



Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice



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ABSTRACT

Triclosan, an antimicrobial agent added to personal care products, can modulate estrogenic actions. We investigated whether triclosan affects concentrations of exogenous and endogenous estradiol. Female mice were given injections of triclosan followed by 1 μ Ci tritium-labeled estradiol. Mice given daily 2-mg triclosan doses (57.9 mg/kg/dose) showed significantly elevated radioactivity in tissues and serum compared to controls. A single dose of 1 or 2 mg triclosan increased radioactivity in the uterus in both cycling and peri-implantation females. We also measured natural urinary estradiol at 2–12 h following triclosan injection. Unconjugated estradiol was significantly elevated for several hours following 1 or 2 mg of triclosan. These data are consistent with evidence that triclosan inhibits sulfonation of estrogens by interacting with sulfotransferases, preventing metabolism of these steroids into biologically inactive forms. Elevation of estrogen concentrations by triclosan is potentially relevant to anti-reproductive and carcinogenic actions of excessive estrogen activity.

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1. Introduction

Triclosan (CAS 3380-34-5) is a synthetic biocide designed to inhibit bacterial reproduction by interacting with enoyl-acyl carrier protein reductase enzymes [1]. It is added to many consumer and household products, including soaps, dish sponges, cosmetics, deodorants, toothpastes, mouthwashes, clothing, and children's toys [2–4]. Dermal contact with these products leads to rapid absorption of triclosan into the body through the skin [5,6], while oral ingestion leads to uptake through the gastrointestinal tract [7]. Based on the 2003–2004 U.S. National Health and Nutrition Examination Survey (NHANES), 74.6% of the 2517 human urine samples contained detectable levels of triclosan, with concentrations ranging from 2.4 to 3790 μ g/l [8]. Detection frequency of urinary triclosan in the U.S. population reached a peak between 2007 and 2008 at 80.8%, but has since fallen to 72.0% as of 2011–2012 [9]. Similarly, mean urinary triclosan concentrations in the U.S. population peaked in 2005–2006 at 18.8 μ g/L but fell to 12.46 μ g/l as of 2011–2012 [9]. Triclosan has also been detected in human serum [10,11], plasma [12], breast milk [12,13], and adipose and liver tissue [14].

Triclosan has known estrogenic effects, including stimulating breast and ovarian cancer cell growth *in vitro* [15,16] and magnify-

ing the effects of ethinyl estradiol in rodent uterotrophic assays [17,18]. However, the mechanisms underlying these effects are not well understood. Triclosan binds to both conventional estrogen receptor (ER) subtypes, ER α and ER β [19,20]. Thus, exposure to triclosan may induce estrogenic effects by directly activating ER. Triclosan also potently inhibits hepatic sulfotransferase activity [21–23], thereby reducing sulfonation of endogenous estrogens such as 17 β -estradiol (E₂) and xenoestrogens such as bisphenol A (BPA) [23]. Thus, exposure to triclosan may potentiate *in vivo* estrogenic effects by preventing metabolism of estrogens to their biologically inactive forms.

Previous work in this laboratory demonstrated *in vivo* interactions between triclosan and BPA. When mice were given a single dose of triclosan ranging from 0.2–18 mg, greater levels of ¹⁴C-BPA were detected in serum and tissues including the heart, lung, muscle, uterus, ovaries, and epididymides, than in animals given ¹⁴C-BPA alone [24]. Other studies indicated that either triclosan or BPA can disrupt blastocyst implantation in inseminated female mice [25–29], and that doses of BPA or triclosan that were insufficient on their own to have effects could disrupt implantation when the two substances were given concurrently [29]. These findings are consistent with the notion that triclosan inhibits BPA conjugation [23], permitting higher levels of BPA to interact with ER in tissues such as the uterus.

Whereas BPA is a weakly estrogenic environmental chemical, E₂ is the most potent natural estrogen. Any deviations from normal E₂ levels might lead to adverse health effects, as estrogen levels

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are tightly regulated and play critical roles in development, fertility, and behavior [30]. Of especial importance to human health is the consistent finding that elevated E_2 , often through hormone-replacement therapy, is associated with an increased risk of breast [31], endometrial [32], and ovarian [33] cancers. Also, in inseminated females, minute elevations in estrogen activity can impede blastocyst implantation, leading to pregnancy failure [30,34]. Given the fact that triclosan exposure is ubiquitous, its potential capacity to modulate estrogen levels or activity *in vivo* is worthy of investigation. Here we investigated the impact of single or repeated triclosan injections on concentrations of exogenous tritium-labeled estradiol ($^3\text{H-E}_2$) and endogenous urinary E_2 . We hypothesized that a single injection of triclosan would elevate $^3\text{H-E}_2$ levels in reproductive tissues of cycling and peri-implantation female mice, and that this effect would be more pronounced with repeated triclosan injections over multiple days. We also hypothesized that triclosan administration would increase endogenous E_2 concentrations as measured in urine.

2. Materials and methods

2.1. Animals and housing

Female mice aged 3–5 months were of CF-1 strain and obtained from Charles River (Kingston, NY). Animals were housed in standard polypropylene cages measuring $28 \times 16 \times 11$ (l \times w \times h) cm with wire tops allowing *ad libitum* access to food (8640 Teklad Certified Rodent Chow; Harlan Teklad, Madison, WI) and water, except where otherwise stated. The colony was maintained at 21°C with a reversed 14 h light:10 h darkness cycle. All procedures adhered to the standards of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

2.2. Chemicals and materials

Triclosan (CAS 3380-34-5, 5-chloro-2-[2,4-dichlorophenoxy]phenol, $\geq 97\%$ purity) was obtained from Sigma-Aldrich, St. Louis, MO. SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, 8 ml midi-vial scintillation vials, and [2,4,6,7- ^3H](N)- E_2 (stock solution in ethanol, $1.0 \mu\text{Ci}/\mu\text{l}$, $81.0 \text{ Ci}/\text{mmol}$) were obtained from PerkinElmer, Waltham, MA. E_2 and creatinine standards were obtained from Sigma-Aldrich, Oakville, ON, Canada. E_2 antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA.

2.3. Experiment 1: measurement of $^3\text{H-E}_2$ in cycling females after repeated triclosan doses

At the onset of darkness on the first day of the experiment, 21 cycling female mice with regular estrous cycles were weighed ($39.4 \pm 5.6 \text{ g}$), individually housed, and each given a subcutaneous (sc) injection of 0, 1, or 2 mg triclosan (corresponding to 0.0 ± 0.0 , 23.2 ± 2.5 , or $57.9 \pm 3.9 \text{ mg triclosan}/\text{kg}$ bodyweight, respectively) dissolved in 0.05 ml peanut oil ($n = 7$ per dose). These injections were repeated at the same time on days 2 through 7, such that each mouse had a total of 7 injections of the same dose. We rotated injection locations of triclosan among the neck, right flank, and left flank to prevent irritation of the injection site. At 24 h after the final triclosan dose, each animal was given an intraperitoneal (ip) injection of $1 \mu\text{Ci } ^3\text{H-E}_2$ (corresponding to $3.36 \text{ ng } E_2$) in $9 \mu\text{l}$ phosphate-buffered saline (PBS). At 1 h after $^3\text{H-E}_2$ administration, each animal was anesthetized with isoflurane and blood was collected via cardiac puncture. Each animal was perfused with 15 ml PBS and tissues were collected in pre-weighed scintillation vials. Tissue samples

taken include the heart, lung, liver, superficial adductor muscle from the hind leg, the whole uterus, both ovaries, and a cross-section of the kidney encompassing both the medulla and cortex. Vials were re-weighed following tissue collection to determine the sample wet mass.

We administered triclosan via sc injection in all experiments, as the presence of triclosan in personal care products can lead to transdermal absorption. Percutaneous penetration is incomplete compared to sc injection; one estimate suggests that transdermal absorption of triclosan is $6.3 \pm 1.1\%$ in humans and $22.8 \pm 4.6\%$ in rats within 24 h [5]. The 1 and 2 mg triclosan doses were chosen based on the lowest effective doses in a previous study showing an interaction between triclosan and $^{14}\text{C-BPA}$ [24]. $^3\text{H-E}_2$ was given ip to facilitate systemic distribution of the steroid [35], and the $1 \mu\text{Ci}$ dose of $^3\text{H-E}_2$ represents just a small fraction of the animals' endogenous E_2 [35,36]. Finally, the 24-h latency between triclosan injections and between the final triclosan injection and $^3\text{H-E}_2$ administration, were chosen to exceed the $10.8 \pm 6.3 \text{ h}$ terminal elimination half-life of dermally administered triclosan [6].

Blood and tissue samples were processed for liquid scintillation counting following previously published procedures used in this laboratory [24,37]. In brief, blood samples were centrifuged at $1,500\text{g}$ for 10 min and $10 \mu\text{l}$ serum was added to a scintillation vial containing 5 ml Ultima Gold. Tissue samples were solubilized by adding 1 ml SOLVABLE to each vial and placing vials in a 50°C water bath for 4–5 h until completely dissolved. Following the addition of 5 ml Ultima Gold, vials were agitated to promote mixing of the sample and scintillation cocktail. Each vial was stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer (PerkinElmer) for 5 min to eliminate background noise in the form of heat and luminescence. Radioactivity was measured for 5 min per vial and final adjusted estimates for the amount of radioactivity per sample in disintegrations per minute (dpm) were automatically calculated by the accompanying Quanta-Smart software package. The dpm measures were then reported as either dpm/mg tissue or dpm/ μl serum. A reported concentration of 1 dpm/mg tissue or 1 dpm/ μl serum is equivalent to $1.52 \text{ pg}/\text{g}$ tissue or $1.52 \text{ pg}/\text{ml}$ serum, respectively.

2.4. Experiment 2: measurement of $^3\text{H-E}_2$ in cycling females after a single triclosan dose

At the onset of darkness, 20 cycling female mice were weighed ($40.7 \pm 4.1 \text{ g}$), individually housed, and each given a single sc injection of 0, 0.6, 1, or 2 mg triclosan (respectively 0.0 ± 0.0 , 14.6 ± 1.7 , 23.6 ± 2.9 , or $53.9 \pm 3.4 \text{ mg}/\text{kg}$) dissolved in 0.05 ml peanut oil ($n = 5$ per dose). At 30 min after triclosan administration, each animal was given an ip injection of $1 \mu\text{Ci } ^3\text{H-E}_2$ ($3.36 \text{ ng } E_2$) in $9 \mu\text{l}$ PBS. At 1 h after $^3\text{H-E}_2$ administration, each animal was anesthetized with isoflurane. All other procedures, including perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those of Experiment 1. The 30-min latency between triclosan and $^3\text{H-E}_2$ and the 1-h latency between $^3\text{H-E}_2$ administration and tissue collection were chosen based on a previous study demonstrating an interaction between triclosan and $^{14}\text{C-BPA}$ [24]. A 1-h latency between $^3\text{H-E}_2$ administration and tissue collection permits systemic distribution of $^3\text{H-E}_2$ [38].

2.5. Experiment 3: measurement of $^3\text{H-E}_2$ in inseminated females after a single triclosan dose

We examined the influences of triclosan upon peri-implantation inseminated females given the potential relevance to blastocyst implantation failure. This should also produce less within-condition variance as endogenous E_2 dynamics are less variable than among cycling females. Sexually naïve females were each

paired with a male mouse aged 5–6 months and their hindquarters were inspected four times daily for the presence of a vaginal copulatory plug. Following detection of a copulatory plug, which marked gestation day 0, females were weighed (28.5 ± 1.6 g) and individually housed. At the onset of darkness on gestation day 3, females were each given a single sc injection of 0 or 2 mg triclosan (respectively 0.0 ± 0.0 or 69.9 ± 3.0 mg/kg) dissolved in 0.05 ml peanut oil. In Experiment 3A, each animal was given an ip injection of $1 \mu\text{Ci}$ $^3\text{H-E}_2$ (3.36 ng E_2) in $9 \mu\text{l}$ PBS at 1 h after triclosan administration ($n = 10$ per dose). In Experiment 3B, each female was given $^3\text{H-E}_2$ at 7 h after triclosan administration ($n = 10$ per dose). In both Experiments 3A and 3B, each animal was anesthetized with isoflurane at 1 h after $^3\text{H-E}_2$ administration. All other procedures, including perfusion, blood and tissue collection, sample processing, and scintillation counting were identical to those of Experiment 1. Here we also collected select brain areas including the olfactory bulbs, a caudal portion of the cerebellum, a section of the cortex, and a portion of the hypothalamic region (posterior to the optic chiasm and anterior to the pituitary stalk on the ventral surface of the brain). Neural tissue samples were processed and measured for radioactivity as were peripheral tissues in Experiment 1. We selected the 2 mg triclosan dose for use in peri-implantation females, as this dose was most effective in Experiments 1 and 2. The 1- and 7-h latencies between triclosan and $^3\text{H-E}_2$ administrations were chosen to determine whether the effects of a single triclosan injection on $^3\text{H-E}_2$ concentrations persisted for several hours after exposure. Neural tissue samples were collected for this experiment to contrast female reproductive tissues with regions of the brain expressing high concentrations of ER.

2.6. Experiment 4: measurement of urinary E_2 in cycling females given triclosan

Twenty female mice were weighed (29.9 ± 2.2 g) and individually placed in a Plexiglas apparatus measuring $21 \times 15 \times 13$ ($l \times w \times h$) cm with a wire-mesh grid floor with squares measuring 0.5 cm^2 . The wire-mesh grid floor was raised approximately 1 cm above a Teflon-coated stainless-steel surface covered with wax paper, which permitted non-invasive collection of urine. Mice were allowed to acclimate to the novel cages for 3 days prior to the start of the experiment. At the onset of darkness on the first day of the experiment, each female received a single sc injection of 0.05 ml peanut oil. Given the effects of a single triclosan exposure on $^3\text{H-E}_2$ concentrations for up to 7 h in Experiment 3, urine was collected at 2, 4, 6, 8, 10, and 12 h post-injection. At the onset of darkness on day two, each mouse received a sc injection of 1 or 2 mg triclosan (respectively 33.5 ± 2.1 or 67.4 ± 5.8 mg/kg) dissolved in 0.05 ml peanut oil ($n = 10$ per dose). Urine was once again collected at 2, 4, 6, 8, 10, and 12 h post-injection.

All urine samples were placed into labeled vials and frozen at -20°C at the time of collection. Full procedures and validations for enzyme immunoassays for mouse urine were previously reported [39]. Cross-reactivities for anti- E_2 are: E_2 100%, estrone 3.3%, P_4 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids $<0.1\%$. Urinary E_2 levels were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and reported as ng/mg creatinine and ng/ml urine, respectively.

2.7. Experiment 5: measurement of urinary E_2 in inseminated females given triclosan

Thirty female mice were each paired with a male mouse and their hindquarters inspected as in Experiment 3. Following detection of a copulatory plug, which marked gestation day 0, females were weighed (28.3 ± 1.5 g) and placed in a Plexiglas apparatus

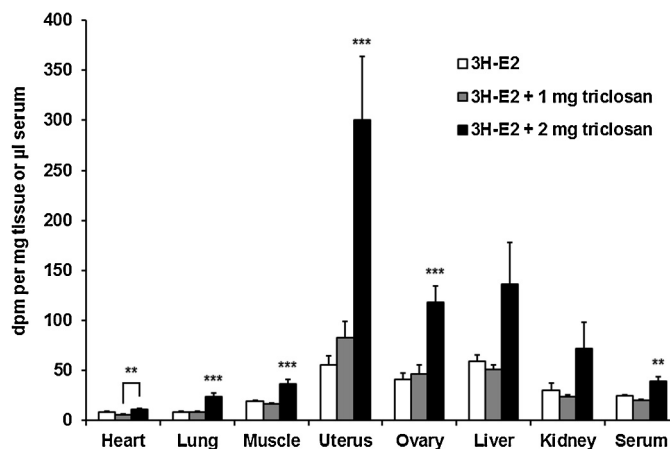


Fig. 1. Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/ μl serum) in the heart, lung, muscle, uterus, ovary, liver, kidney, and serum of cycling females following 7 daily sc injections of 0, 1, or 2 mg triclosan and subsequent ip injection of $1 \mu\text{Ci}$ $^3\text{H-E}_2$ ($n = 7$ per dose) in Experiment 1. Unless otherwise noted, asterisks indicate that a condition differs significantly from the 0 mg and 1 mg triclosan treatments in the same tissue: ** $p < 0.01$; *** $p < 0.001$.

with a wire-mesh grid floor, then permitted to acclimate to the cages for 3 days. At the onset of darkness on gestation day 3, each female received a single sc injection of 0 or 2 mg triclosan (respectively 0.0 ± 0.0 or 71.0 ± 3.7 mg/kg) dissolved in 0.05 ml peanut oil ($n = 15$ per dose). Urine was collected at 2, 4, 6, 8, 10, and 12 h post-injection. All other procedures, such as urine sample collection and storage, as well as enzyme immunoassay measurements, were identical to those of Experiment 4.

2.8. Statistical analyses

All statistical analyses were performed using the R software environment [40]. Data are presented as mean + standard error (SE). A comparison-wise error rate of $\alpha < 0.05$ was employed for all statistical tests. In Experiment 1, differences between $^3\text{H-E}_2$ treatments were analyzed by univariate analysis of variance (ANOVA) for each tissue, with Holm-Bonferroni adjustments to correct for the number of tissues [41]. Significant effects in ANOVA were followed by pair-wise Newman-Keuls multiple comparisons. Given that triclosan had especially strong influences in the uterus on the presence of $^3\text{H-E}_2$ in Experiment 1 and the presence of $^{14}\text{C-BPA}$ in a previous study [24], we focused statistical comparisons on this tissue in subsequent experiments. In Experiments 2 and 3, concentrations of $^3\text{H-E}_2$ in the uteri of animals given triclosan were compared to those of animals given an oil injection by two-tailed independent-samples t -test. In Experiments 4 and 5, differences between urinary E_2 levels were analyzed by ANOVA comparing the effect of treatment on the volume-based and creatinine-corrected urinary E_2 concentrations. Significant effects in ANOVA were followed by t -tests at each urine collection time point, with Holm-Bonferroni adjustments to correct for the number of time points.

3. Results

3.1. Experiment 1: measurement of $^3\text{H-E}_2$ in cycling females after repeated triclosan doses

Radioactivity was measured in the peripheral tissues of cycling females that received daily sc injections of triclosan followed by an ip injection of $1 \mu\text{Ci}$ $^3\text{H-E}_2$ (Fig. 1). The impact of triclosan on $^3\text{H-E}_2$ concentrations was most prominent in the reproductive tissues of females; mice given 2 mg triclosan had mean radioac-

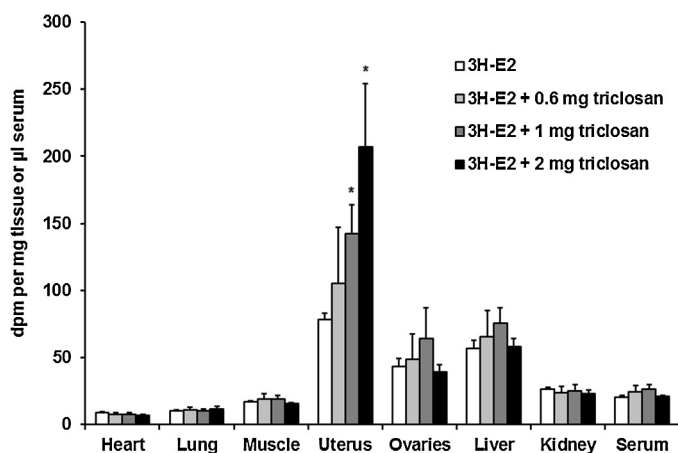


Fig. 2. Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/ μ l serum) in the heart, lung, muscle, uterus, ovary, liver, kidney, and serum of cycling females following a single sc injection of 0, 0.6, 1, or 2 mg triclosan and subsequent ip injection of 1 μ Ci 3 H-E₂ (n = 5 per dose) in Experiment 2. Significant difference from 0 mg treatment in the same tissue: * p < 0.05.

tivity levels that were 5.4 and 2.9 times higher than those of controls in the uterus and ovaries respectively. Statistical comparisons were made among the three treatments for each of eight tissues. ANOVA followed by Holm-Bonferroni correction produced significant effects of treatment for the heart, $F(2,18) = 6.05$, $p = 0.029$; lung, $F(2,18) = 13.51$, $p = 0.002$; muscle, $F(2,18) = 17.23$, $p < 0.001$; uterus, $F(2,18) = 12.02$, $p = 0.002$; ovary, $F(2,18) = 14.82$, $p = 0.001$; and serum, $F(2,18) = 10.42$, $p = 0.004$. Multiple comparisons revealed that the 2 mg triclosan dose differed from the 0 and 1 mg triclosan doses for the lung, muscle, uterus, ovary, and serum. The 2 mg triclosan dose also differed from the 1 mg triclosan dose for the heart.

3.2. Experiment 2: measurement of 3 H-E₂ in cycling females after a single triclosan dose

Radioactivity was measured in the peripheral tissues of cycling females that received a single sc injection of triclosan followed by an ip injection of 1 μ Ci 3 H-E₂ (Fig. 2). Unlike repeated injections over several days, a single exposure of triclosan did not significantly modulate 3 H-E₂ concentrations in serum or peripheral tissues other than the uterus. Although 3 H-E₂ concentrations were greater in the uteri of mice given 2 mg triclosan, the magnitude of elevation was less than that in Experiment 1; mean radioactivity levels were 2.6 times (versus 5.4 times in Experiment 1) higher than those of controls in the uterus. Statistical comparisons were made between the 0 mg triclosan treatment and the 0.6, 1, or 2 mg triclosan treatments for the uterus. Two-tailed independent-samples *t*-test of treatment effects showed that, compared to animals given 0 mg triclosan, the level of radioactivity in the uterus was significantly greater in animals given 1 mg triclosan, $t(8) = 2.90$, $p = 0.020$, and in animals given 2 mg triclosan, $t(8) = 2.70$, $p = 0.027$.

3.3. Experiment 3: measurement of 3 H-E₂ in inseminated females after a single triclosan dose

Radioactivity was measured in the peripheral tissues of inseminated females on gestation day 3 that received a single sc injection of triclosan followed by an ip injection of 1 μ Ci 3 H-E₂ (Fig. 3A and B). As in Experiments 1 and 2, the uterus showed the most pronounced effects; mean radioactivity levels were 1.4 and 2.1 times greater respectively at 1- and 7-h between triclosan and 3 H-E₂ exposures. Statistical comparisons were made between the 0 mg and 2 mg tri-

closo treatments for the uterus. Two-tailed independent-samples *t*-test of treatment effects showed that, when females were given 3 H-E₂ at 1 h after injection of vehicle or triclosan (Experiment 3A), the level of radioactivity in the uterus was significantly greater in animals given 2 mg triclosan, $t(18) = 2.46$, $p = 0.024$. When females were given 3 H-E₂ at 7 h after triclosan administration (Experiment 3B), the level of radioactivity in the uterus was once again significantly greater in animals given 2 mg triclosan, $t(18) = 3.62$, $p = 0.002$.

3.4. Experiment 4: measurement of urinary E₂ in cycling females given triclosan

Urinary E₂ levels of cycling females were measured the day prior and the day of a single injection of triclosan (Fig. 4A–D). Generally, the effect of triclosan on urinary E₂ concentrations was most prominent at around 6–10 h after injection. Repeated-measures ANOVA produced significant effects of treatment for the creatinine-corrected 1 mg triclosan dose, $F(1,53) = 11.66$, $p = 0.001$; the volume-based 2 mg triclosan dose, $F(1,53) = 17.59$, $p < 0.001$; and the creatinine-corrected 2 mg triclosan dose, $F(1,53) = 26.09$, $p < 0.001$. For the creatinine-corrected 1 mg triclosan dose, repeated-measures *t*-tests followed by Holm-Bonferroni correction produced significant effects of treatment at 6 h after injection, $t(9) = 3.66$, $p = 0.032$, and 10 h after injection, $t(6) = 2.59$, $p = 0.041$. For the volume-based 2 mg triclosan dose, significant effects of treatment were observed at 6 h after injection, $t(9) = 3.38$, $p = 0.040$, and 8 h after injection, $t(9) = 3.91$, $p = 0.021$. Similarly, for the creatinine-corrected 2 mg triclosan dose, significant effects of treatment were observed at 6 h after injection, $t(9) = 4.12$, $p = 0.013$, and 8 h after injection, $t(9) = 4.23$, $p = 0.013$.

3.5. Experiment 5: measurement of urinary E₂ in inseminated females given triclosan

Urinary E₂ levels of inseminated females on gestation day 3 were measured after a single sc injection of 0 or 2 mg triclosan (Fig. 5A and B). The effect of triclosan on urinary E₂ concentrations was evident throughout the 2–12 h collection window; however, the effect was most prominent at around 10–12 h after injection. ANOVA produced significant effects of treatment for the volume-based measures, $F(11,64) = 27.87$, $p < 0.001$, and the creatinine-corrected measures, $F(11,64) = 59.25$, $p < 0.001$. For the volume-based 2 mg triclosan dose, *t*-tests followed by Holm-Bonferroni correction produced significant effects of treatment at 4 h after injection, $t(27) = 3.39$, $p = 0.013$, and 10 h after injection, $t(28) = 2.92$, $p = 0.034$. For the creatinine-corrected 2 mg triclosan dose, significant effects of treatment were observed at 2 h after injection, $t(28) = 3.00$, $p = 0.017$; 4 h after injection, $t(27) = 3.35$, $p = 0.010$; 8 h after injection, $t(27) = 2.90$, $p = 0.017$; 10 h after injection, $t(28) = 3.98$, $p = 0.002$; and 12 h after injection, $t(26) = 4.09$, $p = 0.002$.

4. Discussion

To the best of our knowledge, these data are the first to show that triclosan modulates the concentrations of exogenous and endogenous E₂ *in vivo*. We have shown this using two strategies. First, we found enhanced uptake of 3 H-E₂ in the uterus and other tissues of female mice that received triclosan. Second, after triclosan administration, we observed an elevation in endogenous E₂ as reflected in urinary measures. We measured urinary E₂ given evidence that enzyme immunoassay cannot reliably quantify E₂ in mouse serum due to extensive binding to carrying molecules in blood [42]. Moreover, whereas previous evidence found very low concentrations of

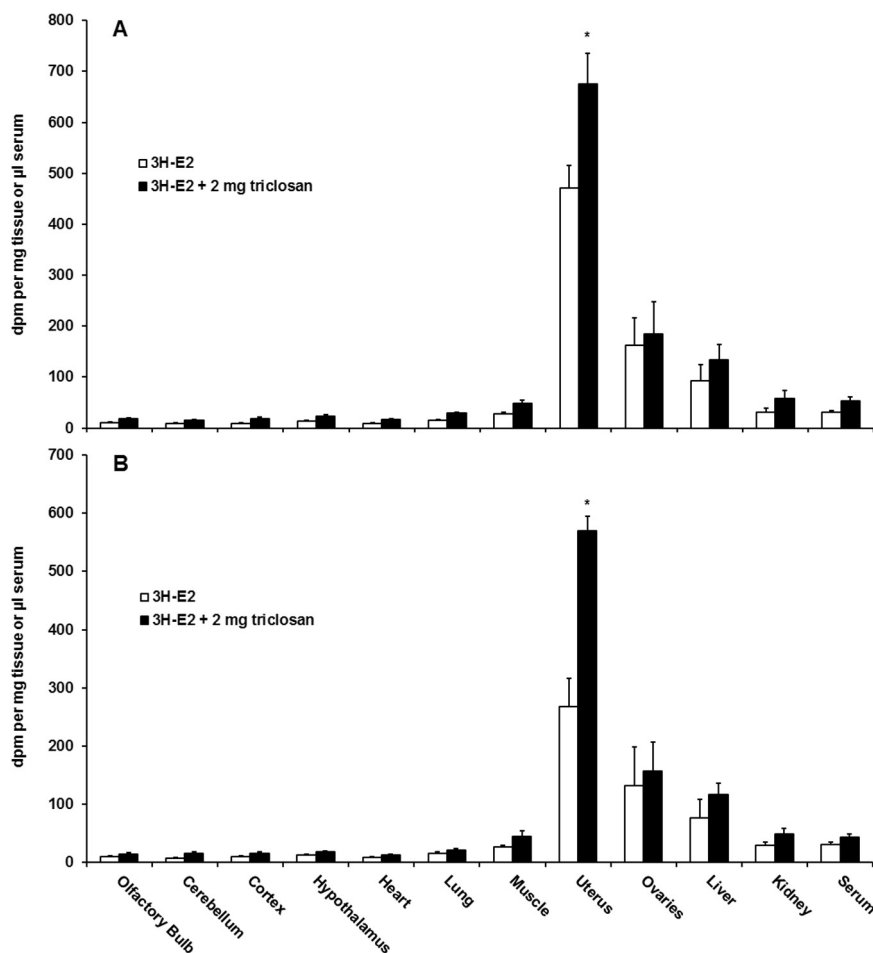


Fig. 3. Mean (+SE) concentration of radioactivity (dpm/mg tissue or dpm/ μ l serum) in the olfactory bulb, cerebellum, cortex, hypothalamus, heart, lung, muscle, uterus, ovary, liver, kidney, and serum of inseminated females on gestation day 3 following ip injection of 1 μ Ci 3 H-E₂ at (A) 1 h or (B) 7 h after a single sc injection of 0 or 2 mg triclosan (n = 10 per dose per time point) in Experiment 3. Significant difference from 0 mg treatment in the same tissue: * p < 0.05.

estrogen conjugates in mouse urine [39], unconjugated E₂ is abundant there and it generally reflects systemic trends [39,43–46].

There are several potential mechanisms through which triclosan and E₂ could interact, including actions at estrogen receptors, blood binding factors, and enzymes involved in steroid metabolism. Although there is some evidence that triclosan can interact with estrogen receptors, such action is competitive with binding of E₂ [19,20]. That would suggest an opposite effect to the finding in our data that triclosan magnified 3 H-E₂ presence in the uterus and other tissues. Similarly, competition for blood binding factors would presumably reduce E₂ presence in serum and tissues. A strong possibility is that our findings arise from triclosan's actions on sulfotransferase. In *in vitro* human liver fractions, low nM to μ M concentrations of triclosan can potently inhibit estrone and E₂ sulfonation by non-competitively interacting with various sulfotransferase isoforms, including SULT 1E1, 1B1, and 1A1 [21,22]. Given that a considerable portion of triclosan present in the body is localized within the liver [14], we suggest that triclosan may interact with hepatic sulfotransferases to inhibit the conjugation of exogenous and endogenous E₂.

We found that triclosan magnified radioactivity in the heart, lungs, muscle, uterus, ovaries, and blood serum of females given 3 H-E₂. The chosen dose of 3 H-E₂ represented just a small fraction of the animals' endogenous E₂ [35]. The interaction of triclosan with exogenous 3 H-E₂ may be relevant to human estrogen supplementation, albeit with some caveats. Whereas we injected 3 H-E₂ ip, human estrogen supplementation usually involves E₂ derivatives

such as ethinyl estradiol [47] administered via an oral pill, transdermal patch, or vaginal ring [47]. However, triclosan can potentiate the estrogenic effects of ethinyl estradiol in rats [17,18]. In all experiments involving 3 H-E₂ administration, the uterus showed the highest concentrations in vehicle-treated animals. In addition, we observed the greatest impact of triclosan exposure on the uterus, with the 2-mg triclosan dose elevating concentrations by 140 to 540% across experiments. Whereas repeated triclosan doses increased concentrations of 3 H-E₂ in serum and most peripheral tissues, only concentrations in the uterus were significantly elevated following a single triclosan injection. The localization of 3 H-E₂ to the female reproductive tract is consistent with the high expression of ER α and ER β in these tissues [48,49]. In humans, higher household incomes correlate with higher urinary triclosan concentrations [8] and greater use of hormonal contraceptives [50]; study of the effects of triclosan on estrogen supplementation in contraceptives and hormone-replacement therapy may be warranted.

We also showed elevated levels of endogenous urinary E₂ following triclosan administration. This effect was greater in recently inseminated females, spanning between 2 and 12 h after triclosan injection in the creatinine-corrected measures, compared to between 6 and 10 h in cycling females. This may be due to the reduced variance in urinary E₂ measures among inseminated females. Even slight elevations in E₂ levels can lead to adverse health and reproductive outcomes in human and other mammalian females. In humans, it is well established that persistently elevated E₂ levels correlate with an increased risk of breast [31,51], endome-

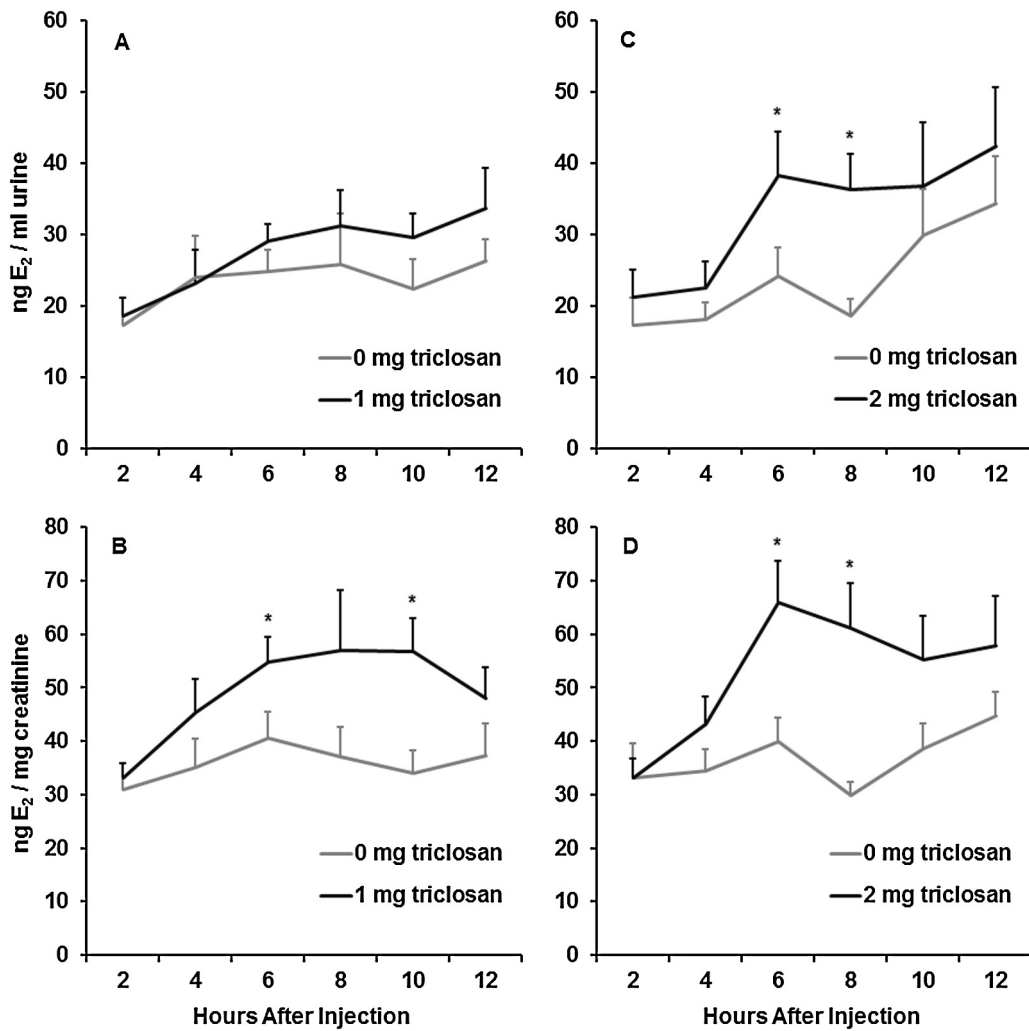


Fig. 4. Mean (+SE) level of urinary E₂ (ng E₂/ml urine and ng E₂/mg creatinine) before and after a single sc injection of (A, B) 1 mg or (C, D) 2 mg triclosan in cycling females (n = 10 per dose) in Experiment 4. Significant difference from 0 mg treatment at the same time point: * p < 0.05.

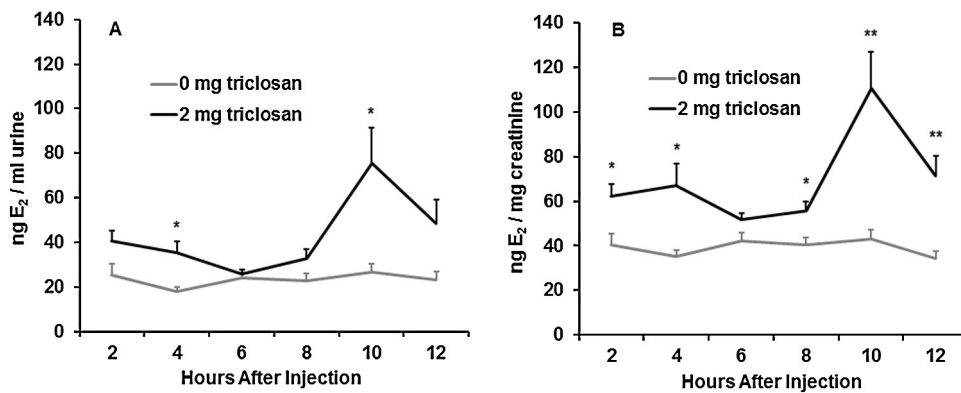


Fig. 5. Mean (+SE) level of urinary E₂ (A: ng E₂/ml urine, B: ng E₂/mg creatinine) following a single sc injection of 0 or 2 mg triclosan in inseminated females on gestation day 3 (n = 15 per dose) in Experiment 5. Significant difference from 0 mg treatment at the same time point: * p < 0.05; ** p < 0.01.

trial [32], and ovarian cancer [33]. In mice, elevated E₂ levels can disrupt pregnancy by preventing intrauterine blastocyst implantation [45]. These data are also consistent with the finding that triclosan can have estrogenic effects in the uterus, increasing uterine mass and expression of genes upregulated by natural estrogens [52].

At doses as low as 1 or 2 mg triclosan, we showed elevated concentrations of exogenous ³H-E₂ in reproductive tissues and natural E₂ in urine. The doses of triclosan in the present study are arguably higher than those to which most humans are exposed. Urinary measures from large samples of the U.S. population indicate widespread exposure to triclosan [8,9], but there are few published attempts to quantify this exposure. One study estimated that the average

daily intake of triclosan in U.S. individuals is 350 μg [53], which is equivalent to 5 $\mu\text{g}/\text{kg}$ for a 70 kg adult. No estimate of occupational exposure levels exists, which may be important considering the wide variance in human urinary triclosan concentrations, where the highest measured concentration was nearly 300 times greater than the geometric mean [8].

Our data are concordant with a mechanism whereby the non-competitive binding of triclosan to sulfotransferase and/or other metabolic enzymes inhibits E_2 metabolism. Our data do not support interaction of triclosan with ER as an agonist or antagonist, or interaction of triclosan with blood binding factors, which would produce opposing effects to those observed here. A number of environmental chemicals and their metabolites with chemical structures similar to that of triclosan have been shown to inhibit estrogen sulfotransferase, including polychlorinated biphenyls [54,55], polyhalogenated hydrocarbons [56], brominated flame retardants [57], and parabens [58]. Accordingly, concurrent exposure to multiple toxicants might lead to summation or other interactions of their effects. Given the potential carcinogenic and adverse reproductive influences of excessive estrogenic activity, further research is needed on such interactions and indirect estrogenic influences.

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