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Administration of low dose triclosan to pregnant ewes results in placental uptake and reduced estradiol sulfotransferase activity in fetal liver and placenta

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Abstract

Sulfonation is a major pathway of estrogen biotransformation with a role in regulating estrogen homeostasis in humans and sheep. Previous *in vitro* studies found that triclosan is an especially potent competitive inhibitor of ovine placental estrogen sulfotransferase, with K_{ic} of <0.1 nM. As the placenta is the main organ responsible for estrogen synthesis in pregnancy in both women and sheep, and the liver is another site of estrogen biotransformation, this study examined the effects of triclosan exposure of pregnant ewes on placental and hepatic sulfotransferase activity. Triclosan, 0.1 mg/kg/day, or saline vehicle was administered to late gestation fetal sheep for two days either by direct infusion into the fetal circulation or infusion into the maternal blood. On the third day, fetal liver and placenta were harvested and analyzed for triclosan and for cytosolic estradiol sulfotransferase activity. Placenta contained higher concentrations of triclosan than liver in each individual sheep in both treatment groups. There was a negative correlation between triclosan tissue concentration (pmol/g tissue) and cytosolic sulfotransferase activity (pmol/min/mg protein) towards estradiol. These findings demonstrated that in the sheep exposed to very low concentrations of triclosan, this substance is taken up into placenta and reduces estrogen sulfonation.

Keywords

Triclosan infusion; Estradiol sulfonation; Placenta; Sulfotransferase Inhibition

1. Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy) phenol (Figure 1), a halogenated diphenyl ether, is an antibacterial agent that was added to a number of personal care products

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including hand soaps, toothpastes, mouthwashes, bandages and clothing (Yueh and Tukey, 2016). As a result of its widespread use, triclosan has been found in wastewater, sewage treatment plants and sewage sludge around the world (Bester, 2005; Dhillon et al., 2015; Fernandes et al., 2011; Hua et al., 2005). The main routes of human exposure are thought to be buccal absorption, ingestion and dermal absorption (Bagley and Lin, 2000; Lin, 2000). Studies have documented the presence of triclosan in between 50 and 100 % of human blood and urine samples, depending on the population studied (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Arbuckle et al., 2015a; Arbuckle et al., 2015b; Calafat et al., 2009; Calafat et al., 2008; Geens et al., 2012; Liu et al., 2014; Philippat et al., 2013; Provencher et al., 2014; Pycke et al., 2014; Sandborgh-Englund et al., 2006; Velez et al., 2015; Weiss et al., 2015; Woodruff et al., 2011). Triclosan has also been measured in autopsy samples of liver, brain and adipose tissue (Geens et al., 2012).

The antibacterial properties of triclosan derive from its potent inhibition of the enoyl-acyl carrier protein reductase, an enzyme essential in fatty acid synthesis in bacteria (Levy et al., 1999; McMurry et al., 1998). By blocking the active site of the enzyme, triclosan prevents the bacteria from synthesizing fatty acid, which is necessary for reproducing and building cell membranes. Humans do not express this enzyme, so arguments were made that triclosan was unlikely to pose a risk to people (Dayan, 2007; Jones et al., 2000; Rodricks et al., 2010).

It has been found that triclosan exhibits biological activities other than its antibacterial function. Potential health concerns related to the use of triclosan include endocrine disruption, effects on drug- and hormone-metabolizing enzymes, the potential for development of antibiotic resistance, and the formation of carcinogenic by-products during triclosan degradation (Crofton et al., 2007; Gee et al., 2008; Levy, 2000, 2001; Schweizer, 2001; Stoker et al., 2010; Veldhoen et al., 2006; Wang et al., 2004; Zorrilla et al., 2009). Administered to pregnant sheep, triclosan was found to alter transcriptomics in the fetal hypothalamus (Rabaglino et al., 2016). Triclosan directly inhibits and indirectly induces certain phase II UDP-glucuronosyltransferase (UGT) and PAPS-sulfotransferase (SULT) enzymes (James et al., 2010; Wang et al., 2004; Wang and James, 2006; Zorrilla et al., 2009). Administration of triclosan (0 to 1000 mg/kg) to rats for 4 days induced dose-dependent increases in *Ugt1a1* and *Sult1c1* mRNA expression, and increased liver microsomal glucuronidation activity toward thyroxine, but did not affect mRNA expression of *Ugt1a6* or *2b5* and reduced mRNA expression of *Sult1b1* (Paul et al., 2010). It was shown that triclosan serves as a substrate and inhibitor of human hepatic glucuronidation and sulfonation detoxification pathways with drug and xenobiotic substrates (Wang et al., 2004). Triclosan was found to be an exquisitely potent competitive inhibitor of sheep placental estrogen sulfotransferase activity toward 17 β -estradiol with a K_{ic} value of 0.09 nM (James et al., 2010). Triclosan also inhibited estrone sulfonation, exhibiting an IC_{50} value of 0.6 nM (James et al., 2010).

Sulfoconjugation is an essential reaction in the phase II biotransformation of various endogenous and foreign substances, including drugs, toxic chemicals, steroid hormones, and neurotransmitters (Strott, 2002). Sulfonation plays a vital role in the transport of steroids. Natural estrogens are steroid hormones that, while present in both males and females, are usually present at considerably higher levels in females of reproductive age. The steroid sex

hormone, 17 β -estradiol (E2) is one of the three main estrogens naturally produced in the body. E2 has two hydroxyl groups in its molecular structure, while estrone has one (E1) and estriol has three (E3). The sulfated, inactive forms of E1 and dehydroepiandrosterone (DHEA) are the primary “transport forms” of steroids and serve as the precursors of androgen and estrogen biosynthesis (Hobkirk, 1985; Morato et al., 1965; Mortola and Yen, 1990). Estrogen sulfotransferase, SULT1E1, is the major isoform responsible for E1 and E2 sulfonation at physiological concentrations (Zhang et al., 1998), though other SULTs can catalyze estrogen sulfonation at lower efficiencies (Wang and James, 2005).

The pregnant sheep has been used extensively as a model to study physiology in pregnancy, in particular pathways of estrogen homeostasis (Purinton and Wood, 2000; Wood, 2005; Wood et al., 2003). In this study, pregnant ewes and their fetuses were administered an environmentally relevant dose of triclosan by infusion and the effects on estrogen sulfotransferase investigated.

2. Materials and methods

2.1 Chemicals

[3H]-17 β -estradiol (E2), >97% pure, 60 Ci/mmol, was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). Triclosan used for sheep dosing was purchased from TCI America (Portland, Oregon) and shown to be >99.8% pure by reverse-phase HPLC analysis with UV detection at 280 nm. Triclosan sulfate was purchased from Toronto Research Chemicals, North York, Ontario. The internal standard for LC/MS/MS analysis was 2,2',5'-trichloro-biphenyl-4-ol (4'-OH-CB18), purchased from Accustandard (New Haven, CT). Water utilized in these experiments was purified by Milli-Q water system to 18 M Ω . *Helix pomatia* beta-glucuronidase containing sulfatase activity (G0751) was obtained from Sigma-Aldrich, St. Louis, MO. Molecular weight standards, 12% mini-Protean TGX gels and nitrocellulose membranes were purchased from BioRad, Hercules, CA. All other chemicals and solvents were of the highest grade available and obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

2.2 Animal treatment

Experiments were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the American Physiological Society's Guiding Principles for Research Involving Animals and Human Beings. The administered triclosan dose was calculated to be comparable to exposure of an adult to personal care products such as toothpaste containing 0.3% triclosan. Time-dated pregnant ewes between 120 and 130 days gestation were administered 0.1 mg triclosan/kg/day for 2 days either through direct infusion into the fetal circulation or through administration to the ewe. On the third day, hepatic and placental tissues were harvested from the late gestation fetuses. Other tissues from these animals were examined for genomic effects of triclosan and a full description of the exposure protocol has been published (Rabaglino et al., 2016). Briefly, for direct exposure, chronically-catheterized fetal sheep were intravenously infused with vehicle (dimethylsulfoxide:saline, 1:1) or triclosan solution, whereas indirect fetal exposure was achieved through intravenous infusion of vehicle or triclosan into the maternal

circulation (Rabaglino et al., 2016). Eight fetuses being carried by six ewes received triclosan directly, and maternal exposure was to three ewes carrying five fetuses. After infusion was complete, the pregnant ewes and fetuses were humanely euthanized with an overdose of sodium phenobarbital. Upon confirmation of cardiac arrest, fetal tissues were rapidly removed and snap frozen in liquid nitrogen. Tissue samples were stored at -80°C until use in these experiments. Portions of the tissue samples were analyzed for triclosan or processed into subcellular fractions for measurement of enzyme activity.

2.3 Preparation of subcellular fractions

Subsamples of liver and placental cotyledon from individual sheep, 0.2 to 1.5 g, were homogenized in 4 volumes of homogenizing buffer containing 0.25 M sucrose, 0.05 M Tris base, pH 7.4, 5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged as described in previous work to separate microsomes and cytosol fractions (James et al., 2010). Protein concentrations in the subcellular fractions were determined by the bicinchoninic acid method, with bovine serum albumin as a standard (Thermo, Rockford, IL).

2.4 Analysis of triclosan in tissues

Triclosan concentrations in liver and placenta were measured by LC/MS/MS. All glassware was rinsed with acetone and deionized water before use. We maintained a triclosan-free area of the laboratory for all analytical work, to avoid accidental contamination of samples. Samples of all tissues were analyzed for total triclosan following hydrolysis of tissue homogenates with glucuronidase/sulfatase and sample cleanup. Duplicate tissue samples, 0.5 g, were homogenized with a bio-homogenizer (Biospec products, Inc., Bartlesville, OK) in 1.5 ml 0.1 M ammonium acetate buffer pH 5 and transferred to a glass tube. The vessel was rinsed with 2.5 ml buffer that was added to the tube together with 0.025 ml of a 0.6 μM methanol solution of 4'-OH-CB18 as internal standard. To this was added 1 ml of *Helix pomatia* β -glucuronidase containing sulfatase (15,000 units) and the mixture was incubated for 24 h at 37°C . Additional duplicate samples from fetal liver and placenta of fetally-infused sheep were analyzed without hydrolysis, and these samples were taken to the next step without incubation. Preliminary studies with biosynthesized triclosan sulfate and triclosan glucuronide standards demonstrated that hydrolysis of these conjugates to triclosan was not complete before 24h. After hydrolysis, acetonitrile containing 1% formic acid, 2 ml, was added to each tube and vortex-mixed. The tube was centrifuged at 3,000 g for 10 min and the supernatant transferred to a clean tube. The pellet was extracted twice more and the supernatants pooled. The pooled supernatants were centrifuged again to remove particulates prior to application to a 500 mg Hybrid SPE-phospholipid cartridge to remove phospholipids and proteins (Supelco, Bellefonte, PA). The eluent was collected in a glass test tube and diluted with Milli-Q water to 15% acetonitrile. The diluted sample was applied to a pre-conditioned 200 mg 3 cc C_{18} Sep-Pak Vac cartridge with reservoir (Waters, Milford, MA). The cartridge was washed with 15% methanol then eluted with 1 ml 100% methanol. The methanol eluent was filtered in Spin-X centrifuge device with 0.22 μ nylon filter (Corning Costar, Corning, NY) prior to LC/MS/MS analysis.

LC-MS/MS analyses were performed using a Shimadzu LC system with auto-sampler coupled to an AB Sciex 3200QTRAP (Foster City, CA) mass spectrometer using electrospray ionization (ESI) operated in negative-ion mode. The chromatographic separation of 10 μ L tissue extracts was achieved with a Luna 5 μ C8 (50 mm x 4.6 mm) column (Phenomenex, Torrance, CA) and a methanol:water gradient using a flow rate of 0.25 mL/min. The gradient conditions consisted of holding 80% methanol for 5 min, applying a linear gradient to 100% methanol over 20 min, holding at 100% methanol for 4 min then returning to initial conditions. The gases for the mass spectrophotometer were delivered using a Parker Balston gas generator. The curtain gas was maintained at 20 psi, nebulizer gas at 50 psi and the turbo gas at 40 psi, which was held at 400 °C. The negative-ion mode spray voltage was set at -4500 V. Triclosan was quantified in multiple reaction monitoring (MRM) scan mode, monitoring the fragmentation transition of Cl^- loss from the parent compound (286.8 $m/z \rightarrow$ 35.0 m/z). A compound similar to triclosan that also contains three chloride atoms, 4'-OH-CB18, was used as an internal standard. The fragmentation monitored for the internal standard was 270.8 m/z (M-H^-) $\rightarrow$ 234.7 m/z (M-H-Cl^-). Triclosan eluted from the LC at 9.6 min and the internal standard eluted at 7.9 min. The ESI compound parameters were optimized to achieve maximum sensitivity for each ion pair. Calibration standard curves were analyzed using a range of triclosan concentrations of 0–6 pmoles with 4'-OH-CB18 at a fixed concentration of 0.15 pmoles in an injection volume of 10 μ L. Unknown concentrations were calculated from a standard curve that was analyzed along with each set of extracted tissue samples. The LC-MS/MS system was controlled by Analyst 1.4.2 software and the spectra were integrated using the IntelliQuan algorithm in the software.

2.5 Estrogen Sulfotransferase Assays

The individual sheep placental and hepatic cytosol fractions were assayed for E2 sulfonation activity (James et al., 2010). Reaction mixtures with a total volume of 0.5 mL contained 0.1 M Tris-Cl, pH 7.4, 10 mM MgCl_2 , 1 nM [^3H]-17 β -estradiol, 20 μ M 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and 0.5 μ g of cytosolic protein, added last to start the reaction. After a 5 min incubation at 37 °C, 0.2 mL of an ice-cold solution of trichloroacetic acid (TCA), 3% w/v, was added to the samples (at the same timed interval the protein was added) to stop the reaction. Blanks had TCA added before protein. The amount of cytosolic protein added was such that less than 20% of the substrate was consumed in the assay, and product formation was linear. Water-saturated methylene chloride, 1 mL, was added to each tube and the tubes were vortex-mixed prior to a 5 min centrifugation to separate the phases. The methylene chloride (lower phase) was pipetted out and the extraction was repeated twice more to remove unreacted E2. Scintillation cocktail was added to a 0.2 mL aliquot of the upper aqueous phase and was subject to liquid scintillation counting for quantification of the sulfate conjugate. Activities as pmoles of estradiol-3-sulfate produced/min/mg cytosolic protein were corrected for the non-incubated blanks.

In some experiments, placental cytosols from triclosan-treated sheep were treated to remove small molecules. Samples of cytosol were added to 10,000 nominal molecular weight cutoff centrifugal filter units (Amicon Ultra, Merck Millipore, Ireland) with 2 volumes of homogenizing buffer and the units were centrifuged until two thirds of the volume passed

through the filter. Clean homogenizing buffer was added and the washing procedure was repeated twice. The washed samples were assayed for protein concentration and estrogen sulfotransferase as described above.

The effect of triclosan sulfate on estrogen sulfotransferase activity was determined by conducting assays with control sheep placental cytosol in the presence and absence of triclosan sulfate, 1 μ M final concentration.

2.6 Western blot against hSULT1E1

Samples of sheep placental and hepatic cytosol fractions (100 or 10 μ g protein respectively), purified human SULT1E1, 0.005 μ g protein, and pre-stained molecular weight standards were denatured in buffer containing sodium dodecyl sulfate, separated on 12% polyacrylamide gels and the proteins transferred to nitrocellulose membrane. Monoclonal antibody to human SULT1E1 was purchased from the University of Iowa Developmental Studies Hybridoma Bank (CPTC-SULT1E1, Ames, IA) and used according to their recommendations with a final concentration of 0.2 μ g/ml for the primary antibody and a dilution of 1:7500 for the secondary antibody, peroxidase-conjugated AffiniPure goat anti-mouse IgG, (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). This antibody cross-reacted with proteins in sheep placental or hepatic cytosol with similar migration to expressed recombinant human SULT1E1.

2.7 Data analysis

Data was analyzed with Excel software (Microsoft, Redmond, WA) and GraphPad software (version 6.0; GraphPad Software, Inc., San Diego, CA). Linear regression analyses were used to examine the relationship between estrogen sulfotransferase activity and triclosan tissue concentration. One-way ANOVA with Tukey's multiple comparisons post-test was used to compare tissue concentrations of triclosan. Ratio paired t-tests were used to evaluate placental triclosan versus fetal liver triclosan concentrations in each treatment group. Values were considered significant with $p < 0.05$.

3. Results

3.1 Triclosan in tissues

Using LC-MS/MS, tissue concentrations of total triclosan (pmol/g tissue) following hydrolysis of conjugates were measured in fetal liver and placenta from all treated sheep. Maternal liver was analyzed for total triclosan in the maternally-infused ewes. Tissues from saline-treated controls did not exhibit triclosan signals and were used to prepare standard curves. The limit of detection was 2 pmol/g tissue. ANOVA showed that fetal placenta had higher concentrations of triclosan following direct fetal infusion with triclosan, as compared with samples where the ewe was administered triclosan (Table 1). In both fetally-infused and maternally-infused sheep, triclosan was detected in the placenta at higher concentrations than in fetal liver (Table 1). Values for the ratio of triclosan concentration in placenta relative to fetal liver were calculated from each individual fetal sample by both exposure routes. The ratio of triclosan concentration in placenta to that in fetal liver was 7.32 ± 2.66 (mean $\pm$ S.E.M., $n=10$). Ratio paired t-test analysis of concentrations in placenta and fetal liver from

all animals (fetally-infused and maternally-infused) showed significantly higher concentrations in placenta, $p=0.0012$.

Samples of fetal liver and placenta from fetally-infused sheep were analyzed without hydrolysis. In placenta the free triclosan concentration was 176 ± 37.1 pmol/g (mean $\pm$ S.E., $n=6$) and in fetal liver was 34.9 ± 16 (n=6).

3.2 Estrogen sulfotransferase activity

Triclosan administration to sheep resulted in lower estrogen sulfotransferase activity in liver and placenta. When tissue concentrations of triclosan in the placenta were plotted against measured estrogen sulfotransferase activity in placental cytosol, a clear negative correlation was observed (Fig. 2). The negative correlation was significant, $p=0.02$. Activity in the placental vehicle-treated controls was 32.5 ± 4.1 pmol/min/mg protein (mean S.D., $n=4$), while the samples with levels of triclosan greater than 200 pmol/g tissue exhibited rates of 19 to 23 pmol/min/mg protein. A similar negative correlation of triclosan concentration versus estrogen sulfotransferase activity was found in fetal liver cytosol, $p=0.01$ (Fig 2). Comparing estrogen sulfotransferase in placenta and fetal liver by paired t-test we found that activity in fetal liver cytosol fractions from vehicle-treated animals, 13.7 ± 1.3 (mean $\pm$ S.D., $n=4$) was lower than activity in placental cytosol from the same animals, $p=0.0037$.

Three samples of placental cytosol of triclosan-treated sheep that were passed through a 10,000 nominal molecular weight cut-off filter and exchanged into clean buffer had the same estrogen sulfotransferase activity as the unfiltered samples. This experiment showed that removing triclosan or triclosan metabolites from the cytosol samples did not affect activity.

Addition of triclosan sulfate, 1 μ M, to estrogen sulfotransferase assays with placental cytosol had no effect on measured activity. Higher concentrations were not studied.

3.3 Sulfotransferase expression

Western blots of sheep liver cytosol (Figure 3) revealed very strong cross-reactivity against the monoclonal antibody to hSULT1E1 in triclosan-treated and control sheep, much stronger than observed in placenta. As expected the hSULT1E1 expressed recombinant enzyme showed strong cross-reactivity. In placental cytosol, more protein was needed to obtain a signal, and several non-specific bands were observed. In each tissue, there was no difference in band intensity between triclosan-treated sheep and controls.

4. Discussion

Due to the rising concern regarding the risk of adverse reproductive health effects during fetal development from exposure to environmental chemicals, the present study sought to provide insight into the possible adverse effects that triclosan can pose to placental function, in a sheep model.

These studies examined pregnant sheep that were treated with environmentally relevant concentrations of triclosan either directly infused into the fetus or infused into the mother. Analysis of human liver total triclosan concentrations from autopsy samples reported one

liver contained 29 ng/g and the mean of 11 samples was 0.44 ng/g (Geens et al., 2012). This is in the same order of magnitude as we found in the maternal liver of maternally exposed sheep, 29 pmol/g, i.e. 8.5 ng/g (Table 1), demonstrating the relevance of our findings to environmental exposures. A separate study of the same sheep demonstrated that this low-dose triclosan treatment resulted in changes in gene expression in the fetal hypothalamus (Rabaglino et al., 2016). Several genes related to feeding, reproduction and cell cycle were upregulated while genes related to chromatin modification and the metabolism of steroids, lipoproteins, fatty acids and glucose were downregulated compared with controls. These changes suggested possible developmental effects of triclosan. Although this short-term (2 days) low dose (0.1 mg/kg/day) exposure study was not designed to determine the effects of triclosan on pregnancy success, the finding reported herein that low dose exposure to triclosan affects estrogen sulfotransferase activity has implications for placental function. The physiological functions of the placenta in providing estrogen to the developing fetus have been examined in studies using the pregnant sheep animal model (Wood, 2005). Estrogen sulfates formed in the placenta are transported into the fetus and taken up by target tissues such as the brain where they are hydrolyzed by sulfatase to the active hormone (Wood, 2005). Previous *in vitro* experiments showed that triclosan was an exquisitely potent inhibitor of sheep placental estrogen sulfotransferase (James et al., 2010), results that suggested the possibility that triclosan exposure to pregnant sheep may endanger pregnancy by reducing total placental estrogen secretion and disrupt subsequent estrogen action in target tissues. Results from this study indicate that triclosan is present in placenta and treated animals have lower estrogen sulfotransferase activity, suggesting a cause and effect relationship. The detection of triclosan in fetal tissues upon indirect exposure via the pregnant ewe confirms that triclosan crossed the placental barrier and was taken up by fetal tissues.

Expression of the estrogen sulfotransferase enzyme, assessed with an antibody to hSULT1E1, did not reveal changes in the amount of enzyme present in control or triclosan-treated sheep. The antibody used was not selective for sheep SULT1E1 and as the observed signal was much stronger in liver than placenta, while estrogen sulfotransferase activity was higher in placenta, it is likely that other hepatic SULT1 family isoforms are cross-reacting with this human antibody, so this finding does not provide conclusive evidence that SULT1E1 expression is unaffected by triclosan. While the amount of triclosan in whole placental tissue of treated animals (Table 1) is sufficient to potentially inhibit estrogen sulfotransferase (James et al., 2010), it must be remembered that a very dilute sample is used to assay activity, so the amount in activity assays will be very small, calculated at less than 0.005 nM, or one-twentieth of the K_i . Furthermore, we showed that removing small molecules by filtering through a 10,000 nominal molecular weight cutoff membrane did not change measured activity, thus ruling out a direct inhibition by triclosan present in the cytosol. At this time, we are unable to explain why estrogen sulfotransferase activity was lower in the triclosan-treated animals, and further studies are needed to explore the mechanism of reduction in activity.

Another important finding of this study was that after either direct exposure of fetal sheep to triclosan or indirect exposure through the mother, the placenta attained higher concentrations of total triclosan than fetal liver. Our analysis showed that most of the triclosan was in the

unconjugated form in placenta and fetal liver (Table 1 and section 3.1). We previously showed that glucuronidation of triclosan does not occur in the placenta, and sulfonation proceeds slowly (James et al., 2010), so finding mainly free triclosan in placenta is not surprising. Fetal liver also contained mainly free triclosan. This could be because conjugating enzymes are expressed at low levels in the fetal sheep, as has been demonstrated for glucuronosyltransferases in humans (Hines, 2008), or because once formed, conjugates are quickly eliminated from liver cells by transporters. Although it is lipophilic, triclosan does not accumulate in people because it is readily converted in the liver to triclosan sulfate and triclosan glucuronide, which are eliminated in urine (Queckenberg et al., 2010; Wang et al., 2004). The toxicology of triclosan conjugates has not been studied, however in this work we showed that triclosan sulfate, 1 μ M, had no effect on estrogen sulfotransferase activity. Our finding of high free triclosan levels in placenta of exposed sheep is particularly important because of the critical role of the placenta in maintaining pregnancy. A recent study showed that women who lost their pregnancy in mid-gestation had 11-fold higher levels of triclosan in their urine than women who carried their pregnancy to term (Wang et al., 2015). Another study showed that pregnant rats exposed to triclosan accumulated higher levels in placenta than six other tissues (Feng et al., 2016), suggesting preferential uptake of triclosan by placenta.

Although in September 2016, the US Food and Drug Administration issued a final rule banning the use of triclosan in consumer antiseptic washes in the USA, a rule that took effect in September 2017, triclosan is still used in other countries so it is likely that triclosan will persist in the environment for some time (Halden et al., 2017). As discussed in the Florence Statement (Halden et al., 2017), triclosan already present in the environment is poorly degraded by bacteria in sewage treatment plants, and is partially converted to methyltriclosan, which is more lipophilic and can be taken up by animals and converted to triclosan (James et al., 2012). If sewage sludge contaminated with triclosan is applied as fertilizer to fields where sheep graze, there may be consequences for ovine reproduction. Furthermore, although the use of triclosan in soaps and body washes has been discontinued in the USA, the Food and Drug Administration permitted continued use in toothpaste (Kux, 2016). Thus, toothpaste and toothbrushes will be a continued source of environmental triclosan (Han et al., 2017).

5. Conclusions

This study focused on triclosan due to the widespread human exposure to this substance. The results of the study revealed that triclosan is readily taken up by placenta, where it reduces estradiol sulfonation. In hepatic and placental tissues, the rate of estradiol sulfoconjugation was significantly greater in the controls than in the triclosan treated samples, confirming that estrogen sulfotransferase activity can be reduced by triclosan in both the fetal liver and placenta.

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References

- Adolfsson-Erici M, Pettersson M, Parkkonen J, Sturve J. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere*. 2002; 46:1485–1489. [PubMed: 12002480]
- Allmyr M, Adolfsson-Erici M, McLachlan MS, Sandborgh-Englund G. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *Sci Total Environ*. 2006; 372:87–93. [PubMed: 17007908]
- Arbuckle TE, Marro L, Davis K, Fisher M, Ayotte P, Belanger P, Dumas P, LeBlanc A, Berube R, Gaudreau E, Provencher G, Faustman EM, Vigoren E, Ettinger AS, Dellarco M, MacPherson S, Fraser WD. Exposure to free and conjugated forms of bisphenol A and triclosan among pregnant women in the MIREC cohort. *Environ Health Perspect*. 2015a; 123:277–284. [PubMed: 25494523]
- Arbuckle TE, Weiss L, Fisher M, Hauser R, Dumas P, Berube R, Neisa A, LeBlanc A, Lang C, Ayotte P, Walker M, Feeley M, Koniecki D, Tawagi G. Maternal and infant exposure to environmental phenols as measured in multiple biological matrices. *Sci Total Environ*. 2015b; 508:575–584. [PubMed: 25483107]
- Bagley DM, Lin YJ. Clinical evidence for the lack of triclosan accumulation from daily use in dentifrices. *Am J Dent*. 2000; 13:148–152. [PubMed: 11763951]
- Bester K. Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters. *Arch Environ Contam Toxicol*. 2005; 49:9–17. [PubMed: 15959704]
- Calafat AM, Weuve J, Ye X, Jia LT, Hu H, Ringer S, Huttner K, Hauser R. Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants. *Environ Health Perspect*. 2009; 117:639–644. [PubMed: 19440505]
- Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Urinary concentrations of triclosan in the U.S. population: 2003–2004. *Environ Health Perspect*. 2008; 116:303–307. [PubMed: 18335095]
- Crofton KM, Paul KB, DeVito MH, Hedge JM. Short-term in vivo exposure to the water contaminant triclosan: evidence for disruption of thyroxine. *Environ Toxicol Pharmacol*. 2007; 24:194–197. [PubMed: 21783810]
- Dayan AD. Risk assessment of triclosan [Irgasan] in human breast milk. *Food Chem Toxicol*. 2007; 45:125–129. [PubMed: 17011099]
- Dhillon GS, Kaur S, Pulicharla R, Brar SK, Cledon M, Verma M, Surampalli RY. Triclosan: current status, occurrence, environmental risks and bioaccumulation potential. *Int J Environ Res Public Health*. 2015; 12:5657–5684. [PubMed: 26006133]
- Feng Y, Zhang P, Zhang Z, Shi J, Jiao Z, Shao B. Endocrine Disrupting Effects of Triclosan on the Placenta in Pregnant Rats. *PLoS One*. 2016; 11:e0154758. [PubMed: 27149376]
- Fernandes M, Shareef A, Kookana R, Gaylard S, Hoare S, Kildea T. The distribution of triclosan and methyl-triclosan in marine sediments of Barker Inlet, South Australia. *J Environ Monit*. 2011
- Gee RH, Charles A, Taylor N, Darbre PD. Oestrogenic and androgenic activity of triclosan in breast cancer cells. *J Appl Toxicol*. 2008; 28:78–91. [PubMed: 17992702]
- Geens T, Neels H, Covaci A. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. *Chemosphere*. 2012; 87:796–802. [PubMed: 22277880]
- Halden RU, Lindeman AE, Aiello AE, Andrews D, Arnold WA, Fair P, Fuoco RE, Geer LA, Johnson PI, Lohmann R, McNeill K, Sacks VP, Schettler T, Weber R, Zoeller RT, Blum A. The Florence Statement on Triclosan and Triclocarban. *Environ Health Perspect*. 2017; 125:064501. [PubMed: 28632490]
- Han J, Qiu W, Campbell EC, White JC, Xing B. Nylon Bristles and Elastomers Retain Centigram Levels of Triclosan and Other Chemicals from Toothpastes: Accumulation and Uncontrolled Release. *Environ Sci Technol*. 2017; 51:12264–12273. [PubMed: 29067803]
- Hines RN. The ontogeny of drug metabolism enzymes and implications for adverse drug events. *Pharmacol Ther*. 2008; 118:250–267. [PubMed: 18406467]
- Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can J Biochem Cell Biol*. 1985; 63:1127–1144. [PubMed: 3910206]

- Hua W, Bennett ER, Letcher RJ. Triclosan in waste and surface waters from the upper Detroit River by liquid chromatography-electrospray-tandem quadrupole mass spectrometry. *Environ Int.* 2005; 31:621–630. [PubMed: 15910958]
- James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. *Environ Int.* 2010; 36:942–949. [PubMed: 19299018]
- James MO, Marth CJ, Rowland-Faux L. Slow O-demethylation of methyl triclosan to triclosan, which is rapidly glucuronidated and sulfonated in channel catfish liver and intestine. *Aquat Toxicol.* 2012; 124–125C:72–82.
- Jones RD, Jampani HB, Newman JL, Lee AS. Triclosan: a review of effectiveness and safety in health care settings. *Am J Infect Control.* 2000; 28:184–196. [PubMed: 10760227]
- Kux, L. Administration, F.a.D. Federal Register. Vol. 81. Federal Register; Washington, DC: 2016. Safety and Effectiveness of Consumer Antiseptics: Topical Antimicrobial Drug Products for Over-the-Counter Human Use; p. 25pp
- Levy CW, Roujeinikova A, Sedelnikova S, Baker PJ, Stuitje AR, Slabas AR, Rice DW, Rafferty JB. Molecular basis of triclosan activity. *Nature.* 1999; 398:383–384. [PubMed: 10201369]
- Levy SB. Antibiotic and antiseptic resistance: impact on public health. *Pediatr Infect Dis J.* 2000; 19:S120–122. [PubMed: 11052402]
- Levy SB. Antibacterial household products: cause for concern. *Emerg Infect Dis.* 2001; 7:512–515. [PubMed: 11485643]
- Lin YJ. Buccal absorption of triclosan following topical mouthrinse application. *Am J Dent.* 2000; 13:215–217. [PubMed: 11763935]
- Liu LP, Xia TW, Zhang XQ, Barr DB, Alamdar A, Zhang J, Tian MP, Huang QY, Shen HQ. Biomonitoring of infant exposure to phenolic endocrine disruptors using urine expressed from disposable gel diapers. *Analytical and Bioanalytical Chemistry.* 2014; 406:5049–5054. [PubMed: 24924209]
- McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature.* 1998; 394:531–532. [PubMed: 9707111]
- Morato T, Lemus AE, Gual C. Efficiency of Dehydroepiandrosterone Sulphate as an Estrogen Precursor. *Steroids.* 1965; 58(SUPPL 1):59–74.
- Mortola JF, Yen SS. The effects of oral dehydroepiandrosterone on endocrine-metabolic parameters in postmenopausal women. *J Clin Endocrinol Metab.* 1990; 71:696–704. [PubMed: 2144295]
- Paul KB, Hedge JM, DeVito MJ, Crofton KM. Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats. *Toxicol Sci.* 2010; 113:367–379. [PubMed: 19910387]
- Philippat C, Wolff MS, Calafat AM, Ye X, Bausell R, Meadows M, Stone J, Slama R, Engel SM. Prenatal exposure to environmental phenols: concentrations in amniotic fluid and variability in urinary concentrations during pregnancy. *Environ Health Perspect.* 2013; 121:1225–1231. [PubMed: 23942273]
- Provencher G, Berube R, Dumas P, Bienvenu JF, Gaudreau E, Belanger P, Ayotte P. Determination of bisphenol A, triclosan and their metabolites in human urine using isotope-dilution liquid chromatography-tandem mass spectrometry. *J Chromatogr A.* 2014; 1348:97–104. [PubMed: 24835763]
- Purinton SC, Wood CE. Ovine fetal estrogen sulfotransferase in brain regions important for hypothalamus-pituitary-adrenal axis control. *Neuroendocrinology.* 2000; 71:237–242. [PubMed: 10773743]
- Pycke BF, Geer LA, Dalloul M, Abulafia O, Jenck AM, Halden RU. Human fetal exposure to triclosan and triclocarban in an urban population from Brooklyn, New York. *Environ Sci Technol.* 2014; 48:8831–8838. [PubMed: 24971846]
- Queckenberg C, Meins J, Wachall B, Doroshyenko O, Tomalik-Scharte D, Bastian B, Abdel-Tawab M, Fuhr U. Absorption, pharmacokinetics, and safety of triclosan after dermal administration. *Antimicrob Agents Chemother.* 2010; 54:570–572. [PubMed: 19822703]

- Rabaglino MB, Chang EI, Richards EM, James MO, Keller-Wood M, Wood CE. Genomic Effect of Triclosan on the Fetal Hypothalamus: Evidence for Altered Neuropeptide Regulation. *Endocrinology*. 2016; 157:2686–2697. [PubMed: 27145008]
- Rodricks JV, Swenberg JA, Borzelleca JF, Maronpot RR, Shipp AM. Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. *Crit Rev Toxicol*. 2010; 40:422–484. [PubMed: 20377306]
- Sandborgh-Englund G, Adolfsson-Erici M, Odham G, Ekstrand J. Pharmacokinetics of triclosan following oral ingestion in humans. *J Toxicol Environ Health A*. 2006; 69:1861–1873. [PubMed: 16952905]
- Schweizer HP. Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol Lett*. 2001; 202:1–7. [PubMed: 11506900]
- Stoker TE, Gibson EK, Zorrilla LM. Triclosan exposure modulates estrogen-dependent responses in the female wistar rat. *Toxicol Sci*. 2010; 117:45–53. [PubMed: 20562219]
- Strott CA. Sulfonation and molecular action. *Endocr Rev*. 2002; 23:703–732. [PubMed: 12372849]
- Veldhoen N, Skirrow RC, Osachoff H, Wigmore H, Clapson DJ, Gunderson MP, Van Aggelen G, Helbing CC. The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquat Toxicol*. 2006; 80:217–227. [PubMed: 17011055]
- Velez MP, Arbuckle TE, Fraser WD. Female exposure to phenols and phthalates and time to pregnancy: the Maternal-Infant Research on Environmental Chemicals (MIREC) Study. *Fertil Steril*. 2015; 103:1011–1020. e1012. [PubMed: 25681860]
- Wang LQ, Falany CN, James MO. Triclosan as a substrate and inhibitor of 3'-phosphoadenosine 5'-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions. *Drug Metab Dispos*. 2004; 32:1162–1169. [PubMed: 15269185]
- Wang LQ, James MO. Sulfotransferase 2A1 forms estradiol-17-sulfate and celecoxib switches the dominant product from estradiol-3-sulfate to estradiol-17-sulfate. *J Steroid Biochem Mol Biol*. 2005; 96:367–374. [PubMed: 16011896]
- Wang LQ, James MO. Inhibition of sulfotransferases by xenobiotics. *Curr Drug Metab*. 2006; 7:83–104. [PubMed: 16454694]
- Wang X, Chen X, Feng X, Chang F, Chen M, Xia Y, Chen L. Triclosan causes spontaneous abortion accompanied by decline of estrogen sulfotransferase activity in humans and mice. *Sci Rep*. 2015; 5:18252. [PubMed: 26666354]
- Weiss L, Arbuckle TE, Fisher M, Ramsay T, Mallick R, Hauser R, LeBlanc A, Walker M, Dumas P, Lang C. Temporal variability and sources of triclosan exposure in pregnancy. *Int J Hyg Environ Health*. 2015; 218:507–513. [PubMed: 26009209]
- Wood CE. Estrogen/hypothalamus-pituitary-adrenal axis interactions in the fetus: The interplay between placenta and fetal brain. *J Soc Gynecol Investig*. 2005; 12:67–76.
- Wood CE, Gridley KE, Keller-Wood M. Biological activity of 17beta-estradiol-3-sulfate in ovine fetal plasma and uptake in fetal brain. *Endocrinology*. 2003; 144:599–604. [PubMed: 12538622]
- Woodruff TJ, Zota AR, Schwartz JM. Environmental chemicals in pregnant women in the United States: NHANES 2003–2004. *Environ Health Perspect*. 2011; 119:878–885. [PubMed: 21233055]
- Yueh MF, Tukey RH. Triclosan: A Widespread Environmental Toxicant with Many Biological Effects. *Annu Rev Pharmacol Toxicol*. 2016; 56:251–272. [PubMed: 26738475]
- Zhang H, Varlamova O, Vargas FM, Falany CN, Leyh TS. Sulfuryl transfer: the catalytic mechanism of human estrogen sulfotransferase. *J Biol Chem*. 1998; 273:10888–10892. [PubMed: 9556564]
- Zorrilla LM, Gibson EK, Jeffay SC, Crofton KM, Setzer WR, Cooper RL, Stoker TE. The effects of triclosan on puberty and thyroid hormones in male Wistar rats. *Toxicol Sci*. 2009; 107:56–64. [PubMed: 18940961]

Highlights

- Low-dose triclosan was administered to pregnant ewes
- Higher triclosan concentrations were present in placenta than liver
- Estrogen sulfotransferase activity was reduced by triclosan treatment

Figure 1.
Structure of triclosan

Figure 2.

Estrogen sulfotransferase activity in fetal hepatic and placental cytosol from saline-treated or triclosan-treated sheep.

Each point represents the mean of duplicate determinations from an individual. Results are shown from sheep in which triclosan was infused directly into the fetus or into the maternal circulation. Estrogen sulfotransferase activity showed a significant negative correlation with Pearson's r of -0.65 and -0.78 in liver and placenta respectively, $p=0.023$ for liver and 0.005 for placenta. The lines drawn indicate a linear relationship.

Figure 3.

Western blot of placental cytosol

Western blots of fetal hepatic and placental cytosol, showing cross-reactivity to a monoclonal anti-human SULT1E1 antibody. Lanes 1–4 contained 10 μ g hepatic cytosolic protein from saline-treated sheep; Lane 5 and 20 were pre-stained molecular weight standard; Lanes 6–9 contained 10 μ g hepatic cytosolic protein from triclosan-treated sheep; Lanes 10 and 15 show hSULT1E1 standard, 5 ng; Lanes 11–14 contained 100 μ g protein from placental cytosol of saline-treated sheep; Lanes 16–19 contained 100 μ g protein from placental cytosol of triclosan-treated sheep.

Table 1

Tissue concentrations of total triclosan in placenta and fetal and maternal liver following exposure to triclosan.

Sample	Fetal-infused	Maternal-infused
Maternal liver	N.D.	29.3 ± 11.4 (3) ^A
Fetal liver	51.5 ± 16.9 (6) ^A	4.57 ± 2.02 (4) ^A
Placenta	206 ± 44.9 (6) ^B	33.0 ± 13.3 (5) ^A

Values for tissue concentrations are pmol triclosan/g tissue, mean ± S.E.M. (n). ANOVA showed significant differences in concentrations of triclosan in tissues with different letters, $p < 0.02$

Results shown for fetal infusion are of six fetuses from five ewes. In the maternal exposure group, three ewes carrying five fetuses received triclosan infusions. One fetal liver sample from the maternally-infused group was not analyzed. N.D. – not determined.