of exposure, duration, and dose range. However, these differences are unlikely to explain the facts that Kumar et al. (2009) observed TCS-induced suppression of male reproduction while Zorrilla et al. (2009) exhibited little or no adverse effect. Although the duration of exposure employed by Kumar et al. (2009) is twice as long as that of Zorrilla et al. (2009), the latter study exposed animals during pubertal development and employed maximal doses of TCS more than 10-fold that of the former, conditions that would tend to increase the probability of producing an inhibitory effect on the male reproductive system.

The observations of Kumar et al. (2009) that TCS inhibits male reproductive function, not only contrasts the data of Zorrilla et al. (2009) and those of Axelstad et al. (2013) (Table 3) but also the prevailing data obtained from one- or two-generation reproductive and developmental studies conducted in mice, rats, hamsters, and rabbits exposed to high doses of TCS (150 mg/kg/day and above) which reveal little evidence of adverse effects on male reproductive function, as reviewed elsewhere by Rodricks et al. (2010). Some spermatogenic abnormalities and reduced spermatozoa number were reported in a study of male Syrian hamsters treated for 95 weeks with 250 mg/kg/day of TCS but not with 75 mg/kg/day, although general toxicity at this high dose of the compound could not be excluded as a factor (Chambers 1999, Rodricks et al. 2010).

A recent review conducted by the Scientific Committee of Consumer Safety (SCCS 2011) also raised concerns about the study conducted by Kumar et al. (2009). Doubts were expressed by the reviewers concerning "non-careful writing" (misstatement in a figure legend) as well as the purity of the TCS employed (which was 98%). In fact, Axelstad et al. (2013) suggest that the observations of Kumar et al. (2009) could reflect impurities in the TCS they used, since dioxin and furan were reported previously in TCS samples produced in India and China (Menoutis and Parisi 2002) and TCS employed by

Kumar et al. (2009) was produced in India. The SCCS (2011) also noted that the decrements in testicular weight at TCS doses of 10 and 20 mg/kg/day observed by Kumar et al. (2009) are not consistent with studies in rats that failed to exhibit adverse effects on reproductive parameters at doses as high as 600 mg/kg/day for 90 days (study not referenced) or in a two-generation study where F0 rats were treated with TCS dose at 176 mg/kg/day (Morseth 1988).

One explanation for the differences between the observations of Kumar et al. (2009) and the findings of others may reside in the animals employed. The animals used by Kumar et al. (2009) exhibit atypically low body weights. At 10 and 18 weeks of age, the mean body weights of these animals are 168 g and 187 g, respectively. In contrast, the rats from the same strain in the study by Zorrilla et al. (2009) are 300 g at 7–8 weeks of age. These differences in body weight are also reflected in the weights of the testes and sex accessory organs. The fact that the rats employed by Kumar et al. (2009) are of low body weight is also evident when comparing them to body weights of historical controls for male Wistar rats at 10 weeks (373 g) and 18–19 weeks (about 500 g) (Powers 2002). The low body weight exhibited by Kumar et al. (2009) could reflect underlying illness or stress in these animals. Accordingly, the observed suppression of male reproductive function could be an artifact of these underlying conditions. Suppression of serum testosterone secretion from the testes mediated by stress-induced elevations glucocorticoid, is well documented (Cooke et al. 2004).

In conclusion, the data are not compelling with regard to adverse effects of TCS on male reproduction. The fact that TCS is an AR ligand that exhibits diverse response patterns in cell-based assay systems is not unexpected in light of the promiscuity of steroid receptors and complexity of target cell steroid signaling pathways. TCS-induced suppression of testosterone production observed in Leydig cell-based assays should be considered with caution in view of the limited applicability of in vitro systems, concerns about the doses of TCS employed, and sources of cell used in both studies. Furthermore, two of the three in vivo studies examining effects of TCS on male reproduction do not support adverse effects, consistent with data from one- or two-generation reproductive/developmental studies in a variety of mammalian species. Finally, concern exists about the source of animals employed in the study of Kumar et al. (2009) reporting an adverse effect of TCS on male reproduction.

Effects of TCS on female reproduction

In Vitro studies

TCS has been shown to bind weakly to the ER from MCF-7 human breast cancer cell cytosol as well as recombinant human ER (Gee et al. 2008). Testing for biological activity using a variety of cell-based systems reveal a diversity of profiles with regard to exhibiting agonist and antagonist activity, depending upon the system employed. Among these profiles are: (1) estrogenic with a yeast-based galactosidase expression system (YES; Svobodová et al. 2009) and rat GH3 pituitary cells transiently transfected with estrogen responsive element (ERE) luciferase reporter construct (Jung et al. 2012); (2) both estrogenic and anti-estrogenic as measured by cell proliferation of MCF7 BOS human breast cancer cell (Henry and Fair 2013); (3) anti-estrogenic activity in the absence of estrogenic activity in MCF-7 human breast cancer cells transfected with an ERE – CAT reporter (Gee et al. 2008), and human ovarian cancer cells transfected with an ER responsive luc reporter (Ahn et al. 2008); and (4) neither estrogenic nor anti-estrogenic with T47D-KBluc, an estrogen transactivational assay derived from human breast cancer cells (Louis et al. 2013) and yeast-based bioluminescent assay (Svobodová et al. 2009).

In Vivo studies

In addition to studying TCS effects on thyroid function as discussed previously, Stoker et al. (2010) employed two protocols to study the effects of TCS on female reproduction in female Wistar rats, namely the pubertal design and uterotrophic design. In the pubertal assay which utilized the EDSP protocol, female Wistar rats received varying doses of TCS by oral gavage (0, 9.375, 37.5, 75, or 150 mg/kg/day) from PNDs 22 to 42. At 150 mg/kg/day, TCS advanced the age of vaginal opening from PNDs 32.7 to 30.4 and increased the wet uterine weight by about 18%. At this dose, mean day of first estrus (as indicated by cornified vaginal epithelial cells) was decreased from 37 to 35 days but did not achieve statistical significance. TCS produced a dose-related decrease in serum estradiol (32 to 55%) between doses of 37.5 and 150 mg/kg/day without any effect on serum LH or PRL (Table 4). In the uterotrophic assay protocol rats were given varying doses of TCS by oral gavage (1.2, 2.3, 4.7, 9.4, 18.75, 37.5, 75, 150, and 300 mg/kg/day) from PNDs 19 to 21. TCS alone failed to produce an uterotrophic effect of TCS. However in the presence of ethinyl estradiol (EE) at 3 μg/kg/day, TCS at doses from 4.7 to 37.5 mg/kg/day produced a dose-related amplification of the estrogenic effect on uterine weight and uterine histology (Table 4). Two mechanisms were suggested by the authors for this potentiation by TCS of the estrogenic effect on the uterus: (1) TCS could be influencing the interaction of estrogen with the ER and (2) TCS could be retarding clearance of estrogen (i.e., prolonging its half-life).

A study from the same laboratory (Louis et al. 2013) further characterized this interaction between EE and TCS. In this study Wistar female rats were dosed through oral gavage from PND 19 to PND 21 either with EE alone (0.125, 0.25, 0.5, 1, 2, and 3 μg/kg/day) or each EE dose in combination with a series of TCS doses (2.3, 4.7, 9.4, and 18.75 mg/kg/day). To validate estrogen specificity, some combinations were also administered along with the estrogen antagonist, ICI 182,780 (ICI) at 10 mg/kg/day. The endpoints examined were uterine weight, uterine epithelial cell height, and the mRNA expression of the two isoforms of ER α and β, and uterine markers for an estrogenic response, CaPB-9k and IGF-1. TCS at doses between 4.7 and 18.75 mg/kg/day enhanced the dose-dependent effect of EE on uterine weight and histology consistent with amplification (Table 4). Amplification of the uterotrophic effect of EE with TCS was demonstrable with EE doses as low as 0.25 μg/kg/day. While TCS alone exhibited no effects on uterine mRNA nor increased uterine weight, it influenced the changes induced by EE in a diverse fashion, such as amplification of EE-induced decreases in ERα and ERβ, a nonsignifi-

Table 4. Effects of TCS on female reproduction in rats in vivo.

Study	Experimental design	Results
Stoker et al. (2010)	W females, TCS (0, 9.4, 37.5, 75, 150 mg/kg/day) PND 22 to PND 42, oral gavage (pubertal design)	Age of vaginal opening advanced (PND 32.7 to PND 30.4) at 150 mg/kg/day Uterine weight increased (about 18%) at 150 mg/kg/day No statistically significant effect on day of first estrus (vaginal cornification) Dose-related decrease in serum estradiol (32 to 55%) at doses of 37.5 to 150 mg/kg/day No effect on serum LH or prolactin
	W females, TCS (0, 1.2, 2.3, 4.7, 9.4, 18.75, 37.5, 75, 150, 300 mg/kg/day) PND 19 to PND 21, oral gavage (uterotrophic design)	No uterotrophic effect of TCS alone Dose-related amplification of estradiol-induced uterotrophic effect (weight and histology) at TCS doses of 4.7 to 37.5 mg/kg/day
Louis et al. (2013)	W females, ethinyl estradiol (EE) alone (0, 0.125, 0.25, 0.5, 1, 2, 3 mg/kg/day) ± TCS (2.3, 4.7, 9.4, 18.75 mg/kg/day), PND 19 to PND 21	TCS (4.7 to 18.75 mg/kg/day) dose-dependently enhanced the effect of EE on uterine weight, histology, and other endpoints Effects reversed by ICI TCS alone produced no effect on uterine endpoints
Rodriguez and Sanchez (2010)	W adult females, TCS (0, 1, 10, 50 mg/kg/day) 8 days pre-gestation to PND 21, drinking water Above treatment extended in female offspring from PND 22 to PND 50 (pubertal cohort) and from PND 22 to PND 24 (uterotrophic cohort)	Delayed vaginal opening (4 + days) in female offspring treated up to PND 21 as well as in pubertal cohort at all doses of TCS No evidence of stimlatory effect of TCS on uterine weight in female offspring
Jung et al. (2012)	Sprague–Dawley female rats, TCS (0, 7.5, 37.5, 187.5 mg/kg/day) PND 19 to PND 21, oral gavage	TCS increased uterine weight, C3 (mRNA), CaBP-9 K (mRNA, protein) dose-dependently TCS effects inhibited by ICI or RU 486

cant amplification of EE-induced increases on CaPB-9k, and inhibition of EE-induced increases in IGF-1. The effects associated with EE + TCS were reversed by cotreatment with ICI (Table 4). In order to gain further insight regarding the interaction between TCS and EE, Louis et al. (2013) employed the ER transactivation assay involving T47D-KBluc cells derived from human breast cancer cells expressing both isoforms of ER. Exposure of these cells to varying doses of TCS in vitro (0.03–100 μM) exhibited neither agonist activity, antagonist activity, nor replicated the in vivo observation of enhancement of an estrogenic effect.

Louis et al. (2013) note that while TCS amplifies the estrogenic effects within the contraceptive estrogenic dose range (between 0.1 and 1.4 μg/kg/day), the doses of TCS required to produce this enhancement (4.7 mg/kg/day) exceed estimates of TCS exposure in humans (average: 0.0045–0.13 mg/kg/day). The interaction between TCS and EE in vivo appears to be very complex, particularly, because it cannot be replicated with an ER transactivational assay in vitro. The authors note that the ability of ICI to block this interaction in vivo is consistent with the involvement of an ER-mediated pathway. They also acknowledge that the effect of TCS on EE induced down regulation of both isoforms of ER suggests that TCS "may modify ER activity or the downstream signaling response through indirect interactions with transcription factors". Furthermore, they also note that the reversal using TCS of an EE stimulation of IGF-1 mRNA expression, which is opposite in direction to the effects of TCS on EE-induced changes in ER and CaPB-9k, suggests that TCS may also act via an ER-independent pathway. Although Louis et al. (2013) propose several possible explanations for TCS–EE interaction, such as involvement of other receptors, hormone biosynthesis, hormone metabolism, hormone availability to tissues, and hormone clearance, none were discussed in detail. As indicated by the authors, hormone biosynthesis is unlikely because the animals in this study were sexually immature. As an explanation for the lack of TCS–EE interaction in vitro, Louis et al. (2013) suggest target tissue differences (mammary versus uterine) or the limitations of the in vitro model for the examination of in vivo phenomena (i.e., the absence of peripheral metabolism).

In addition to examining the effects of TCS on thyroid function of dams through gestation and lactation, as described previously, Rodríguez and Sanchez (2010) also examined the effect of TCS exposure on pregnancy outcome and on the female offspring post weaning. Wistar rat dams received various doses of TCS (0, 1, 10, and 50 mg/kg/day) ad libitum through drinking water from 8 days pre-mating to PND 21. Female offspring also received TCS via drinking water in one of two protocols: (1) a pubertal cohort in which animals received TCS orally from PNDs 22 to 50; and (2) a uterotrophic cohort in which TCS was administered orally from PNDs 22 to 24. TCS exposure delayed vaginal opening from Day 32 to Days 36 or 37 at all three doses without any evidence of a dose–response relationship in offspring treated in utero and during lactation, and in females additionally exposed until the age of puberty. No evidence of a stimulatory effect on uterine weight was observed in female offspring exposed to TCS in utero and during lactation as well as until puberty or as a result of the 3-day treatment regimens (Table 4). Endpoints related to gestation, postnatal survival, and development were also examined and will be discussed later in the section dealing with developmental toxicity.

Jung et al. (2012) tested for potential estrogenic activity of TCS in vivo in immature female SD rats. TCS was administered through oral gavage (0, 7.5, 37.5, and 187.5 mg/kg/day) from PNDs 19 to 21. Estrogenicity was assessed by uterine weight and the gene expression of complement 3 (C3) as well as the gene and protein expression of CaBP-9K in the uterus. In order to establish the specificity of the response, either ICI (anti-estrogen) or RU 486 (anti-progestin/glucocorticoid) was also administered through oral gavage. TCS increased uterine wet weight in a dose-dependent fashion (by 50–100% at 7.5–37.5 mg/kg/day). Estrogenicity of TCS was also evident by elevations in C3 mRNA and CaBP-9K mRNA and protein at 37.5 mg/kg/day and above. TCS-induced effects on uterine weight and C3 and CaBP-9K responses were inhib-

ited by co-administration of ICI and RU 486 (Table 4). The inhibitory effects of ICI seemed more pronounced than that of RU 486. As alluded to earlier, Jung et al. (2012) also examined the effects of TCS in vitro using rat pituitary GH3 cells transiently transfected with plasmids containing estrogen response element (ERE) or progesterone response element (PRE) luciferase reporter constructs. TCS at concentrations of 10^{-9} M, 10^{-7} M, and 10^{-5} M activated luciferase activity in GH3 cells containing the ERE-luciferase construct. This response was blocked by co-incubation with ICI. No response to TCS was evident in cells transfected with the PRE-luciferase construct. TCS stimulated CaPB-9k mRNA and protein expression, but only at the high concentration (10^{-5} M). This response was also blocked by co-incubation with ICI. Based upon their in vivo and in vitro data, Jung et al. (2012) conclude that TCS acts via ER signaling pathways. The TCS-induced uterotrophic response, stimulation of C-3 and CaBP-9k expression, and the activation of the ERE-luciferase construct but not the PRE-construct, as well as the inhibitory effects of ICI support these conclusions. However, the inhibitory effect of RU 486 on TCS action in uterus is unexplained. The authors suggest the involvement of a glucocorticoid pathway, because RU 486 is both a glucocorticoid and progestin receptor antagonist.

Analysis and conclusions regarding TCS effects on female reproduction

In vitro data reveal a situation between TCS and the ER that is similar to that discussed previously for the relationship between TCS and the AR, low binding affinity and a diversity of profiles with regard to agonistic and/or antagonistic responses observed using cell-based screening assays. Again, these observations are not unexpected since the promiscuity of the ER and the diversity of ligand-induced responses in vitro are well-documented (Witorsch 2002).

On the other hand, the discrepancies that have been observed with regard to estrogenic activity of TCS in vivo are not that easily explained because they involve the same target organ, the uterus, in the same species of mammal, the rat. Of the four recent studies that have tested for uterotrophic effects, three report no such activity (Stoker et al. 2010, Louis et al. 2013, Rodríguez and Sanchez 2010) while the other does (Jung et al. 2012). Stoker et al. (2010) failed to observe a uterine weight increase in Wistar rats treated with TCS for 3 days (PNDs 19–21) at a dose up to 300 mg/kg/day. In a follow-up to this report, Louis et al. (2013) reported that TCS treatment (18.75 mg/kg/day) of Wistar rats from PNDs 19 to 21 failed to increase not only uterine weight, but also responses of uterotrophic markers (e.g., ER, CaPB-9k, and IF-1). Rodríguez and Sanchez (2010) reported no uterine weight increase in Wistar rats treated with TCS (up to 50 mg/kg/day) in a pubertal cohort (PNDs 22–50) and uterotrophic cohort (PNDs 22–24). In contrast, Jung et al. (2012) report that treatment of SD rats with TCS for 3 days (PNDs 19–21) (7.5–187.5 mg/kg/day) increased uterine weight and the expression of uterotrophic markers (C3 and CABP-9K). These responses were blocked by co-treatment with the estrogen antagonist, ICI and RU 486, a progestin/glucocorticoid antagonist (Table 4). As discussed above, the effect of RU 486 is unexplained. Louis et al. (2013) suggest that the qualitative differences between their results and those of Jung et al. (2012) could reflect rat strain differences or dietary factors.

The observations of Stoker et al. (2010) and Louis et al. (2013), of a potential TCS–estrogen interaction are provocative and appear complex. While TCS alone exerts no effect on the uterus, it appears to influence the action of EE on the uterus, amplifying EE-induced uterine enlargement and influencing EE effects on other endpoints (ER, CABP-9K, and IGF-1). The ability to block this interaction with ICI is not unexpected because the ER mediates the action of EE. The fact that the effects of TCS on EE-induced changes in gene expression are diverse points to the complexity of this relationship. On the other hand, the inability to replicate this interaction in vitro using a cell line derived from human breast cancer cells suggests either the involvement of tissue specificity in this interaction or in vivo conditions yet to be identified. While Louis et al. (2013) acknowledge that TCS can amplify effects of therapeutic dose levels of EE, they also note that the amount of TCS required for this amplification (4.7 mg/kg/day) exceeds estimates of TCS intake by humans by 30- to 1000-fold. It is also noteworthy, that there are conflicting data with regard to the influence of TCS on vaginal opening advancement reported by Stoker et al. (2010) and delay reported by Rodríguez and Sanchez (2010).

Overall, the recent data are conflicting with regard to the effects of TCS on female reproduction with three of four studies not supporting an estrogenic effect. The possibility of an interaction between TCS and estrogen, while intriguing, is of questionable relevance to the issue of health effects of TCS in humans because of the high dose level of TCS necessary for this interaction. Finally, as reported elsewhere, reproductive and developmental studies provide little or no evidence of effects of TCS on female reproduction endpoints such as uterine parameters or number of corpora lutea (Rodricks et al. 2010).

Effects of TCS on gestation and postpartum development

In Vitro studies

One study examined the interaction between TCS and enzymatic processing of estrogen in placenta using a cell-free system. James et al. (2010) examined TCS as a potential inhibitor of estrogen sulfonation (sulfotransferase) using cytosol fractions from sheep placenta with estradiol and estrone as substrates. Incubation with TCS at sub-nM to nM concentrations of TCS markedly inhibited estradiol sulfonation. Analysis of the kinetics of this inhibition revealed that TCS exhibited a "mixed" inhibition, having both competitive and noncompetiitive components, at low and high concentrations of TCS, respectively. The primary component of this inhibition is competitive, which assumes that TCS competes with estradiol for the substrate binding site. A minor noncompetitive component of this inhibition is suspected of being a form of "partial substrate inhibition" where the molecule interacts with the enzyme substrate complex interfering with product formation. While the authors of this study speculate on the health implications of this finding particularly as it relates to the in utero condition, any implications other than the dynamics of the interaction between TCS and natural estrogens at

a substrate site and its effect on enzyme activity are very premature. This study involves the interaction between TCS and a cytosol sub-fraction derived from disrupted cells in the absence of the structural relationships that normally exist in an intact organism.

Honkisz et al. (2012) examined the effects of TCS in vitro on the secretion of estradiol, progesterone, and beta-human chorionic gonadotropin (β-hCG) in JEG-3 cells, a human choriocarcinoma derived cell line. In addition, this study also investigated TCS effects on cell markers for viability, proliferation, and apoptosis. JEG-3 cells were cultured with increasing doses of TCS (0, 1, 10, 50, and 100 nM and 1, 10, 50, and 100 μM) for 24, 48, and 72 h. At 24 and 48 h, a statistically significant elevation in estradiol output was observed at 1 and 10 μM TCS. At 72 h, statistically significant elevations were observed with 1, 10, and 100 nM (not 50 nM), and 1 μM (not 10 μM) of TCS. All elevations in estradiol were about 15% in magnitude and not dose-dependent. At the highest TCS concentrations (50 and 100 μM) estradiol production was markedly decreased by 70% or more, indicative of cytotoxicity. Progesterone output was slightly increased between 1 nM and 10 μM without evidence of dose dependency at 24 h. Small increases in progesterone were observed between 100 nM and 10 μM at 48 and 72 h. As seen for estradiol, sharp depressions in progesterone output were observed at 50 and 100 μM TCS at 24, 48, and 72 h. TCS produced similar patterns of β-hCG output at 24 and 72 h, generally modest decreases from 1 or 10 nM to 1 μM with a marked increase at 10 μM (1.6- to 1.7-fold), while at 48 h significant declines in β-hCG were observed between 50 nM and 10 μM without an upward spike at the higher dose. At 72 h the suppression of β-hCG seemed rather robust (between 1 nM and 1 μM TCS). At any of the time intervals inhibition of β-hCG did not appear to be dose dependent. At 50–100 μM TCS, there was no detectible β-HCG at 24, 48, and 72 h, indicative of cytotoxicity. At all three incubation periods, JEG-3 cell proliferation appeared to be slightly suppressed at most dose levels of TCS up to 50 μM with a sharp decline in proliferation at 100 μM. Caspase-3 activity, a marker for cell apoptosis, was elevated 30–60% at TCS doses ranging from 1 nM to 1 μM above which there was a decline as cytotoxicity increased. Apoptotic bodies were evident in cells exposed to 1 and 100 nM TCS for 24 h. Honkisz et al. (2012) suggest that their observations on the effects of TCS on hormone production and cell viability of this choriocarcinoma cell line has adverse health implications with regard to interference of placental function and the success of pregnancy.

The health-related implications of the observations of Honkisz et al. (2012) appear overstated. When relating to health risks in general, in vitro models have major limitations with regard to their lack of the pharmacokinetic and pharmacodynamic components of the in vivo condition. In addition, immortalized neoplastic cell lines have the additional limitation, due to genetic modification, of exhibiting responses to xenobiotics that may differ from those of normal cells (Gentry et al. 2010). The responses to TCS themselves raise questions about their toxicological significance. The TCS-induced hormonal changes, namely the elevations in estradiol and progesterone and decrease in β-hCG were, for the most part, modest in magnitude and not dose dependent. Occasionally, the effective TCS concentrations overlap with those found in human maternal and umbilical cord serum, 10 nM or less (Honkisz et al. 2012). In some instances, the response pattern seemed erratic, if not implausible, as reflected in the spikes in β-hCG levels at 10 μM TCS observed at 24 and 72 h but not 48 h. Other effects, such as the slight inhibition of cell proliferation and activation of caspase-3 (which do not appear to be dose related), seem more like adaptive responses to xenobiotics rather than severe adverse effects. Furthermore, the marked changes observed at high doses of TCS (10 μM or above) seem representative of cytotoxic responses as is typical under in vitro conditions. Finally, as will be discussed later, the suggested health implications of Honkisz et al. (2012) are inconsistent with the prevailing in vivo data in animals which indicate that, except for extremely high doses, exposure to TCS during pregnancy has little or no adverse effect on the course of gestation and well-being of offspring.

Animal studies in vivo

In addition to examining the effects of TCS on thyroid function in dams and offspring exposed during gestation and lactation, Paul et al. (2010b, 2012) examined gestation and postpartum development in Long Evans dams and offspring exposed to TCS by oral gavage (0, 30, 100, and 300 mg/ml) from GD 6 to PND 21. No treatment-related clinical signs of toxicity were observed in dams or offspring at any time after TCS exposure. In addition, no effect of TCS was observed on the number of fetuses, number of implantation sites in GD 20 dams, gestation length, litter size, viability index, male:female sex ratio, day of eye opening, or gross malformations in pups. No effect of TCS was observed neither on dam body weight during gestation nor on pup body weight at PND 4, PND 14, or PND 21.

Rodríguez and Sanchez (2010) also evaluated the effects of TCS on the course of pregnancy and postpartum development in their previously discussed study of thyroid and reproductive function. Wistar rat dams were exposed to TCS (0, 1, 10, and 50 mg/kg/day) ad libitum via drinking water for 50 days, from 8 days pre-mating through PND 21. For this phase of the study, the endpoints of interest were pregnancy rate, gestation length, litter size, number of implantations, live birth index, 6-day survival of offspring, male:female sex ratio, weaning index, and pup weight on PNDs 1, 5, 10, 15, and 20. Dams treated with TCS showed no external signs of toxicity. No effects of TCS were observed on pregnancy rate, gestation length, litter size, number of implantation sites, or weaning index. The high dose of TCS was associated with a decline in live birth index (14%) and in 6-day survival (30%). In addition, a decrease in male:female sex ratio (from 1.3 to 0.6) was observed at all doses of TCS without any evidence of a dose–response relationship. TCS exposure at all doses also reduced pup weight at PND 20 by about 30%. Rodríguez and Sanchez (2010) suggest that growth retardation in pups may reflect an indirect effect of thyroid disruption, possibly through impairment of the milk ejection reflex in dams, thus compromising the normal feeding of pups.

Maternal and fetal development were also followed in the previously described study of Axelstad et al. (2013) where Wistar dams were treated with varying doses of TCS (0, 75, 150, and 300 mg/kg/day) through gavage from GD 7 to PND 16. Maternal weight gain from GD 7 to the day before birth (GD 21) exhibited no significant effect. However, when fol-

lowed after delivery (to PND 1), a statistically significant dose-dependent downward trend and decrease were seen at 300 mg/kg/day indicating that the high dose of TCS produced maternal toxicity. This toxicity appeared transient as dam body weights at PND 16 were not affected by TCS exposure. TCS exposure of the dams during gestation and lactation did not reveal fetotoxicity or developmental toxicity as evidenced by the absence of effects on gestation, gender distribution, post-implantation loss and litter size, neonatal deaths and offspring postnatal body weights or, as discussed previously, on anogenital distance, nipple retention, prostate and thyroid weights and prostate histopathology. Furthermore, postnatal oral TCS exposure (0, 50, and 150 mg/kg/day) from PNDs 3 to 16, caused no significant effects on pup body weights or body weight gains during the exposure period (Axelstad et al. 2013).

Crawford and deCatanzaro (2012) examined the influence of TCS on pregnancy outcome in CF-1 female mice dams treated with TCS subcutaneously (0, 87, 262, 523, or 795 mg/kg/day) on GDs 1–3. Under these conditions, the two highest doses of TCS (523 and 785 mg/kg/day) reduced the number of implantation sites on GD 6 in a dose-related fashion (27–40%). In addition, a single injection of TCS at the above doses on GD 2 or GD 3 reduced implantation sites. No such effect of TCS was observed when administered on GD 0 or GD 1. According to Crawford and deCatanzaro (2012), the effect of TCS on implantation is indirect evidence of estrogenicity of the compound or its ability to prolong action of endogenous estrogen in accordance with the in vitro observation of James et al. (2010) discussed earlier. An estrogenic mechanism seems premature speculation at this time and the health implications of this observation are questionable. The doses of TCS between 500 and 800 mg/kg/day are enormous. In addition, it is noteworthy that, unlike most studies discussed thus far, TCS was administered to mice in this study by a parenteral route. Because subcutaneous administration avoids the pass through the liver that occurs with oral administration, it represents a significantly higher exposure to the xenobiotic than occurs through the dietary route. The adverse effects on implantation observed in the study could reflect direct toxicity associated with TCS administered subcutaneously at enormous doses or a nonspecific response to the stress associated with such high exposures. As reviewed in detail by Rodricks et al. (2010) and summarized below, several studies conducted in a variety of mammalian species have found little evidence that TCS produces reproductive and developmental toxicity or interferes with gestation, except for an occasional weight loss in dams and pups at high doses. In a two generation reproductive and development study, F0 and F1 male and female CRL:CD (SD)Br rats received dietary TCS (0, 15, 50, and 150 mg/kg/day) from 10 weeks pre-mating through gestation and lactation (Morseth 1988). No treatment-related effects were observed on estrus cycling, pregnancy, gestation length in F0 or F1 adult rats at any dose tested. F1 pups were examined at PNDs 0, 4, 7, 14, and 21. No treatment-related clinical signs of toxicity or effects on viability, sex ratio, and weaning index were observed. In the F1 generation, some retardation of body weight gain was observed at PND 14 as well as weight gain during premating, gestation, and lactation, particularly at the high dose. In the F2 generation no effect of TCS was observed on viability or weaning indices, and body weight, nor was there evidence of clinical toxicity. Upon terminal sacrifice, no evidence was observed for treatment-related gross lesions in F0, F1, and F2 generations (Morseth 1988, Rodricks et al. 2010).

One-generation developmental studies testing the effects of TCS were conducted in mice, rats, hamsters, and rabbits. CD1 mice were given TCS through the diet in varying doses (0, 10, 25, 75, or 350 mg/kg/day) from GDs 6 to 15 and were sacrificed and necropsied on GD 18. No significant effects were observed on the number of corpora lutea, implantations, resorptions, and litter size. Offspring of dams given TCS at doses of 75 or 350 mg/kg/day exhibited decreased fetal body weight and a significant increase in the incidence of irregular ossification of the skull. These skeletal alterations were considered to be transient, developmental delays related to the decreased fetal body weights (Christian and Hoberman 1992, Rodricks et al. 2010). Pregnant CD rats received TCS through oral gavage from GDs 6 to 15 (15, 50, or 150 mg/kg/day). No consistent, treatment-related maternal toxicity was detected at any of the TCS doses, nor were effects reported for such uterine parameters as number of corpora lutea, implantations, resorptions, or live fetuses. At a TCS dose of 150 mg/kg/day, the only observation suggestive of a treatment-related effect was delayed ossification of certain skeletal bones (Schroeder and Daly 1992a, Rodricks et al. 2010). In another developmental study, TCS was administered to Colworth Wistar rats (30, 100, or 300 mg/kg/day) from GDs 6 to 15. The high dose reduced maternal body weight gain transiently that was not evident at termination on GD 21. No effects of TCS were observed on maternal uterine parameters or on fetal survival and development (Denning et al. 1992, Rodricks et al. 2010). A developmental study conducted in New Zealand white rabbits involved administration of TCS (15, 50, or 150 mg/kg/day) through gastric intubation from GDs 6 to 18. At the highest dose of TCS maternal body weights, weight gain, and food consumption were significantly decreased. However, no effects on uterine parameters or fetal effects were reported at any dose (Schroeder and Daly 1992b, Rodricks et al. 2010). Finally, Syrian hamsters received TCS through oral gavage (4, 8, 16, 40, or 80 mg/kg/day) from GDs 6 to 10 and were sacrificed and necropsied at GD 16. No consistent effects related to TCS treatment in dams or fetuses were reported (Piekacz 1978, Rodricks et al. 2010).

Epidemiologic studies

Several human epidemiologic studies have examined the relationship between urinary TCS levels and reproductive and developmental endpoints, largely with negative results. Among these were the absence of associations between maternal urinary TCS and birth outcomes, such as birth weight, birth length, gestational age, and head circumference (Wolff et al. 2008, Philippat et al. 2012), and male genital cryptorchidism (Chevrier et al. 2012). No significant associations were observed between urinary TCS in girls aged 6–8 and BMI (Wolff et al. 2007) and that of girls aged 12–16 (NHANES) and age at menarche (Buttke et al. 2012). The relationship between urinary TCS in girls aged 6–9 and breast and pubic hair stage revealed a small inverse association with pubic hair stage that was marginal and nonmonotonic, as well as no association for breast stage (Wolff et al. 2010). Finally, a case-control study involving 877 cases and 713 controls

revealed no statistical relationship between urinary levels of TCS and idiopathic male infertility (Chen et al. 2013).

Analysis and conclusions regarding TCS effects on gestation and postpartum development

The two in vitro studies addressing the effects of TCS on gestation are of limited value with regard to their relevance to health effects in humans. The report of James et al. (2010) which examined TCS effects on sufotransferase activity from sheep placenta is mainly an enzyme kinetic study employing cytosol from disrupted cells. While it might be of value in gaining insights about substrate-enzyme interactions, placental cytosol lacks, as a model, the necessary elements of the living organism or even the interface between the extracellular fluid and the intact cell. Honkisz et al. (2012) observed modest changes in estradiol, progesterone, and β-hCG secretion as well as what appear to be adaptive responses in human choriocarcinoma cells at a wide range of TCS concentrations, some of which are approaching that of human maternal and umbilical serum. However, it should be borne in mind that this cell line is an in vitro model derived from neoplastic cells and does not represent the normal in utero environment.

Most of the available in vivo literature does not convincingly support the concept that TCS adversely affects gestation and postpartum development. Paul et al. (2010b, 2012) observed no evidence of adverse effects to dams and offspring through gestation involving exposures of TCS as high as 300 mg/kg/day. Most of the endpoints examined by Rodríguez and Sanchez (2010) in dams and offspring after exposure to TCS up to 50 mg/kg/day (e.g., pregnancy rate, gestation length, litter size, number of implantation sites, and weaning index) were unaffected, although a decline in live birth index, 6-day survival, male:female sex ratio, and pup weight were observed. While no data were presented supporting their hypothesis that TCS might adversely affect the milk ejection reflex, the suggestion that TCS might impair the thyroid system in offpsring is not consistent with the available evidence. As discussed previously, Paul et al. (2012) reported that the modest hypothyroxinemia is a transient event in TCS-exposed offspring at PND 4 which is restored to normal at PNDs 14 and 21. Paul et al. (2012) also reported that serum TCS levels declined postpartum consistent with the fact that lactation is an inefficient delivery system for the xenobiotic. The observations of Axelstad et al. (2013) with regard to maternal toxicity, fetotoxicity, and developmental toxicity are, for the most part, in agreement with the data of Paul et al. (2012). The observation of Crawford and deCantanzaro (2012) of reduced implantation sites in pregnant mice on GD 6 required treatment on GDs–3 with enormous doses of (500–800 mg/kg/day) of TCS administered parenterally. In addition, a variety of studies in several mammalian species provided little evidence that TCS adversely affects the process of gestation and postpartum development of offspring with the exception of transient decreases in pup body weights and delayed ossification at high doses. Finally, the epidemiologic literature does not support a statistical relationship between urinary TCS levels and adverse effects on gestation and development consistent with animal data.

Overall summary and conclusion

The animal studies reviewed herein reveal that TCS exposure in vivo consistently produces a decrease in serum T4 in rats without any consistent change in other thyroid-related endpoints. There is no consistent evidence of adverse effects resulting from this hypothyroxinemia. The evidence supporting the model proposed to explain this hypothyroxinemia in the rat, accelerated hepatic clearance of T4 mediated by PXR and CAR pathways is not conclusive. In view of marked species differences between rats and humans with regard to sensitivity of the thyroid to xenobiotics, it is unlikely that TCS-induced hypothyroxinemia occurs in humans. Furthermore, human data show no evidence of adverse effects of TCS exposure through personal care products use and thyroid disruption. Further research in rats comparing the effects TCS with other xenobiotics (e.g., 3-MC and Aroclor 1254) might add insight regarding mechanisms responsible for hypothyroxinemia independent of changes in serum TSH.

The fact that TCS binds with low affinity to both the AR and ER in vitro and evokes a diverse array of modest response profiles is not unexpected and indicative of the promiscuity of the steroid receptor. The ability of a xenobiotic to interact with a receptor for an endogenous substance does not necessarily mean that it represents an environmental risk. The data suggesting adverse effects of TCS on male or female reproduction are inconsistent, while the prevailing historical literature shows little evidence that TCS adversely affects the male or female reproductive systems. Because only a limited number of recent studies of TCS effects on male and female reproduction have been conducted (Tables 3 and 4, respectively), further studies of this sort might resolve any inconsistencies. Additional research may also provide further insight as to the physiological significance, if any, of possible interactions between TCS and androgens and estrogens at target cells.

In vitro data dealing with TCS effects in utero have little relevance to either the in vivo condition or human health risk. The majority of animal laboratory studies, both reviewed herein or reviewed in detail elsewhere show little evidence that TCS exposure adversely affects gestation or postpartum development of offspring. This is consistent with the epidemiologic literature which does not support an adverse effect of TCS exposure in utero or postpartum.

Finally, doses of TCS required to produce hypothyroxinemia, and occasional effects on male and female reproduction, gestation, and offspring in animal studies are several orders of magnitude greater than estimated exposure levels of TCS in humans. On the basis of this analysis of the literature, it is concluded that TCS does not present a risk of endocrine disruptive health effects through exposure to personal care products.

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