Urinary triclosan concentrations and early outcomes of *in vitro* fertilization-embryo transfer

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Abstract

Triclosan (TCS) exists ubiquitously in the environment. Several *in vitro* and *in vivo* studies have demonstrated that TCS exerts endocrine disruptive effects on reproduction, but data from human populations are limited and conflicting. The objective of our study was to investigate whether high urinary TCS concentration is adversely associated with early reproductive outcomes in women undergoing *in vitro* fertilization-embryo transfer (IVF-ET). This prospective cohort study was conducted from September 2015 to June 2016, including 156 infertile women undergoing their first IVF-ET cycle. Two spot urine samples were collected prior to oocyte retrieval for TCS detection using solid-phase extraction (SPE) and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Linear regression model and binary logistic regression model were used to evaluate the association between urinary TCS concentrations and IVF outcomes. The intake of aquaculture food may have positive influences on urinary TCS concentrations. After adjustment for age, body mass index (BMI), baseline follicle-stimulating hormone (FSH), antral follicle count (AFC) and smoking status, a significant decrease of top quality embryo formation and implantation rate was observed in patients with urinary TCS concentration greater than or equal to the median level (0.045 µmol/mol Cr). We concluded that TCS exposure may exert negative effects during early stages of human reproduction.

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Introduction

Triclosan (TCS) is a broad-spectrum antimicrobial agent being widely used in pharmaceuticals and personal care products with concentration around 0.1–0.3% (w/w) (Dhillon *et al.* 2015). Previously, TCS had not been considered as a chemical pollutant, leading to widespread use without proper regulation. Recently, because of its ubiquitous existence in the environment and human population (Calafat *et al.* 2008, Wang & Tian 2015), especially with a molecular structure similar to anthropogenic estrogens (Dhillon *et al.* 2015, Wang & Tian 2015), increasing attention has been diverted to potential toxic effects of this chemical. In humans, ingestion is the major route of TCS absorption (Moss *et al.* 2000, Sandborgh-Englund *et al.* 2006), and various reports have noted its occurrence in plasma, breast milk and urine (Allmyr *et al.* 2006a,b, Li *et al.* 2013). Approximately 80% of the orally administered dose in volunteers is excreted in the urine during the first 4 days (Sandborgh-Englund *et al.* 2006), and the elimination half-life of TCS is estimated to be

approximately 10–20 h (Sandborgh-Englund *et al.* 2006, Queckenberg *et al.* 2010). Even though, there has been evidence demonstrating its bioaccumulation after daily use (Mustafa *et al.* 2003).

In vitro studies using various cell lines indicated both estrogenic (Gee *et al.* 2008, Huang *et al.* 2014) and androgenic (Ahn *et al.* 2008, Christen *et al.* 2010) activities of TCS. The reproductive endocrine-disrupting effects of TCS might be mediated either through sex hormone-related pathways or during steroidogenesis (Kumar *et al.* 2008, Forgacs *et al.* 2012). Besides, the synergistic effect of TCS with BPA (Crawford & Decatanzaro 2012) and ethinylestradiol (Stoker *et al.* 2010) indicates its disruptive effects in sex hormone metabolism.

The estrogenic effect of TCS was also detected in female rats. Being exposed to TCS at the highest dose (i.e. 150 mg/kg/day) for 21 days, a significantly advanced age of vaginal opening and increased uterine weight could be observed (Stoker *et al.* 2010). However, several studies conducted to assess the potential androgenic

effects of TCS produced discordant results. In a study by Zorrilla and colleagues, decreased serum testosterone level was observed in male Wistar rats when exposed to a 200 mg/kg dose of TCS (Zorrilla *et al.* 2009), while Axelstad failed to find any androgenic effects (Axelstad *et al.* 2013).

The evidences of TCS's potential effects in human reproduction are still limited and conflicting. A recent Navigation Guide systematic review indicated that TCS is 'possibly toxic' to reproductive and developmental health (Johnson *et al.* 2016). However, several epidemiological studies indicated a weak association between TCS exposure and the age of first menarche in girls (Buttke *et al.* 2012), or idiopathic male infertility (Chen *et al.* 2013). In our study, we evaluated the association between urinary TCS concentration and early reproductive outcomes in women undergoing *in vitro* fertilization-embryo transfer (IVF-ET), including oocyte maturation rates, fertilization rates, top quality embryo formation and embryo implantation rates. This is one of the first studies assessing TCS endocrine-disrupting effects in IVF-ET, which is the best model to study the potential effects during the early stage of human reproduction.

Materials and methods

Study participants and data collection

In this prospective cohort study, 156 infertile patients undergoing their first IVF/ICSI cycle in the Center for Reproductive Medicine of Nanfang Hospital, Southern Medical University, were recruited from September 2015 to June 2016. Women aged 20–40 years using their own oocytes for IVF were eligible for recruitment. Cryothaw and donor egg cycles were not included in this analysis. The study was approved by the Institutional Review Board for Nanfang Hospital (study number NFEC-2015-106), and all the participants signed written informed consents.

Upon recruitment, a brief questionnaire was assigned to each patient to collect baseline data including demographics, occupation, medical history, lifestyle and residential factors. In the questionnaire, a passive smoker is defined as a person exposed to smoking at work or at home at least 1 day per week for over 15 min. A snack is a portion of food smaller than a regular meal, generally eaten between meals, which comes in a variety of forms including packaged snack foods and other processed foods. Aquaculture food refers to both freshwater and saltwater populations, such as fish, crustaceans and mollusks. The frequency of snack intake, cosmetic use, smoked or grilled food intake and aquaculture food intake was classified as always, sometimes and never. 'Always' refers to ≥ 1 –3 times per week, 'sometimes' refers to ≤ 1 –3 times monthly and 'never' refers to no such use in the recent 3 months. Clinical information was obtained from electronic medical records.

Treatment protocol and clinical IVF measures

Patients were treated with a standard long GnRH-agonist protocol. Briefly, either long- or short-acting GnRH-agonist

was injected in the mid-luteal phase. After pituitary down-regulation, the ovarian stimulation was conducted till at least two follicles had reached a diameter of ≥ 18 mm, followed by human chorionic gonadotropin (HCG) administration to induce final follicular maturation. Oocyte retrieval was performed via trans-vaginal ultrasound-guided follicle aspiration 36 h later. Oocytes were cultured individually from the time of retrieval until the assessment on day 3 pre-transfer. The oocyte maturation rate was defined as the total number of mature oocytes (MII oocytes for ICSI cycle and fertilized oocytes for IVF cycle) divided by the total number of oocytes retrieved. The fertilization rate was defined as the total number of fertilized oocytes divided by the total number of oocytes retrieved. The normal fertilization rate was defined as the total number of normally fertilized oocytes (2PN) divided by the total number of oocytes retrieved. A top quality embryo was characterized by the presence of 4–5 blastomeres on day 2 and 7–8 blastomeres on day 3, the absence of multinucleated blastomeres and $<10\%$ cellular fragments. The top quality embryo formation rate was defined as the total number of top quality embryos divided by the total number of normally fertilized oocytes (2PN).

A maximum of three embryos were transferred on day 3 after retrieval and luteal phase support was given by daily injections of progesterone started from the day of oocyte retrieval continuing until the day of pregnancy test. A positive pregnancy test was defined by a plasma β -HCG concentration >10 IU/L. A clinical pregnancy was defined as an intrauterine gestational sac with a heartbeat 30 days after embryo transfer, and clinical pregnancy rate is defined as the total number of pregnancies divided by the total number of initial cycles. Implantation rate is defined as the total number of gestational sacs divided by the total number of embryos transferred.

Blood samples and hormone assays

Blood samples were taken on day 2–5 of the patients' spontaneous menstrual cycle before any treatment for basic measurements of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2) and progesterone (P4), and serum peak E2 level was measured on the day of HCG administration. Serum hormonal levels were measured by electrochemiluminescence (ECL) immunoassay (Roche).

Urine sample collection and urinary TCS concentrations

Two spot urine samples were collected from each patient, with the first one collected between day 6 of the ovarian stimulation and the day of HCG administration; the second one collected on the day of oocyte retrieval. Both samples were 15 mL fasting samples, and were collected in a sterile clean polypropylene bottle. Each urine sample was divided into aliquots and creatinine was measured immediately. The rest of the aliquots were stored at -20°C and analyzed.

The urinary concentrations of total (free and conjugated) TCS were measured using solid-phase extraction (SPE) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described before (Provencher *et al.* 2014). Briefly, 4 mL urine was treated with β -glucuronidase/sulfatase (Sigma), and TCS was extracted on the Caliper Rapid Trace SPE

workstation (Hopkinton, MA, USA). The analyses by LC–MS/MS were carried out with Agilent 1100 series HPLC system. An Eclipse Plus-C18 reversed-phase column (250 mm × 4.6 mm i.d., 5 µm; Agilent) was applied for LC separation of analytes. The limit of detection (LOD) was 0.005 µg/L and the limit of quantification was 0.015 µg/L. The recovery rate of the target substances from urine samples was detected to be higher than 95%. TCS concentrations were adjusted by creatinine (Cr) concentrations (µmol/L). The arithmetic mean of the Cr-adjusted TCS concentrations (µmol/mol Cr) from two spot urine samples was calculated and used as the TCS concentration of the patient. TCS concentrations below the LOD were assigned a value equal to the LOD divided by the square root of 2 before Cr adjustment.

Statistical methods

Demographic and baseline reproductive characteristics of the female patients are presented using mean ± s.d. or percentages, and TCS concentrations of different groups are presented using median (Q1–Q3). Patients' exposures to TCS were divided into two groups by the median of urinary TCS concentration, with the one below the median considered as the reference group. Demographic and baseline reproductive characteristics between the two groups of urinary TCS concentrations were compared using *t*-test for continuous variables and *chi*-squared tests for categorical variables. The influences of patients' BMI and lifestyles on urinary TCS concentrations were analyzed using non-parametric test. Group comparisons were conducted using contingency table analysis. Linear regression model and binary logistic regression model were used to evaluate the association between urinary TCS concentrations and IVF outcomes. Final models were adjusted for age (continuous), BMI (continuous), baseline FSH (continuous), AFC (continuous) and smoking status (non-smoker vs passive smoker). The variables considered as potential confounders included factors previously related to IVF outcomes in this and other studies. All tests were two-tailed and the level of statistical significance was set at 0.05. Computation was performed using SPSS statistical software version 12.0.1 for Windows.

Results

Demographics

A total of 156 patients were enrolled in the study, and 12 were excluded due to failure of oocyte retrieval (*n* = 4) or only one urine sample collected (*n* = 8). The average age of the participants was 31.4 ± 4.4 years and 61.1% of them were diagnosed to be tubal factor infertility. The average BMI was 21.6 ± 2.8 kg/m², while 17 (11.8%) patients were overweight (BMI > 25 kg/m²). Almost 123 patients went through cleavage stage embryo transfer (ET), while fresh cycle ET was cancelled in 21 patients to prevent ovarian hyperstimulation syndrome. Patients' characteristics are presented in Table 1.

According to the lifestyle questionnaire responses, none of the participants were current or ever-smokers,

Table 1 Baseline characteristics of the enrolled patients.

Characteristics	<i>n</i> (%)
Age (years) mean ± s.d. (range)	31.4 ± 4.4 (21–39)
BMI (kg/m ²) mean ± s.d. (range)	21.6 ± 2.8 (16.8–34.0)
BMI > 25 kg/m ²	17 (11.8%)
Diagnosis	
Female factor	102 (70.8%)
Ovulation disorders	5 (3.5%)
Endometriosis	9 (6.3%)
Tubal factor	88 (61.1%)
Male infertility	37 (25.7%)
Unexplained	5 (3.5%)
Oocyte treatment	
IVF	105 (72.9%)
ICSI	37 (25.7%)
IVF/ICSI	2 (1.4%)

while 31 (21.5%) were passive smokers. Almost 12.5% (18/144) and 20.8% (30/144) of the participants always took overcooked food (smoked or grilled food) and snacks in the recent 3 months. Almost 21.5% (31/144) and 46.5% (67/144) of them were frequent aquaculture food consumers and cosmetic users respectively (Table 2). The intake of aquaculture food (*P* < 0.001) may have positive influences on urinary TCS concentrations as shown in Table 2, while the place of residence or the other lifestyle habits may not be related to TCS exposure (all *P* > 0.05).

Triclosan detection

The detection rate of TCS was 60.4% (87/144) with the concentration ranging from below the LOD to 4.98 µmol/mol Cr. The median (Q1–Q3) and geometric mean was 0.045 (0.004–0.337) and 0.041 µmol/mol

Table 2 Patients lifestyle and urinary TCS concentrations (µmol/mol Cr).

Characteristics	<i>n</i>	Median	Mean (s.d.)	Statistics	<i>P</i>
Place of residence				2.389	0.303
Downtown	82	0.071	0.412 (0.816)		
Countryside	35	0.044	0.226 (0.466)		
Suburb	27	0.010	0.365 (0.727)		
Smoking				1.320	0.187
Non-smokers	31	0.051	0.530 (0.780)		
Passive smokers	113	0.044	0.311 (0.710)		
Smoked or grilled food intake				0.489	0.625
Always	18	0.050	0.317 (0.465)		
Sometimes	126	0.045	0.364 (0.760)		
Never	0				
Aquaculture food intake				15.561	0.000
Always	31	0.236	0.709 (0.941)		
Sometimes	106	0.038	0.279 (0.647)		
Never	7	0.003	0.004 (0.003)		
Cosmetic use				−1.848	0.065
Always	67	0.099	0.444 (0.825)		
Sometimes	77	0.022	0.283 (0.629)		
Never	0				
Snacks intake				−0.428	0.669
Always	30	0.089	0.297 (0.519)		
Sometimes	114	0.034	0.374 (0.775)		
Never	0				

Table 3 Distribution of unadjusted and urinary creatinine-adjusted triclosan (TCS) levels.

	Geometric mean	Arithmetic mean $\pm$ S.D.	25th centile	50th centile	75th centile
TCS ($\mu\text{g/L}$)	0.058	0.628 $\pm$ 1.159	0.004	0.064	0.645
TCS/urinary ($\mu\text{mol/mol Cr}$)	0.041	0.358 $\pm$ 0.728	0.004	0.045	0.337

Cr respectively (Table 3). Patients who had TCS concentrations at or above the median of the sample were significantly younger than those with TCS below the median (30.6 vs 32.2, $P=0.025$), and the former group of patients' total antral follicle count (AFC) was significantly higher (15.4 vs 11.9, $P=0.009$). After adjustment for age, BMI and smoking status using linear regression model, the difference of AFC between the two groups of patients is still statistically significant ($P=0.029$). There were no significant differences between the two groups of patients in body mass index (BMI), menstrual cycle duration and baseline hormone levels (Table 4).

Triclosan concentration and IVF outcomes

TCS concentration and the relevant IVF outcomes are presented in Table 5. We observed a significant increase of peak E2 level in patients with TCS concentrations at or above the median (4129.6 ± 2554.4 vs 3237.9 ± 1871.0 pg/mL, $P=0.046$). As there was a moderate correlation between peak E2 and number of oocyte retrieved (Spearman $r=0.70$, $P<0.000$), without surprising, a slight increased trend was observed in the number of oocyte retrieved in patients with TCS concentrations at or above the median (14.4 ± 6.6 vs

Table 5 Adverse effects of TCS exposure on early reproductive results after adjustment for age, BMI, baseline FSH, AFC and smoking status using Binary Logistic Regression Model.

Outcomes	OR	95% CI	P
Fertilization	1.238	0.990–1.548	0.061
Top embryo formation	1.322	1.046–1.698	0.020
Implantation	1.709	1.024–2.854	0.040

CI, confidence interval; OR, odds ratio.

12.3 ± 7.5 , $P=0.074$). However, when adjusted for age, baseline FSH, BMI, AFC and smoking status using linear regression model, no statistical differences could be observed either in peak E2 ($P=0.298$) or in the number of oocyte retrieved ($P=0.709$).

Although there was no statistical difference in oocyte maturation rate (78.8% vs 79.3%, $P>0.05$), a significant decrease of fertilization rate (73.0% vs 79.9%, $P=0.001$), top quality embryo formation rate (40.9% vs 48.9%, $P=0.006$) and implantation rate (27.6% vs 50.0%, $P<0.001$) could be observed in patients with higher urinary TCS concentration (Table 4). There were no statistical differences in normal fertilization rate and clinical pregnancy rate (per cycle) between the two groups of patients. After adjustment for age, BMI, baseline FSH, AFC and smoking status using binary logistic regression model, no dose–response correlation was noted between TCS concentrations and the outcomes of fertilization (OR=1.238, $P=0.061$), whereas negative dose–response associations could be observed between TCS concentrations and top embryo formation (OR=1.322, $P=0.020$), as well as implantation (OR=1.709, $P=0.040$) (Table 5).

Table 4 Urinary TCS concentrations and stimulation outcomes.

	TCS < median ($n=72$)	TCS $\geq$ median ($n=72$)	P
Mean age (years)	32.2 (4.7)	30.6 (3.9)	0.028
Body mass index (BMI) (kg/m^2)	21.7 (2.7)	21.6 (2.8)	0.772
Menstrual cycle duration (days)	33.7 (15.2)	37.0 (21.7)	0.281
Total antral follicle count	12.0 (6.4)	15.4 (8.6)	0.008
Baseline FSH (IU/mL)	7.6 (3.3)	6.9 (1.7)	0.098
Baseline LH (IU/mL)	5.5 (3.3)	5.9 (4.3)	0.483
Baseline LH/FSH	0.8 (0.4)	0.9 (0.7)	0.163
Baseline E2 (pg/mL)	43.2 (32.4)	46.3 (20.4)	0.489
Baseline testosterone (ng/mL)	1.0 (0.9)	0.4 (0.3)	0.349
Total dose of gonadotrophins (IU)	2511.3 (1211.1)	2232.8 (1070.9)	0.148
E2 on HCG (pg/mL)	3237.9 (1871.0)	4129.6 (2554.4)	0.046
Number of oocytes retrieved	12.3 (7.5)	14.4 (6.6)	0.031
Number of MII oocytes	9.7 (7.7)	11.4 (6.6)	0.037
Number of fertilized eggs	9.8 (7.1)	10.5 (5.2)	0.487
Number of 2PN eggs	7.6 (5.4)	8.3 (4.3)	0.384
Number of top quality embryos	3.7 (3.1)	3.4 (2.7)	0.514
Number of transferred embryos	2.0 (0.4)	2.0 (0.1)	0.745
Oocyte maturation rate (%)	78.8%	79.3%	0.784
Fertilization rate (%)	79.7%	73.0%	0.001
Normal fertilization rate (%)	61.9%	57.8%	0.064
Top quality embryo formation rate (%)	48.9%	40.9%	0.006
Clinical pregnancy rate (%) per cycle	52.8% ($n=62$)	47.2% ($n=61$)	0.401
Implantation rate (%) per embryo	50.0% ($n=62$)	27.6% ($n=61$)	0.000

Discussion

It has been reported that TCS could be detected in approximately 75% of urine samples of US population (Calafat *et al.* 2008) and the geometric mean (GM) concentration was 12.7 µg/g Cr (equivalent to 4.96 µmol/mol Cr). In another survey (Li *et al.* 2013) of 287 participants aged between 3 and 24 years in Guangzhou (China), the detection rate of urinary TCS was 93% and the GM concentration was 3.77 µg/g Cr (equivalent to 1.47 µmol/mol Cr). In our study, the detection rate of TCS was 60.4% (87/144) and the GM concentration was 0.041 µmol/mol Cr, which is lower than the level previously reported. The subjects in our study were subfertile patients seeking assisted reproductive treatments. In view of their subfertility, all patients had been educated and informed by the physicians and nurses from another lower-level hospital before transferring to our institution. They understood the harm of pollutants and made efforts to reduce exposure to possible pollutants, which might explain why the urinary concentration is lower in this specific population.

In our study, aquaculture food intake and cosmetic use were two variables associated with higher TCS exposure. It has been established that TCS is present in personal care products and household items, such as shampoo, toothpaste, dishwashing detergent, antimicrobial agent, cosmetics and so on (Dhillon *et al.* 2015). Such products are externally applied to the human body and pose no immediate threat to one's health as there is no ingestion of TCS. However, the release of such products into domestic wastewater may end up in the accumulation of TCS in aquatic animals and plants, and long-term consumption of these products might be one of the main sources of chronic human exposure to TCS (Dann & Hontela 2010, Escarrone *et al.* 2016, Nadia *et al.* 2016). Indeed, 95.1% (137/144) of the participants in our study were aquaculture food consumers, and a positive dose-response association could be observed between the amount of aquaculture food intake and urinary TCS concentrations ($P < 0.001$).

Due to its molecular structure, which closely resembles anthropogenic estrogens, TCS is highly suspected to be an endocrine-disrupting chemical (EDC) (Dhillon *et al.* 2015, Wang & Tian 2015, Yuan *et al.* 2015). However, the correlation between the concentration of TCS and female infertility remains unclear due to lack of studies available. In our study, although no significant difference was observed in oocyte maturation rate, top embryo formation rate and implantation rate was significantly influenced by higher TCS exposure, and a decreased trend of fertilization rate could be observed. Our results are consistent with some previous reports. In Hwang's study on sea urchin, when both eggs and sperms were exposed to TCS, fertilization rate was affected at 0.5 µM of TCS and when the concentration was higher than 1.25 µM, fertilization could not occur spontaneously.

In the same study, early embryo development was significantly inhibited when exposed to 1.0 µM TCS (Hwang *et al.* 2014). Another *in vivo* study with mice indicated a decrease of implantation sites in the high dose group (18 and 27 mg/animal/day), additionally a combined bisphenol A (BPA) and TCS dosing at individually ineffective doses on gestational day 1–3 significantly inhibited embryo implantation (Crawford & Decatanzaro 2012). In the present study, we also observed a significant increase of AFC in patients who had higher urinary TCS concentrations after age, BMI and smoking status adjustment. It has been reported that EDCs, such as BPA, may play a role in the onset of polycystic ovary syndrome, which could significantly contribute to female infertility due to anovulation (Barrett & Sobolewski 2014). As the molecular structure of TCS is quite similar to that of BPA, we hypothesized that TCS may also exert similar detrimental effects on human reproduction. However, in our study, no statistical difference was observed in the menstrual cycle duration, baseline testosterone or LH/FSH ratio.

The potential limitations of our study are the small sample size and the two spot sampling method. TCS has a short half-life, which could be totally excreted from the human body in less than 24 h after exposure (Sandborgh-Englund *et al.* 2006). In our study, although two spot urine samples were collected, misclassification of TCS exposure is still possible due to patients' highly variable diet and lifestyle habits. Unfortunately, we were unable to assess the TCS exposure profile of the male partners. Therefore, we could only speculate but are unable to determine whether the decreased fertilization rate is due to the toxic effects of TCS on oocyte, sperm or both gametes. To the best of our knowledge, this is one of the first prospective studies designed to evaluate the association between TCS exposure and early IVF outcomes in women with fertility problems.

In conclusion, we observed that there were adverse associations between urinary TCS concentration and early reproductive outcomes of patients undergoing IVF-ET. Women with higher urinary TCS concentration had significantly lower top embryo formation and implantation rates, with a significant increase in baseline AFC after adjustment for age, BMI and smoking status. Further research on plausible mechanisms of TCS reproductive endocrine-disrupting effects is warranted. The estimated TCS exposure for humans (0.13 mg/kg/day (Rotroff *et al.* 2010)) is significantly lower than that used in existing animal studies (300 mg/kg/day). Therefore, studies using significantly lower doses of TCS are required to explore the exact effects of TCS on humans such that appropriate public health messages with accurate facts can be presented, and major lifestyle modifications could be made in subfertile patients depending on the future findings of such investigations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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