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Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with triclosan

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ABSTRACT

Triclosan (TCS), a chlorophenol, is widely used as a preservative in different types of commercial preparations. The reports on TCS-mediated endocrine disruption are controversial and the present study aimed to elucidate the probable mode of action of TCS as an antiandrogenic compound using a robust study design. Male albino rats, *Rattus norvegicus*, were treated with three doses of triclosan for a period of 60 days followed by the analysis of various biochemical parameters. RT-PCR analysis demonstrated a significant decrease in mRNA levels for testicular steroidogenic acute regulatory (StAR) protein, cytochrome $P450_{SCC}$, cytochrome $P450_{C17}$, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and androgen receptor (AR) in TCS treated rats ($p < 0.05$). TCS also induced a perturbed translation of testicular StAR, and AR proteins as shown by Western blot analysis in treated groups of rats. A reduced level of StAR was further indicated by immunohistochemistry in testicular Leydig cells. Further, there was a significant decrease ($p < 0.05$) in the level of serum lutenizing hormone (LH), follicle stimulating hormone (FSH), cholesterol, pregnenolone, and testosterone. *I*n vitro assays demonstrated more than 30% decrease in testicular 3β-HSD and 17β-HSD enzyme activities in treated group of animals. Extensive histopathological malformations were observed in the testis and sex accessory tissues of the treated rats. Overall this study showed that TCS decreased the synthesis of androgens followed by reduced sperm production in treated male rats which could be mediated by a decreased synthesis of LH and FSH thus involving hypothalamo–pituitary–gonadal axis.

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1. Introduction

Endocrine-disrupting chemicals (EDC) comprise a category of environmental contaminants that interferes with the function of endocrine system [1]. An increasing body of evidence reveals an association between various environmental compounds that act as EDC and lead to sex hormone-sensitive disease/disorders [2,3]. Chemicals that mimic the structure of the natural hormones found in the animal/human body may pose species-specific risks that are difficult to investigate because of latent adverse effects [4]. A body of literature exists for various EDC demonstrating potential estrogenic activities which have been identified and classified [2]. Although there are similar health concerns regarding (anti)androgenic EDC that interfere with sperm production, alter genital development and contribute to neurological syndromes in males, the identification and classification of these putative health hazards have progressed comparatively slowly [3]. Recent reports of several non-steroidal compounds that have the ability to alter the androgen dependent functions are of particular concern because many of them are ubiquitously used in our daily life.

A number of antimicrobial agents and preservatives are commonly used in the personal care products such as soaps, shampoos, detergents, disinfectants, cosmetics and pharmaceutical products [5–7]. The continuous use of these chemicals results in their accumulation at detectable concentrations within different parts of our body like blood, milk, and various organs and tissues [5,8–10]. Triclosan (TCS; 2,4,4′-trichloro-2′-hydroxydiphenyl ether; a chlorophenol) is an antimicrobial agent widely used as preservative in toothpastes, soaps, shampoos, and cosmetics [11]. The chemical structure is shown in Fig. 1. In general, TCS has been known to be a highly toxic chemical for aquatic flora and fauna [12] and thus has been included in the probable list of endocrine disruptors on account of its resemblance with known non-steroidal estrogens or its mimetic (e.g. diethylestradiol, bisphenol A). Further, TCS and its chlorinated derivatives are readily converted into various chlorinated dibenzo-p-dioxins by heat and ultraviolet irradiation which may also be harmful for biological systems [13–15]. The mode of action of TCS as an EDC is controversial and various studies indicate it to be of different nature, viz. estrogenic or weak androgenic or

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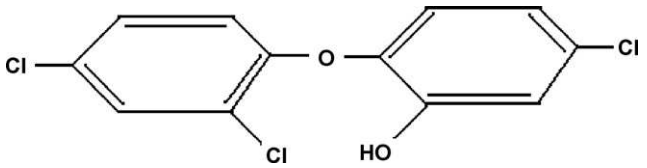

Fig. 1. Chemical structure of the representative test chemical, triclosan, used to treat the animals in the study.

anti-androgenic. Fourteen days TCS exposure in Japanese medaka fry (*Oryzias latipes*) showed a weak androgenic effect [16]. Another study reported that the metabolite of TCS may be a weak estrogenic compound with the potential to induce vitellogenin in male medaka while decreasing the hatchability, as well as delaying the hatching in females [17]. TCS has also been shown to function as an anti-androgen since it inhibits testosterone-induced transcriptional activity [2]. Exposure of TCS to the human may be a consequence of its presence in the cosmetics and other human use products. This chemical has been reported to be absorbed mainly by two routes: either across the skin or through the gastrointestinal tract [8].

This report describes the various targets of TCS toxicity in an effort to help understand its probable mode of action as an (anti)androgenic endocrine disruptor using male albino rats as model. The rats were treated with three dose levels of TCS for a fixed period of time. On completion of treatment serum and tissue samples were analyzed for their (anti)androgenic effects in response to TCS. Further histopathological analysis of testis and sex accessory tissues (SATs) were performed to assess the action of this chemical at the cellular levels. The data presented here demonstrates that some widely used antimicrobial compounds like that of TCS have anti-androgenic properties and warrant further investigation to understand its impact on human reproductive health.

2. Materials and methods

2.1. Animals

The study was carried out on the male Wistar rats, *Rattus norvegicus*, with the approval as well as guidelines of institutional ethical committee. Animals were purchased from All India Institute of Medical Sciences (New Delhi, India) and were in healthy condition at the time of purchasing. They were housed in a well-ventilated animal house with 12 h light:12 h dark schedule. The animals were fed with a balanced animal diet obtained commercially (Ashirwad Animal Feed Industries, Punjab, India) and had free access to drinking water which was the normal tap water filtered through RO system and commonly used for drinking purposes in India. The animals were acclimatized to the animal house condition for 10 days prior to the experiments.

2.2. Dose selection and dosing

The test compound i.e. TCS (about 98% purity, SD Fine Chemicals, Mumbai, India) was tested at three dose levels: 5, 10 and 20 mg/kg of body weight (bw) per day (mg/(kg day)). All the three doses selected were found to be below LD_{50} as demonstrated by earlier studies [18]. A uniform suspension of TCS in phosphate buffer saline (PBS) was made fresh everyday just before intubations and 200 μl of each was used.

2.3. Experimental design

Rats were grouped ($n = 8$) as follows and treatment was initiated when rats were around 10 weeks old:
Group I: Treated with PBS (vehicle treated control)
Group II: Treated with triclosan, 5 mg/(kg day)
Group III: Treated with triclosan, 10 mg/(kg day)
Group IV: Treated with triclosan, 20 mg/(kg day)

The test chemical was administered daily (single time a day) by the intubation and treatment was continued for 60 days. Approximately after 24 h of final treatment, the rats were sacrificed by cervical dislocation under ether anesthesia [19,20]; testes and other accessory sex organs were collected, weighed and processed according to the requirement of the experiment (as described below).

2.4. Serum hormone analysis

For the determination of serum levels of cholesterol, pregnenolone, testosterone, LH and FSH, on completion of the treatment, blood was collected from the decapitated animals by the method of heart puncture from both control as well as rats treated at a dose of 20 mg/(kg day) ($n = 8$). The serum levels of hormones were determined using the commercial enzyme immunoassay kits (DRG Diagnostics, Germany, for steroids, Calbiotech, CA, USA, for LH and FSH and Transasia Biomedical, Mumbai for Cholestrol) as per manufacturer's instructions.

2.5. Daily sperm production (DSP)

Testicular sperm content and DSP/g testis were determined from the freshly removed testis of the animals on completion of the treatment according to the method described previously with slight modifications [21–23]. Briefly, testes of control and 20 mg/(kg day) group were weighed and homogenized for 3 min in 25 ml of physiological saline containing 0.05% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA) using a semimicro-waring blender. Stage 19 spermatids survive this homogenization and their nuclei can then be counted using a hemocytometer to determine average number of spermatids per sample. These values were used to obtain total number of spermatids per testis, this was then divided by the testis weight to determine the number of spermatids per gram of testis. The values for the number of spermatids per testis and spermatids per gram testis were divided by 6.1, which is the duration in days of spermatogenic cycle during which stage 19 spermatids are present in the seminiferous epithelium, to determine DSP and efficiency of sperm production (DSP/g testis), respectively [23].

2.6. Histopathological analysis of sex accessory tissues (SATs)

Prostate, seminal vesicle, vas deferens and cauda epididymis were collected from control and all the three groups of TCS treated animals but only the highest dose group (20 mg/kg/day) was processed further for histopathological analysis. Tissues were fixed in Bouins solution, dehydrated by upgrading from 30 to 100% series of alcohol and then to xylene each for 1 h followed by making sections in paraffin blocks to 5 μm thickness. The sections were then stained in hematoxylin and eosin following the earlier described methods [24].

2.7. Immunohistochemical analysis of steroidogenic acute regulatory (StAR) protein

Paraffin section of the Bouins solution fixed testes obtained from control and TCS (20 mg/kg/day) treated animals were prepared like that of SATs as described earlier. Sections were then deparaffinated by incubating twice for 5 min each time in xylene and then rehydrated by incubating twice, for each time, 3 min in 100% (v/v) ethanol, 3 min in 95% ethanol, 20 min in 70% ethanol containing 1% (v/v) H_2O_2 (to inactivate endogenous peroxidase activity), 20 min in 70% ethanol saturated with Li_2CO_3 (to neutralize picric acid from the fixative), 3 min in 50% ethanol, 10 min in double distilled water, 10 min in phosphate-buffered saline (PBS) (pH 7.2), and 5 min in 300 mM of glycine in PBS. Dehydrated sections were subjected to immunohistochemical analysis as described earlier [25] and without counterstain.

2.8. Steroidogenic enzyme activity

3β-Hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) are the two crucial enzymes in the steroid biosynthesis pathway and their activity has been found to be affected by some of the EDC. These two enzymes were assayed according to the methods described earlier [26]. Briefly, the testis removed from the different groups of intact animals were homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA followed by centrifugation at 10,000 × *g* for 10 min at 4 °C. For 3β-HSD activity, 1 ml aliquot of the supernatant was mixed with 100 μM sodium pyrophosphate buffer (pH 8.9), 0.9 ml double distilled water and 30 μg DHEA making up the incubation mixture to a volume of 3 ml. Enzyme activity was measured at 25 °C after the addition of 0.5 μM of NAD^+ to the mixture against a blank without NAD^+. For the determination of 17β-HSD type III activity, 1 ml aliquot of the above centrifuged supernatant was mixed with 400 μM sodium pyrophosphate buffer (pH 10.2), 25 mg bovine serum albumin, and 0.3 μM testosterone bringing the total reaction volume to 3 ml. Enzyme activity was measured after the addition of 1.1 μM NADP to the mixture against a blank without NADP. By this experiment, the activity of reverse reaction catalyzed by 17β-HSD (conversion of testosterone to androstenedione) was determined. The forward reaction (conversion of androstenedione to testosterone) was performed under almost similar condition using 50 mM phosphate buffer, 30 nM androstenedione and 7 mM NADPH. One unit of enzyme activity was equivalent to a change in the absorbance of 0.001 units/min at 340 nm.

2.9. Semiquantitative RT-PCR

Total RNA was extracted from the testes of the vehicle treated (control) ($n = 8$) and 20 mg/(kg day) TCS treated ($n = 8$) groups of animals according the method described earlier [27]. The extracted RNA samples from individual animals within each groups were pooled, quantified and equal amount of them were transcribed with the help of the RT-PCR kit purchased from Bangalore Genei (Bangalore, India) according to the manufacturer's instruction. Similar patterns of treatment followed by RNA isolation and RT-PCR was carried out at least three times to nullify the inter-assay

Table 1
Primers used for semiquantitative RT-PCR.

Gene	Primer Sequence (5′–3′)	Product size	Cycle used	Annel Temp	Gene bank accession no.
$P450_{SCC}$ (F) $P450_{SCC}$ (R)	CgCTCAgTgCTggTCAAAA TCTggTAgACggCgTCgAT	688	23	55	J_05156
$P450_{C17}$ (F) $P450_{C17}$ (R)	GACCAAGGGAAAGGCGT GCATCCACGATACCCTC	302	24	55	M_22204
3β-HSD (F) 3β-HSD (R)	CCgCAAgTATCATgACAgA CCgCAAgTATCATgACAgA	547	24	55	M_38178
17β-HSD (F) 17β-HSD (R)	TTCTgCAAggCTTTACCAgg ACAAACTCATCggCggTCTT	653	26	55	AF_035156
AR (F) AR (R)	TTACgAAgTgggCATgATgA ATCTTgTCCAggACTCggTg	570	26	55	M_20133
StAR (F) StAR (R)	TTgggCATACTCAACAACCA ATgACACCgCTTTgCTCAg	389	25	58	NM_031558
GAPDH (F) GAPDH (R)	AgACAgCCgCATCTTCTTgT CTTgCCgTgggTAgAgTCAT	207	21	58	NM_017008

variations. PCR was performed by denaturing at 94 °C for 60 s, annealing at various temperatures (depending on primer pairs used) for 30 s and extension at 72 °C for 60 s followed by varying number of cycles for amplification. The primer sequences, annealing temperature and number of cycles for PCR were all designed according the earlier report by Ohsako et al. [30] except for StAR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [28]. Primer sequence for StAR was adopted from earlier report [29]. Primers for GAPDH were designed with the help of Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998, Primer3) and standardized in the laboratory. The PCR products were then separated on 2% agarose gel and visualized in a gel documentation system (Bio Rad, USA). The intensity of the bands on gels was converted into digital image with a gel analyzer and amounts of RT-PCR products were quantified with Scion Images software (Scion Corporation, Fredrick, MD, USA). GAPDH PCR products were used as internal standards and each of the RT-PCR was carried out four times. Primer sequence, product size, annealing temperature, number of cycles used and gene bank accession number of all primers are presented in Table 1.

2.10. Western blot analysis

The testis of the control and 20 mg/(kg day) of TCS treated male rats were homogenized in phosphate buffer saline having 20% (v/v) glycerol and 1 mM EDTA and centrifuged at 10,000 × *g* for 30 min at 4 °C. The pellet was discarded, supernatants were quantified and an equal quantity of protein samples were analyzed by 12% polyacrylamide gel according to the method of described earlier [30]. This was followed by Western blot analysis of the protein samples. Membranes were probed with StAR and androgen receptor (AR) antibodies (dilutions 1:1000 and 1:250 respectively). Color development was performed in 30 ml AP-buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), with 200 μl NBT (50 mg/ml) and 100 μl BCIP (50 mg/ml). The developed blots were subjected to densitometric analysis using the β-actin as internal control.

2.11. Statistical analysis

Origin 6.1 software (Origin Lab Corporation, USA) was used for statistical analysis and data were expressed as mean ± S.E.M. For statistical analysis of data, ANOVA followed by multiple two-tail comparison *t*-test was used and $p < 0.05$ was considered significant.

3. Results

3.1. Body weight and weight of testis and SATs

Treatment of rats with test samples did not induce significant changes in the body weight at any of the test doses (Table 2). Whatever minimal increase in the body weight observed could be attributed to normal aging. Administration of TCS did not cause significant change in the weight of testis and SATs at the 5 mg/(kg day) dosage. In contrast, the higher test doses (10 and 20 mg/(kg day)) induced a significant decrease in the weight of testis and SATs. TCS exposure decreased the weights of testis, epididymis, ventral prostate, vas deferens and seminal vesicles between 20–50% at 10 mg/(kg day) and 35–49% at 20 mg/(kg day) (Table 3) ($p < 0.05$).

Table 2
Effects of three different dosage level of triclosan on the body weights of the animals before and after the completion of 60 days of treatment.

Groups	Body weight (g ± S.E.M.)	
	Initial	Final
Control	168 ± 6.3	187 ± 7.1
Triclosan (5 mg/kg)	166 ± 7.9	185 ± 5.7
Triclosan (10 mg/kg)	169 ± 5.1	188 ± 6.8
Triclosan (20 mg/kg)	165 ± 7.2	183 ± 7.5

Each value denotes mean ± S.E.M. of eight animals.

3.2. Gene expression analysis

Rats treated with 20 mg/(kg day) TCS showed a statistically significant down-regulation in the testicular levels of mRNA for cytochrome $P450_{SCC}$ ($P450_{SCC}$), cytochrome $P450_{C17}$ ($P450_{C17}$), 3β-HSD, 17β-HSD, StAR and AR as compared to control. Level of $P450_{SCC}$, 3β-HSD, 17β-HSD and $P450_{C17}$ mRNA decreased up to 33, 54, 46 and 58% respectively as compared to control ($p < 0.05$) (Fig. 2A). In the case of AR and StAR, the expression decreased up to 33 and 54% respectively as compared to control (Fig. 2B) ($p < 0.05$).

Western blot analysis demonstrated that the rats treated with TCS at a dose of 20 mg/(kg day) showed reduced translation of StAR (30 kDa) and AR (100 kDa) proteins as compared to control (vehicle treated animals) and this decrease was statistically significant (Fig. 3) ($p < 0.05$). The uniform band intensities of β-actin in all the wells indicated equal gel loading.

3.3. Testicular 3β-HSD and 17β-HSD levels in vitro

In vitro spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that the treatment of animals with test chemical caused a statistically significant decrease in the activity of both the testicular steroidogenic enzymes at the two higher dose levels (10 and 20 mg/(kg day)) ($p < 0.05$). The decrease was not significant at a dose of 5 mg/(kg day) for both the enzymes. Doses of 10 and 20 mg/(kg day) TCS decreased 3β-HSD enzyme activity up to 27 and 39% respectively (Fig. 4) while that of 17β-HSD enzyme activity up to 31 and 46% respectively (Fig. 4) as compared to control ($p < 0.05$).

3.4. Serum hormone levels

There was a statistically significant decrease in the serum LH (38.5%), FSH (17%), cholesterol (35%), pregnenolone (31%) and testosterone (41%) levels in male rats treated with a dose of 20 mg/(kg day) as compared to control ($p < 0.05$) (Table 4).

Table 3
Effects of three different dosage level of triclosan on the weights of testis and accessory sex tissues from rats given 60 days treatment.

Groups	Testis (g ± S.E.)	Seminal vesicle (mg ± S.E.)	Ventral prostate (mg ± S.E.)	Epididymis (mg ± S.E.)	Vas defrentia (mg ± S.E.)
Control	2.334 ± 0.089	462.2 ± 15	133.4 ± 4	868.3 ± 10	125.5 ± 6
Triclosan (5 mg/kg)	2.111 ± 0.067	446.3 ± 21	128.4 ± 9	852.2 ± 12	118.5 ± 5
Triclosan (10 mg/kg)	1.709 ± 0.060**	374.3 ± 19**	99.2 ± 5**	651.2 ± 11**	85.2 ± 6**
Triclosan (20 mg/kg)	1.524 ± 0.051**	302.6 ± 22**	74.3 ± 8**	549.3 ± 10**	60.4 ± 7**

Each value denotes mean ± S.E.M. of eight animals.
** Significantly different from control group at $p < 0.05$ level.

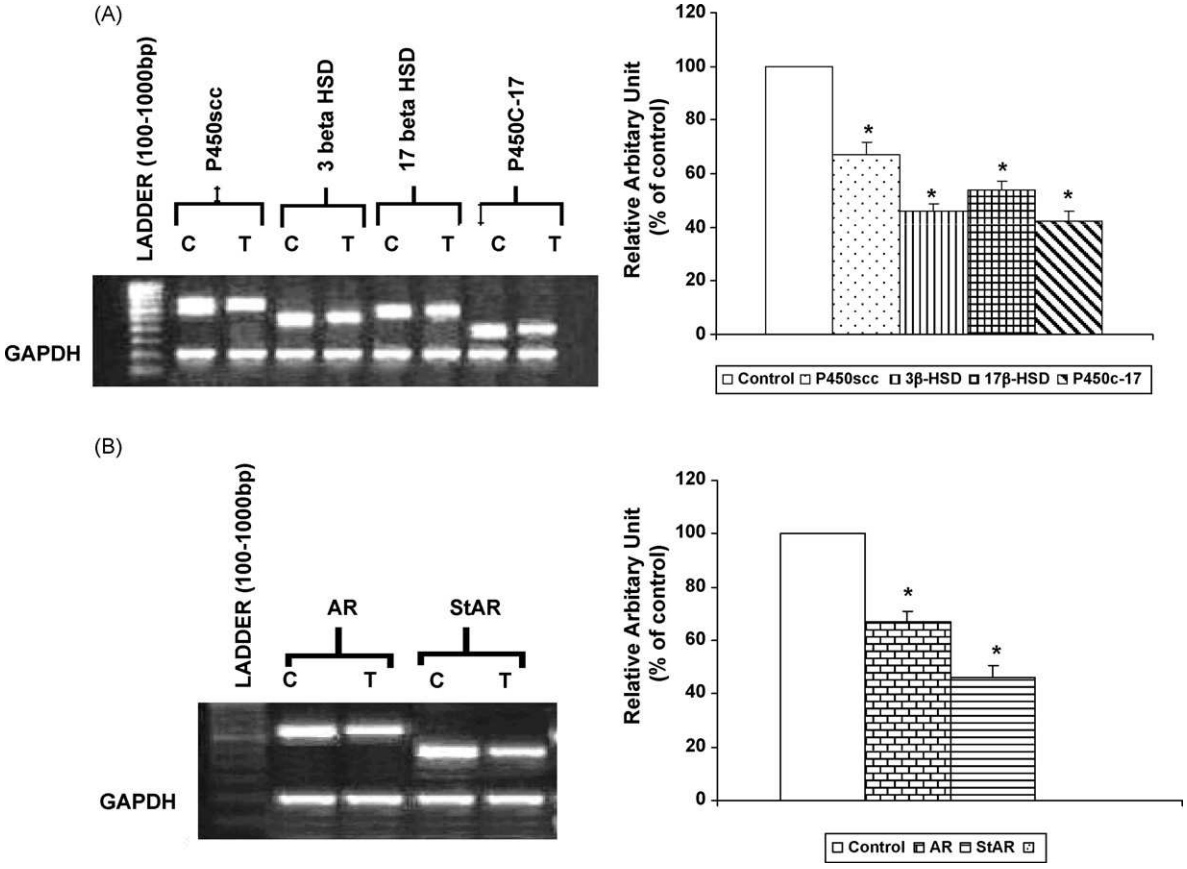

Fig. 2. Changes in the transcriptional profile of some of the testicular genes in response to triclosan. A representative image of RT-PCR products of testicular mRNA for $P450_{SCC}$, 3β-HSD, 17β-HSD and $P450_{C17}$ genes (A), androgen receptor (AR) and StAR genes (B), in rats treated daily either with vehicle or with test chemicals at a dose of 20 mg/(kg day). Corresponding histogram showing mean ± S.E.M. of relative arbitrary units of the bands for three similar experiments conducted with pooled samples from each group expressed as percent of control (which was given a value of 100). C, vehicle treated control; T, treated with 20 mg/(kg day) of triclosan. *Significantly different at $p < 0.05$ vs. control.

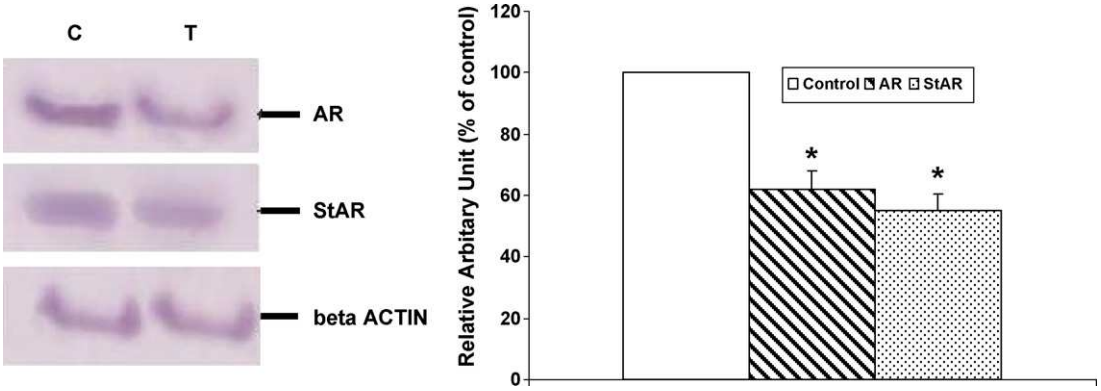

Fig. 3. Changes in the translational profile of some of the testicular genes in response to triclosan. Representative immunoblot showing the levels of testicular AR and StAR proteins in rats treated daily either with vehicle or with test chemicals at a dose of 20 mg/(kg day). Corresponding histogram showing mean ± S.E.M. of relative arbitrary units of the bands for three immunoblots conducted with pooled samples from each group expressed as percent of control (which was given a value of 100). C, vehicle treated control; T, treated with 20 mg/(kg day) of triclosan. *Significantly different at $p < 0.05$ vs. control.

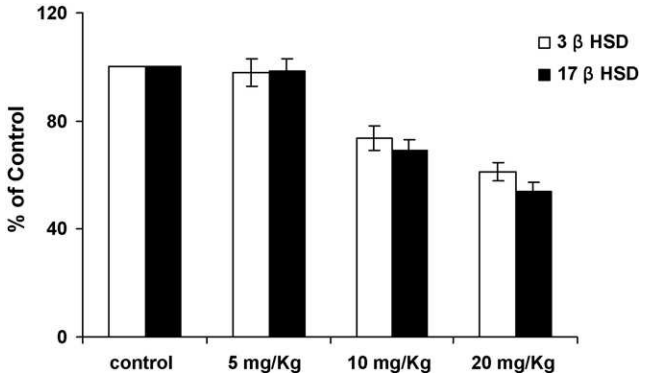

Fig. 4. Effects of triclosan on testicular level of 3β-HSD and 17β-HSD enzyme activity *in vitro*. The crude enzyme extract was isolated from the testes of vehicle treated (control) and triclosan treated rats intubated daily with varying dosages of test chemicals and incubated in the presence of respective substrates as described in materials and methods (n = 8). The results are expressed as fold increase of enzyme activity over vehicle treated groups. Data are mean ± S.E.M. of eight enzymatic reactions for each group for both the enzymes.

Table 4
Serum levels of LH, FSH, cholesterol, pregnenolone and testosterone from untreated (control) and triclosan treated (20 mg/(kg day)) male rats.

	Control	Treated
LH (ng/ml)	1.04 ± 0.054	0.64 ± 0.056**
FSH (ng/ml)	8.12 ± 0.168	6.82 ± 0.045**
Pregnenolone (ng/ml)	0.26 ± 0.008	0.18 ± 0.007**
Testosterone (ng/ml)	6.60 ± 0.130	3.94 ± 0.077**
Cholesterol (mg/dl)	93 ± 7.00	61 ± 5.00**

Each value denotes mean ± S.E.M. of eight animals.
** Significantly different from vehicle treated control group at $p < 0.05$ level.

3.5. Immunohistochemical detection of testicular StAR protein

As shown in Fig. 5, there was a decreased localization of StAR protein in testicular Leydig cells as determined by immunolocalization (Fig. 5C), indicating a reduced expression of this protein in animals treated with TCS as compared to control (Fig. 5A). The reduced expression of StAR could also be correlated to the reduction in Leydig cell number. In order to confirm this correlation, an *in vitro* MTT assay was performed using isolated Leydig cells treated with TCS which did not show any significant cell death even at a concentration of 10 μM TCS treatment (not shown).

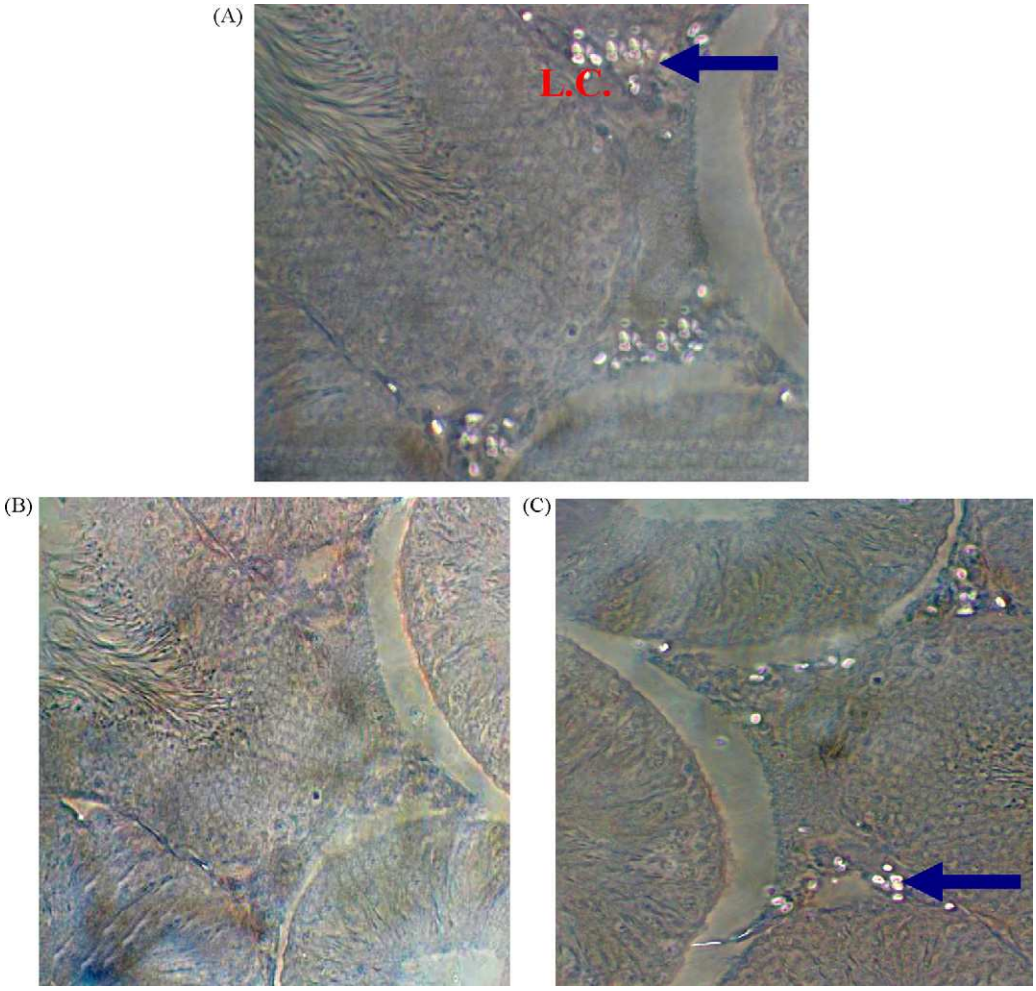

Fig. 5. Immunolocalization of StAR in Leydig cells from testis of control and rats treated daily with triclosan at a dosage level of 20 mg/(kg day). (A) Leydig cells from vehicle treated rats showing expression of StAR (shown by arrow). (B) Testis section from a vehicle treated rat incubated with non-fat milk without primary antibody (negative control). (C) Testis section from the rats treated with triclosan showing a decreased staining of Leydig cells (shown by arrow) indicating a decreased level of StAR. No background staining was provided to the sections and photographs captured in phase contrast mode leading to the appearance of whitish violet colored spot (developed as result of StAR antigen–antibody complex). 40×.

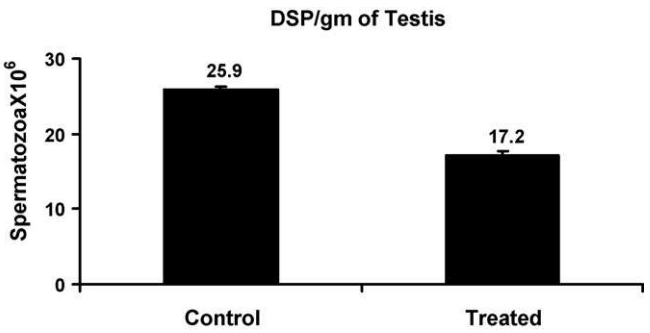

Fig. 6. Daily sperm production/g of testis weight (DSP/g) in vehicle treated (control) and triclosan treated (20 mg/(kg day)) rats. Data are presented as mean ± S.E.M. of duplicate determinations from individual testes; $n = 8$ for control and treated testes. DSP was significantly decreased ($p < 0.05$) in the treated group as compared to control.

3.6. Histopathology of testis and SATs

A number of histopathological malformations were observed in the testis and SATs of 20 mg/(kg day) group as compared to control which probably affected the production and maturation of the sperms. This was supported by a 34% decrease in the DSP/g of testis weight ($p < 0.05$) in treated group as compared to control (Fig. 6). Although the cauda epididymis (CE) from control rats showed a normal structure and sperm density (Fig. 7A), a reduced sperm density was observed in the lumina of epididymal tubule from the treated rats (Fig. 7B).

A normal thickness as well as arrangement of ciliated brush border was observed in vas deference from control rats (Fig. 8A) while several malformations were observed in vas deference from treated rats (Fig. 8B). Lumen of vas deference from the treated rats showed the presence of sterocilia detached from the epithelium and presence of eosinophillic bodies (Fig. 8B). The stereocilia were found to be thin, few or absent in the epithelium of treated rats (Fig. 8B).

In the case of prostate tissues, folliculli appeared to be normal and large sized in the control rats (Fig. 8C) while in the treated rats the folliculli were found to be comparatively degenerated and empty and follicular walls were thinner (Fig. 8D). However, surprisingly the seminal vesicles did not display any noticeable histopathological changes in control and treated rats (data not shown).

4. Discussion

TCS is a synthetic chemical widely used as an antimicrobial agent in different commercial preparations [11]. Since TCS possess a phenolic moiety like many of the common EDC it could be presumed to display similar activities as demonstrated by other EDC of the same chemical family [31,32]. The dosage for the test chemicals used in this study were selected based on LD_{50} values and also some earlier reports where a similar compound, triclocarbon (TCC), was used in rat models [33,34]. In this synthesis it should be noted that TCS is considered as a traditional EDC whereas the group of chemicals represented by TCC may rather act as a new-type of EDC as an "enhancer" through mechanisms that are yet to be identified. Based on these reports, in the present study 5, 10 and 20 mg/(kg day) of TCS were administered by oral routes. Although, 10 and 20 mg/(kg day) showed significant responses against the parameters tested here, we presented the data only for the latter in majority of the experiments since the response were almost similar by those two dosage levels.

Testicular androgenesis involves several crucial steps right from the synthesis of cholesterol, the parent molecule for all the steroid hormones, to its transport within the steroidogenic tissues and then its further metabolism to form steroids. Cholesterol is acquired by steroidogenic cells either by de novo synthesis or from the high density/low density lipoprotein (HDL and LDL) circulating in the blood [35]. In this study, 20 mg/kg/day group demonstrated a decreased level of serum cholesterol. In addition, some of our preliminary data also showed the reduction in the level of SRB-1 expression (data not shown). SRB-1 is a type of lipoprotein receptor present on the surface of steroidogenic cells to recognize HDL and LDL and is responsible for their uptake in those cells. It could be presumed that reduction in SRB-1 level might interfere with the uptake of cholesterol by those cells. Both these events might have been achieved by a reduced LH production (as demonstrated by serum hormone

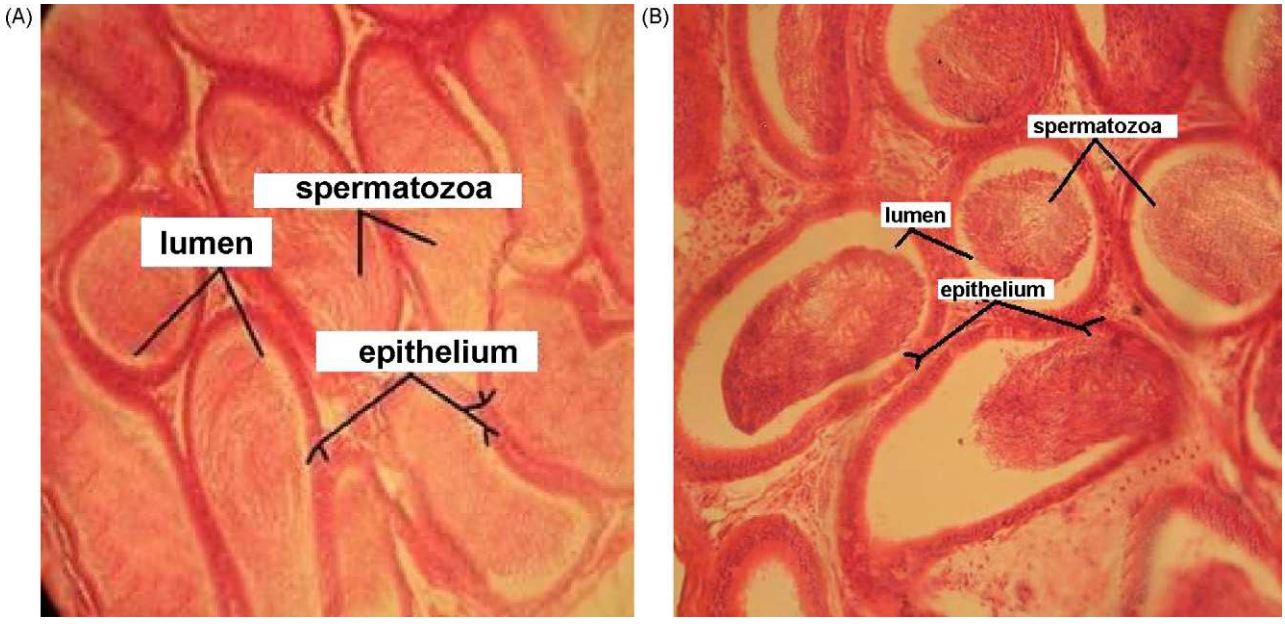

Fig. 7. Photomicrographs of rat cauda epididymes showing reduction in sperm mass as a result of 60 days consecutive triclosan treatment at a dosage level of 20 mg/(kg day). (A) Vehicle treated rat and (B) triclosan treated rats, H&E, 20×.

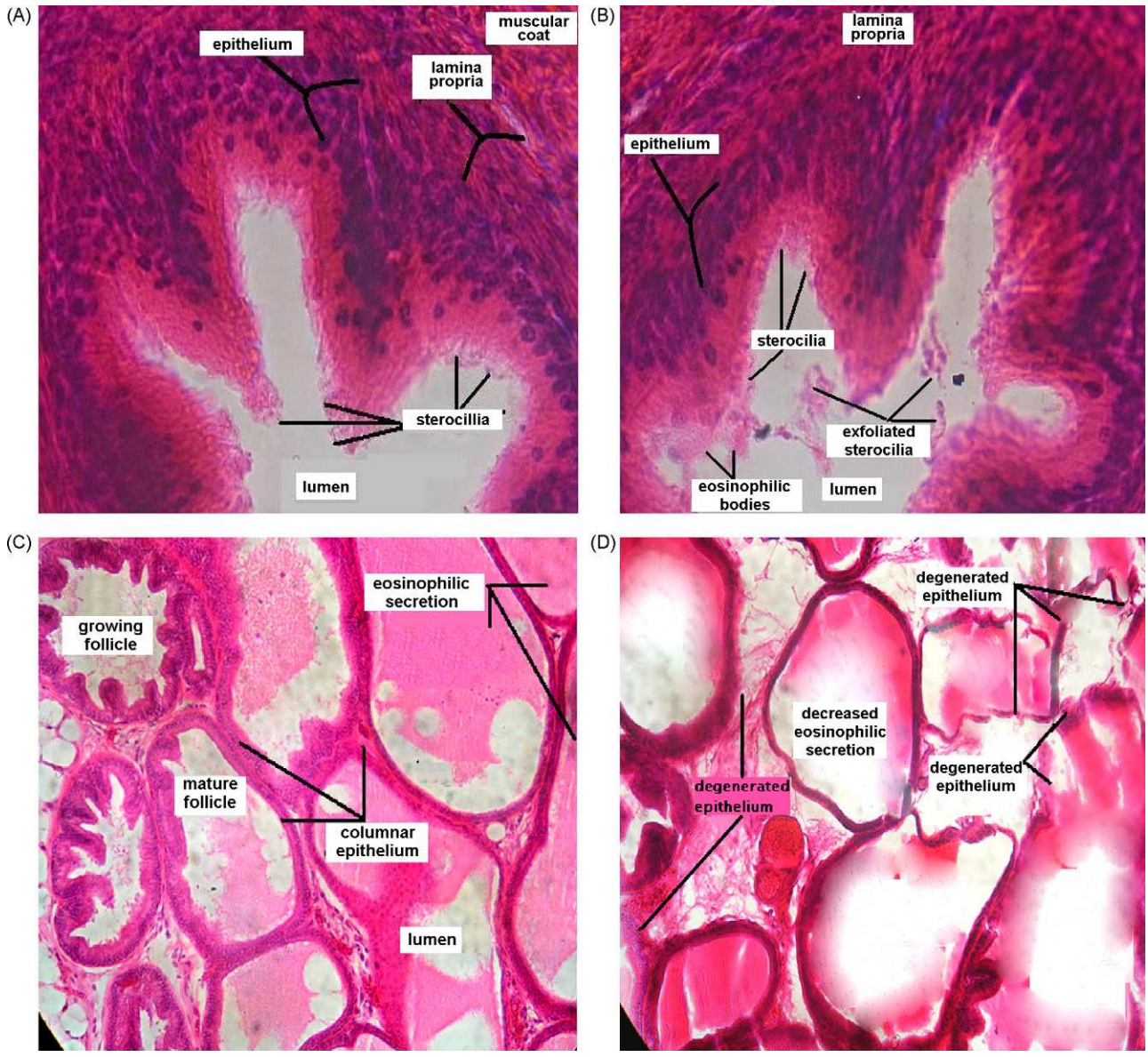

Fig. 8. Structures of vas deferens and prostate in vehicle treated control rats and triclosan treated (20 mg/(kg day)) rats. Sections of vas deferens from (A) control rat and (B) triclosan treated rats and sections of prostrate tissue from (C) control rats and (D) triclosan treated rats, H&E, 63×.

analysis) since this hormone promotes the synthesis of cholesterol by activating the enzymes of cholesterol synthesizing machinery (like cholesterol ester hydrolase) and also regulates the uptake of cholesterol esters by steroidogenic cells by stimulating the expression of receptors to recognize LDL and HDL, e.g. SRB-1 [36]. Serum hormone analysis further demonstrated decreased serum testosterone level which could be attributed to TCS induced decreased synthesis and availability of cholesterol for steroidogenesis. It has been shown elsewhere also that a decreased cholesterol synthesis results in the down-regulation of steroidogenesis [37].

Once the cholesterol is synthesized, StAR is a factor that plays a crucial role in regulating steroidogenesis by transporting cholesterol to inner mitochondrial membrane (IMM) [38,39]. The present findings indicated a decreased transcription and translation of StAR in the testis of 20 mg/(kg day) group (as evident by RT-PCR, immunoblot and immunohistochemical analysis). This might have been caused by a decreased level of serum LH since it regulates steroidogenesis mostly by regulating the level and activity of StAR protein in the steroidogenic cells [29,40]. Several studies have demonstrated that steroidogenesis is severely decreased by a reduced StAR activity [29–31].

In the IMM cholesterol is acted upon by $P450_{SCC}$, one of the major enzymes regulating steroidogenesis, to produce pregnenolone [41,42]. This study showed a significantly reduced level of $P450_{SCC}$ enzyme which might have been caused by a decreased availability of its substrate (cholesterol) in IMM due to reduced level of FSH and LH, since both regulates the level of $P450_{SCC}$ [36]. Thus, a significantly decreased expression of $P450_{SCC}$ enzyme might have been another contributor for attenuating testosterone synthesis. It has been already reported that chemicals like nonylphenol and DBP decreases steroidogenesis by directly inhibiting the expression of StAR and $P450_{SCC}$ enzyme [31,43].

Expression profiles and activity of two major steroidogenic enzymes, 3β-HSD and 17β-HSD, were also found to be significantly decreased in the treated rats. This finding is supported by some recent studies that showed direct effect of endocrine disruptors at enzyme levels [3,44,45]. According to literature, xenobiotics dependent direct up/down-regulation of steroidogenic enzymes and

steroidogenesis can be affected at several levels, viz. direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis, e.g. StAR protein [46] and increasing the stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes [47].

Furthermore, the expression patterns of AR, both at transcription and translation level was found to be decreased in treated groups as demonstrated by RT-PCR and immnunoblot analysis respectively. This might have been achieved by a reduced level of available testosterone although autologous regulation of AR gene in the testes is still a matter of controversy. Several reports exist on both regulation and non-regulation of AR mRNA expression by androgen itself [48–50].

Another interesting result of this study was the development of histopathological abnormalities in SATs of 20 mg/(kg day) group, namely CE, ductus deference and prostate. The decreased testosterone and AR level in treated rats (as indicated above) might have led to the degenerative changes and atrophy in the SATs as supported by their decreased weight and size (data not shown) in this study. Results also demonstrated a decreased sperm count in the testis of treated rats as compared to control, probably due to reduced testicular spermatogenesis induced by TCS [51,52]. This could also be attributed to the reduced level of serum FSH, a hormone directly involved in maintaining spermatogenesis in conjunction with testosterone [53]. Other degenerative changes observed in the cauda were occurrence of epithelial degeneration in the form of nuclear karyolysis and pyknosis. These types of histopathological changes in cauda have been reported by others also [54,55]. Similar degeneration and atrophy were also found in the other SATs like ductus deferens and prostate glands. All these could be attributed to the decrease in the level of androgen and androgen receptors which are known to support the functioning and continuous persistence of these organs.

In conclusion, TCS, a commonly used chemical in various cosmetics and other applications, may act as an endocrine disruptor in male rats and has the potential to impair the pituitary–gonadal pathway at various levels. Inhibition of androgen production by this chemical may be explained by its action at various steps of steroidogenesis: reduction of LH and cholesterol production; depressed StAR expression, one of the crucial protein responsible for cholesterol transport to inner mitochondrial membrane for its utilization by steroidogenic enzymes; and finally down-regulation of several key steroidogenic enzymes ($P450_{SCC}$, $P450_{C17}$, 3β-HSD, and 17β-HSD). Apparently gonadotrophic hormones, steroidogenic enzymes, and StAR, key proteins involved in androgen production and maintenance of SATs, are all potential targets for TCS-mediated impairment of steroidogenesis. To the best of our knowledge this is the first ever report on the anti-androgenic effects of TCS in male rats. However, further studies are needed to designate it as an anti-androgenic EDC since the effects are dependent on several factors like composition of chemicals, animal models, dose and time of treatment to name a few of them. An earlier report exists in the effect of polychlorinated biphenyls on testicular steroidogenesis through oxidative stress [56]. The effects observed here for TCS could involve similar testicular oxidative stress as a mechanism. All these facts reinforces the notion that these common chemicals pose a hazard to human health by acting through various targets in endocrine disruption and warrants further detailed investigation to pin-point the specific target site(s) for these chemical induced toxicity and endocrine disruption.

Conflict of interest

None declared.

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References

[1] Cargouet M, Perdiz D, Mouatassim A, Tamisier S, Levi Y. Assessment of river contamination by estrogenic compounds in Paris area (France). Sci Tot Environ 2004;324:55–66.
[2] Chen J, Ahn KC, Gee NA, Gee SJ, Hammock BD, Lasley BL. Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. Toxicol Appl Pharmacol 2007;221:278–84.
[3] Kumar V, Chakraborty A, Viswanath G, Roy P. Androgenic endocrine disruptors in wastewater treatment plant effluents in India: their influence on reproductive processes and systemic toxicity in male rats. Toxicol Appl Pharmacol 2007;226(1):60–3.
[4] Fenton SE. Endocrine-disrupting compounds and mammary gland development: early exposure and later life consequences. Endocrinology 2006;147(6):18–24.
[5] Darbre PD. Environmental oestrogens, cosmetics and breast cancer. Res Clin Endocrinol Metab 2006;20:121–43.
[6] Cabana H, Jiwan JL, Rozenberg R, Elisashvili V, Penninckx M, Agathos SN, et al. Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 2007;67:770–8.
[7] Lakeram M, Lockley DJ, Sanders DJ, Pendlington R, Forbes B. Paraben transport and metabolism in the biomimetic artificial membrane permeability assay (BAMPA) and 3-day and 21-day Caco-2 cell systems. J Biomol Screen 2006;12:84–91.
[8] Dayan AD. Response to: ethics, data and the pharmaceutical physician. Int J Clin Pract 2006;60:1009.
[9] Heidler J, Sapkota A, Halden RU. Partitioning, persistence and accumulation in digested sludge of the topical antiseptic triclocarban during wastewater treatment. Environ Sci Technol 2006;40:3634–9.
[10] Nakada N, Tanishima T, Shinohara H, Kiri K, Takada H. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Res 2006;40:3297–303.
[11] Black JG, Howes DRT. Percutaneous absorption and metabolism of Irgasan DP 300. Toxicology 1975;3:33–47.
[12] Tatarazako N, Ishibashi H, Teshima K, Kishi K, Arizono. K Effects of triclosan on various aquatic organisms. Environ Sci 2004;11(2):133–40.
[13] Kanetoshi A, Ogawa H, Katsura E, Kaneshima H. Chlorination of Irgasan DP300 and formation of dioxins from its chlorinated derivatives. J Chromatogr 1987;389:139–53.
[14] Kanetoshi A, Ogawa H, Katsura E, Kaneshima H, Miura T. Formation of polychlorinated dibenzo-p-dioxins upon combustion of commercial textile products containing 2,4,4-trichloro-2-hydroxydiphenyl ether (Irgasan DP300). J Chromatogr 1998;442:289–99.
[15] Kanetoshi A, Ogawa H, Katsura E, Kaneshima H, Miura T. Formation of polychlorinated dibenzo-p-dioxins from 2,4,4-trichloro-2-hydroxydiphenyl ether (Irgasan DP300) and its chlorinated derivatives by exposure to sunlight. J Chromatogr 1998 b;454:145–55.
[16] Foran CM, Bennett ER, Benson WH. Developmental evaluation of a potential non-steroidal estrogen: triclosan. Mar Environ Res 2000;50(1–5):153–6.
[17] Ishibashi H, Matsumura N, Hirano M, Matsuoka M, Shiratsuchi H, Ishibashi Y, et al. Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. Aquat Toxicol 2004;67:167–79.
[18] Bhargava HN, Leonard PA. Triclosan: applications and safety. Am J Infect Control 1996;24:209–18.
[19] Biswas NM, Gupta RS, Chattopadhyay A, Choudhury GR, Sarkar M. Effect of atenolol on cadmium induced testicular toxicity in male rats. Reprod Toxicol 2001;15:699–704.
[20] Blesson CS, Awasthi S, Kharkwal G, Daverey A, Dwivedi A. Steroids 2006;71:993–1000.
[21] Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 1978;54:103–7.
[22] Cooke PS, Kirby JD, Porcelli J. Increased testis growth and sperm production in adult rats following transient neonatal goitrogen treatment: optimization of the propylthiouracil dose and effect of methimazole. J Reprod Fertil 1993;97:493–9.

[23] Fernandes GSA, Arena AC, Fernandez CDB, Mercadante A, Barbisan LF, Kempinas WG. Reproductive effects in male rats exposed to diuron. Reprod Toxicol 2007;23:106–12.
[24] Mukherjee KL. Routine biochemical tests and histological techniques. In: Mukherjee K, editor. Medical laboratory technology. New Delhi: Tata McGraw-Hill Publishing Company Limited; 2003. p. 1124–90.
[25] Kroft TL, Patterson J, Won Yoon J, Doglio L, Walterhouse DO, Iannaccone PM, et al. GLI1 localization in the germinal epithelial cells alternates between cytoplasm and nucleus: upregulation in transgenic mice blocks spermatogenesis in pachytene. Biol Reprod 2000;65:1663–71.
[26] Sarkar M, Biswas NM, Ghosh D. Effect of sodium arsenite on testicular 3 and 17 hydroxysteroid dehydrogenase activities in albino rats: dose and duration-dependent responses. Med Sci Res 1991;19:789–90.
[27] Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[28] Ohsako S, Kubota K, Kurosawa S, Takeda K, Qing W, Ishimura R, et al. Alterations of gene expression in adult male rat testis and pituitary shortly after sub acute administration of the antiandrogen flutmide. J Reprod Dev 2003;49:275–90.
[29] Murugesan P, Muthusamy T, Balasubramanian K, Arunakaran J. Effects of vitamins C and E on steroidogenic enzymes mRNA expression in polychlorinated biphenyl (Aroclor 1254) exposed adult rat Leydig cells. Toxicology 2007;232:170–82.
[30] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[31] Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, et al. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 2005;84:249–59.
[32] Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Ann Rev Physiol 2001;63:193–213.
[33] Nollen GA, Dierckman TA. Reproduction and teratogenic studies of 2:1 mixture of 3,4,4′-trichlorocarbanilide and 3-trifluoromethyl-4,4′-dichlorocarbnilide in rats and rabbits. Toxicol Appl Pharmacol 1979;51:417–25.
[34] Chen J, Ahn KC, Gee NA, Ahmed MI, Duleba AJ, Zhao L, et al. Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 2008;149(3):1173–9.
[35] Cao G, Zhao L, Strangle H, Hasegawa T, Richardson JA, Parker KL, et al. Developmental and hormonal regulation of murine scavenger receptor, class B, type 1. Mol Endocrinol 1999;13:1460–73.
[36] Fauser BCJM. Molecular biology of steroidogenesis. In: Molecular biology in the reproductive medicine. New York: Parthenon Publishing; 1999. p. 234–51.
[37] Barlow NJ, Phillips SI, Wallace DC, Gaido WK, Foster PMD. Qunatitative changes in gene expression in fetal rat testis following exposure to di(n-butyl) phathalate. Toxicol Sci 2003;73:431–51.
[38] Hasegawa T, Zhao L, Caron KM, Majdic G, Suzuki T, Shizava S, et al. Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice. Mol Endocrinol 2000;14:1462–71.
[39] Manna PR, Roy P, Clark BJ, Stocco DM, Huhtaniemi IT. Interaction of thyroid hormone and steroidogenic acute regulatory (StAR) protein in the regulation of murine Leydig cell steroidogenesis. J Steroid Biochem Mol Biol 2001;76:167–77.
[40] Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, et al. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem 1997;272(51):32656–62.
[41] Miller WL. Molecular biology of steroid hormone synthesis. Endocr Rev 1988;9:295–318.
[42] Omura T, Morohashi K. Gene regulation of steroidogenesis. J Steroid Biochem Mol Biol 1995;53:19–25.
[43] Arukwe A. Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450scc) in juvenile Atlantic salmon (*Salmo salar*) is a novel aspect of nonylphenol toxicity. Environ Sci Technol 2005;39:9791–8.
[44] Andric NL, Kostic TS, Zoric SN, Stanic BD, Andric SA, Kovacevic RZ. Effect of PCB-based transformer oil on testicular steroidogenesis and xenobiotic-metabolizing enzymes. Reprod Toxicol 2006;22:102–10.
[45] Lyssimachou A, Jessen BM, Arukew A. Brain cytochrome P450 aromatase gene isoforms and activity levels in Atlantic salmon after waterborne exposure to nominal environmental concentrations of the pharmaceutical ethynylestradiol and antifoulant tributyltin. Toxicol Sci 2006;91:82–92.
[46] Rice S, Mason HD, Whitehead SA. Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. J Steroid Biochem Mol Biol 2006;101:216–25.
[47] Lin TC, Chien SC, Hsu PC, Li LA. Mechanistic study of polychlorinated biphenyl 126-induced CYP 11B1 and CYP 11B2 up-regulation. Endocrinology 2006;147:1536–44.
[48] Blok LJ, Barlett JM, Bolt-De Vries J, Themmen AP, Brinkmann AO, Weinbauer GF, et al. Effect of testosterone deprivation on expression of the androgen receptor in rat prostate, epididymis and testis. Int J Androl 1992;15:182–98.
[49] Prins GS, Woodham C. Autologous regulation of androgen receptor messenger ribonucleic acid in the separate lobes of the prostate gland. Biol Reprod 1995;53:609–19.
[50] Shan LX, Bardin CW, Hardy MP. Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. Endocrinology 1997;138:1259–66.
[51] Ku WE, Chapin RE, Wine RN, Gladen BC. Testicular toxicity of boric acid (BA): relationship of dose to lesion development and recovery in the F334 rat. Reprod Toxicol 1993;7:305–19.
[52] Poon R, Rigden M, Chu I, Valli VE. Short-term oral toxicity of pentyl ether, 1,4-diethoxybutane, and 1,6-dimethoxyhexane in male rats. Toxicol Sci 2004;77:142.
[53] Plant TM, Marshall GR. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocr Rev 2001;22(6):764–86.
[54] Chitra KC, Ramachandra KR, Mathur PP. Effect of bisphenol A and co-administration of bisphenol A and vitamin C on epididymis of adult rats: a histological and biochemical study. Asian J Androl 2003;5:203–8.
[55] Narayana K, Prashanthi N, Nayanatara A, Kumard SG, Kumare HHC, Bairy KL, et al. A broad-spectrum organophosphate pesticide O,O-dimethyl O-4-nitrophenyl phosphorothioate (methyl parathion) adversely affects the structure and function of male accessory reproductive organs in the rat. Environ Toxicol Pharmacol 2006;2:315–24.
[56] Murugesan P, Balaganesh M, Balasubramanian K, Arunakaran J. Effects of polychlorinated biphenyl (Arochlor 1254) on steroidogenesis and antioxidant system in cultured adult rat Leydig cells. J Endocrinol 2007;192:325–38.