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Anti-androgenic endocrine disrupting activities of chlorpyrifos and piperophos

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ABSTRACT

The present work describes the screening and characterization of some common endocrine disrupting chemicals for their (anti)androgenic activities. Various chemicals (mostly pesticides and pharmaceuticals) were screened with the NIH3T3 cell line stably expressing human androgen receptor (hAR) and luciferase reporter gene for their ability to stimulate luciferase activity or inhibit the response that was evoked by 0.4 nM testosterone. The most potent anti-androgenic compounds identified in our assay included chlorpyrifos, endosulfan and piperophos. Finally, the chemicals were analyzed for their effects on steriodogenesis in rat Leydig cells. Piperophos and chlorpyrifos showed a significant decrease in testosterone biosynthesis by Leydig cells. RT-PCR studies showed decrease in the expression of key steroidogenic enzymes: cytochrome P450scc, 3β-HSD and 17β-HSD and immunoblot analysis demonstrated a decrease in steroidogenic acute regulatory (StAR) protein expression by both these chemicals. Chlorpyrifos also showed a decrease in LH receptor stimulated cAMP production. In conclusion, we demonstrate that commonly used pesticides like chlorpyrifos and piperophos pose serious threat to male reproductive system by interfering at various levels of androgen biosynthesis.

1. Introduction

Androgens are the hormones that play a pivotal role in the development and maintenance of the male sex characteristic. Their biological effects are mediated by the ubiquitously expressed androgen receptor (AR). The levels of AR change in different pathological conditions such as malignancies or in response to physiological changes of the endocrine system. AR translocates to the nucleus where it binds to the regulatory regions of androgen-responsive genes and subsequently stimulates their transcription [1]. Anti-androgenic compounds, respectively, bind to the AR, but block its transcriptional activity.

In addition to endogenous steroid hormones, an increasing number of pharmaceutics, products in industrial wastes, pesticides, fungicides, water treatment plant effluents have been identified as AR agonists and/or antagonists. These compounds that have the capability to alter male and female sexual functions are generally considered as endocrine disrupting chemicals (EDCs) and have become an important environmental concern [2,3]. Although there are some reports on identifying these chemicals using binding studies with receptors or estimating them chemically, but they mostly fail to show the biological activities of these compounds. This necessitates the development of some in vitro bioassays like that of reporter gene test which can be used to assess the role of these chemicals as endocrine disruptors at cellular levels. For the development of reporter based assays, generally, cell lines lacking the endogenous AR are stably or transiently co-transfected with either human or rat AR expression plasmid in combination with a reporter plasmid containing either chloramphenicol acetyl transferase (CAT) or a luciferase gene under transcriptional control of the MMTV promoter. A wide range of cell lines like CHO-K1, COS1, COS7, CV1, MDA-kb-2, PC3, human U2-OS have been used for this purpose [2,4–10].

Apart from interfering with the hormone binding to the receptor, EDCs have also been known to interfere with the steroid biosynthesis pathway. A wide range of chemicals like plasticizers, pesticides, fungicides, pharmaceuticals were shown to affect steroidogenesis and hypothalamic–pituitary–gonadal axis thereby altering the serum testosterone and luteinizing hormone (LH) levels. Exposure to phthalates like benzylbutyl (BBP), di(n)butyl (DBP), diethylhexyl (DEHP), to fungicides like procloraz, iprodione, ketoconazole, to pesticides like fenarimol, dieldrin, dioxin, decreases testicular testosterone production [11–17]. Studies showed that the decrease in testosterone production in Leydig cells was due

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to the alterations in cholesterol availability in the mitochondria for steroidogenesis, altered steroidogenic enzyme activities and feed back mechanisms. Recently, Chen et al. [18] had shown the interference of triclocarban in cAMP dependent pathway activated by LH receptor. A similar study also demonstrated a cross-talk between LH receptor and triclosan [19]. All these support the notion that these EDCs act at various levels of steroidogenic cascade to disrupt the endocrine system.

The main aim of the present study was to screen different environmental chemicals for their androgenic and anti-androgenic activities using a reporter based bioassay. For this, a stable cell line was made with NIH3T3 cells, transfected with human-AR and the androgen dependent promoter driven luciferase reporter gene. Using this recombinant cell line, chemicals were tested for their (anti)androgenic activities. These chemicals were then studied for their effects on steroid biosynthesis in rat Leydig cell cultures and selected chemicals were further analyzed for their cross-talk with LH receptor mediated functions using a cell-based indirect cAMP assay as was reported earlier [20,21]. The data presented here demonstrates that some widely used pesticides and pharmaceutics have (anti)androgenic properties which are not only restricted through their interactions with AR but also affects the LH receptor and cAMP cascades. All this warrants further investigation to fully understand their potential impact on human reproductive health.

2. Materials and methods

2.1. Chemicals

All the cell culture media were purchased from GIBCO (GIBCO, BRL, Inchinnan, UK) while the antibiotics puromycin and G418 were obtained from Sigma (Sigma, St. Louis, MO, USA) and Promega (Promega Corp., Southampton, UK), respectively. Superfect transfection reagent was purchased from QIAGEN (Qiagen, Valencia, CA). Among the tested chemicals salbutamol and acetazolamide were a kind gift from Dr. R. Husain of Glenmark Pharmaceuticals Ltd., Mumbai, India. Testosterone, estrogen, progesterone, dexamethasone, danazol, spirinolactone and endosulfan were kindly provided by Professor Ilpo Huhtaniemi, Imperial College, London, UK. Chlorpyrifos, piperophos and mancozeb were purchased from Rankem (Rankem, Mumbai, India).

2.2. Cell lines

NIH3T3 and CHO cells were grown in DMEM medium with or without phenol red (GIBCO, BRL, Inchinnan, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (10^5 U/l) and streptomycin (100 mg/ml) (GIBCO, BRL, Inchinnan, UK) in a humidified 5% CO_2 incubator. All the treatments of the cells were performed with charcoal-stripped FCS to reduce the contaminating steroids from the serum.

2.3. Stable transfection

Stable transfections were performed by the lipofection method according to the manufacturer's recommendation (Superfect transfection reagent; QIAGEN, Valencia, CA). Plasmids pMMTV–neomycin-luciferase (MMTV-Neo-Luc) and pSG5-hAR-puro, pSG5-human luteinizing hormone receptor (hLHR) and pADneo2-BGL containing 6× CRE-luciferase (CRE-Luc) were kindly provided by Professor Ilpo Huhtaneimi, Imperial College London, UK. For stable cell lines, the NIH3T3 cells were transfected with hAR and MMTV-Neo-Luc constructs using transfection reagent as per the manufacturer's instruction (Qiagen, Valencia, CA). Forty-eight hours after that the cells were trypsinized and fresh cells were plated on a 150-mm diameter plate and selected in medium containing 1 g/l of neomycin (G 418) and 1 mg/l puromycin. The medium was changed two times a week. After about 2–3 weeks, some neomycin and puromycin-resistant clones appeared in the plates. They were picked using cloning rings and replated in 24-well culture plates. During the assay, about 10,000 cells per well were plated on 96-well plates in 200 μl of DMEM medium without phenol red and with 10% FCS. The following day the cells were washed with PBS and the medium was changed to 200 μl DMEM with 10% charcoal-stripped FCS. After about 3 h, the test compounds were added to the wells in the required concentrations keeping the final concentration of ethanol in the incubations to be 1%. The cells were incubated with the compounds for another 24 h. In case of antagonists, the cells were incubated with the test compound in the presence of 0.4 nM testosterone (half maximally stimulating concentration of testosterone). Luciferase activity was measured using the constant light signal luciferase reaction kit (Roche, East Sussex, UK) according to the manufacturer's instruction. The light produced in the reaction was measured in a multilabel plate reader (BMG Labtech, Germany).

2.4. Leydig cell culture

Leydig cells were isolated according to the method as described earlier [22]. Briefly, the testes collected from freshly sacrificed rats were decapsulated and digested in collagenase (type I) (0.25 mg/ml) containing DMEM-F12 medium at 37 °C for 15 min in a shaking water bath. On completion of the incubation, the tubes were gently shaken, and then 10 ml of DMEM-F-12 medium without collagenase was added and allowed to stand for 15 min. The supernatant was aspirated and transferred to a sterile tube and the procedure was repeated again. The crude Leydig cell preparation obtained was further purified on discontinuous percoll gradients. The purity of Leydig cells was assessed by immunocytochemical staining of 3β-HSD according to the methods described earlier [23] with some modifications. Briefly, the fixed cells were first allowed to dry for 20 min at 37 °C followed by at RT for 1–2 h. The cells were then treated with formamide and successively incubated at RT, 75 °C and 4 °C for various times. The cells were then incubated with blocking buffer and followed by 3β-HSD antibody for 30 min at RT. Finally the cells were treated with fluorescein conjugated secondary antibody (Genei, Bangalore, India) for 1 h at RT and then washed 3 times with distilled water and observed under fluorescence microscope (Axiovert25 CFL, Carl Zeiss, USA). The cell viability was determined by trypan blue dye exclusion method. The purity was 85–90% and viability was 90%. Purified Leydig cells were plated in culture plates containing DMEM-F12 medium with 2% FBS. After 24 h, cells were washed twice with FBS-free medium and starved for an hour at 34 °C. After starvation, the medium was replaced with fresh medium containing various concentrations of test compounds (0.1–100 μM) and incubated for another 24 h at 34 °C. Leydig cells were used to assess the cell viability in response to the test chemicals by microtiter assay according to the method described earlier [24]. Briefly, to the test chemical treated cells, 50 μl of 5 mg/ml of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) was added and incubated at 37 °C for 4 h. The MTT-containing medium was then aspirated and 200 μl of DMSO (Himedia, Mumbai, India) and 25 μl of Sorensen glycine buffer (0.1 M glycine and 0.1 M NaCl, pH 10.5) were added to lyse the cells and solubilize the water insoluble formazone. Absorbances of the lysates were determined on a Fluostar optima (BMG Labtech, Germany) microplate reader at 570 nm. The percentage inhibition was calculated as

$$\frac{\text{Mean OD of vehicle treated cells (negative control)} - \text{mean OD of treated cells}}{\text{Mean OD of vehicle treated cells (negative control)}} \times 100$$

Table 1
Primers used for semi-quantitative RT-PCR.

Gene	Primer sequence No.	Product size	Gene Bank accession
P450scc-F P450scc-R	CGCTCAGTGCTGGTCAAAA TCTGGTAGACGGCGTCGAT	688	J0_5156
3β-HSD-F 3β-HSD-R	CCGCAAGTATCATGACAGA CCGCAAGTATCATGACAGA	547	M_38178
17β-HSD-F 17β-HSD-R	TTCTCAAGGCTTTACCAGG ACAAACTCATCGGCGGTCTT	653	AF_035156
AR-F AR-R	TTACGAAGTGGGCATGATGA ATCTTGTCCAGGACTCGGTGG	570	M_20133
GAPDH-F GAPDH-R	AGACAGCCGCATCTTCTTGT CTTGCCGTGGGTAGAGTCAT	207	NM_017008

To analyze testosterone producing efficacy, Leydig cells were exposed to test compounds with and without LH (100 ng/ml) for 24 h at 34 °C, then the culture media were collected for testosterone assay. The assays were performed using the commercial enzyme immunoassay kits as per manufacturer's instructions (Omega Diagnostics, UK). A fresh batch of Leydig cells were treated with 10 nM concentrations of chlorpyrifos and piperophos (that showed a significant decrease in testosterone), for the isolation of RNA and protein which was used for the analysis of steroidogenic enzymes and StAR protein expression profiles, respectively.

2.5. Indirect estimation of cAMP in CHO-K1 cell lines

The cAMP was estimated by an indirect method using the luciferase driven cAMP response element according the method described earlier [20]. Briefly, CHO-K1 cells were transiently transfected with 1 μg each of hLH receptor and CRE-luc constructs (as described earlier) using transfection reagent as per the manufacturer's instruction (Qiagen, Valencia, CA, USA). Twenty-four hours after transfection the cells were treated with hCG (10 ng/ml) or other test chemicals and incubated further for another 15 h. The media were then removed and cells were lysed using the lysis buffer and then assayed for reporter gene expression. Luciferase activity was determined using the luminescence kit (Roche, East Sussex, UK).

2.6. RNA isolation and semi-quantitative RT-PCR

Total RNA from the cells was isolated using TRI reagent (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. The total RNA was quantified using spectrophotometer at 260 nm. Equal amount of RNA was used for the semi-quantitative RT-PCR studies and was performed according to the manufacturer's instructions (Genei, Bangalore, India). The primer sequences were designed according to earlier report [19] and are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as internal standard.

2.7. Western blot analysis

The Leydig cells were cultured in a petridish and treated with the test compounds for 24 h. The cells were then lysed directly in the dish by adding lysis buffer (160 mM Tris, pH 6.9, 200 mM DTT, 4% SDS, 20% glycerol, 0.004% bromophenol blue) in the presence of a protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The lysate was collected into a microfuge tube and boiled for 10 min and cellular debris was pelleted at 13,000 × *g*. The extracts were immunoblotted with a polyclonal antibody for StAR (Kindly donated by Professor D.M. Stocco, Texas Technical University, USA). The signal was detected using the alkaline phosphatase conjugated secondary antibody purchased from Genei (Bangalore, India).

2.8. Statistical analysis

The values shown are the mean ± S.D. from three independent experiments performed in quadruplicates for each treatment. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons test or Student's t test when appropriate. The level of significance was set at $p < 0.05$. The median effective concentration (EC_{50}) and the median inhibitory concentration (IC_{50}) values were calculated from the dose response curve considering best-fit values using GraphPad Prism, version 5.02 software (GraphPad Software Inc., CA, USA).

3. Results

3.1. Development of cell-based androgen reporter assay

About 12 neomycin and puromycin-resistant clones were obtained after selection in the presence of 10 nM testosterone. Some of the clones were found to be highly active in the presence of androgen. Out of all these clone number 4 was found to be the most active causing about 26-folds inductions in the presence of testosterone and was picked up for further studies and was referred as NIH3T3-AR-Luc cell. As shown in Fig. 1A, the testosterone induced luciferase activity increased in a dose dependent manner from 0.001 nM and finally saturating at 10 nM, with an EC_{50} value of 0.4 nM. The expression levels of AR were analyzed until passage 18, and they remained stable, even after freezing. Thereafter, the cells showed gradual reduction in AR activity.

To examine the specificity of the NIH3T3-AR-Luc cell line, the cells were treated with estradiol, progesterone and dexamethasone. All these steroids showed <3% transactivation (Fig. 1B) at a concentration of 100 nM which was insignificant even at a concentration 1000-fold higher than the 100% transactivation obtained with testosterone ($p < 0.05$). However, dexamethasone was marginally more active than other two steroids although statistically not significant.

3.2. Effects of various chemicals on luciferase activity of NIH3T3-AR-Luc cells

NIH3T3-hAR-Luc cells were treated with increasing concentrations of three known testosterone agonists, dihydroxytestosterone (DHT), nandrolone and danazol (Fig. 1C). These compounds showed an EC_{50} value of 0.19, 0.3 and 0.75 nM, respectively.

Different classes of chemicals – acetazolamide, salbutamol, piperophos, spironolactone, endosulfan, chlorpyrifos and mancozeb were then tested in NIH3T3-AR-Luc cells. Among the tested chemicals, while salbutamol and acetazolamide showed androgen agonistic activity (data not shown), the other latter five compounds, piperophos, spironolactone, endosulfan, chlorpyrifos and mancozeb, showed anti-androgenic activity (Fig. 2) when the cells were treated with increasing concentrations of the chemicals in the presence of 0.4 nM testosterone. Piperophos showed a strong antagonistic activity with an IC_{50} value of 1.4 μM while spirinolactone, endosulfan, chlorpyrifos and mancozeb although exhibited significant anti-androgenic activities ($p < 0.05$) but with a higher IC_{50} values in the range of 5–10 μM.

3.3. Effect of pesticides on Leydig cell viability and steroidogenesis

MTT assay was carried out to check the effect of some common pesticides as tested above on the Leydig cell viability. As shown in Fig. 3, there is no significant reduction in the cell viability when the

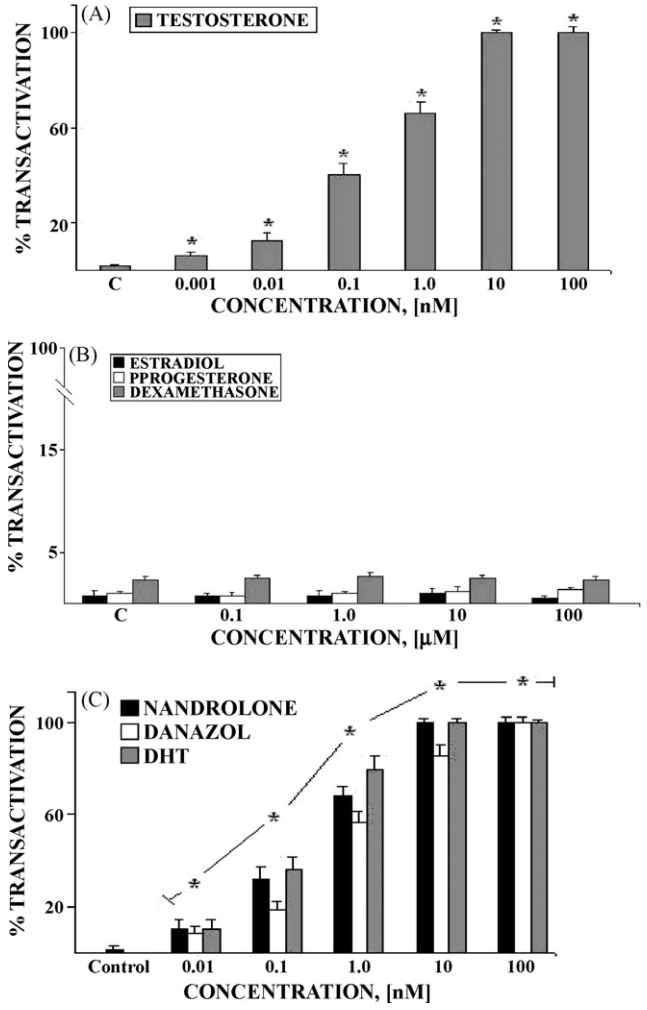

Fig. 1. Characterization of the transactivation assay in NIH3T3-AR-Luc cells. (A) Dose dependent luciferase induction by increasing concentration of testosterone. (B) Determination of ligand specificity. (C) Determination of the androgenic activities of nandrolone, danazol and DHT. Luciferase activities were expressed as percentage of that obtained with 10 nM testosterone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates. *Significant luciferase activity as compared to vehicle treated cells ($p < 0.05$).

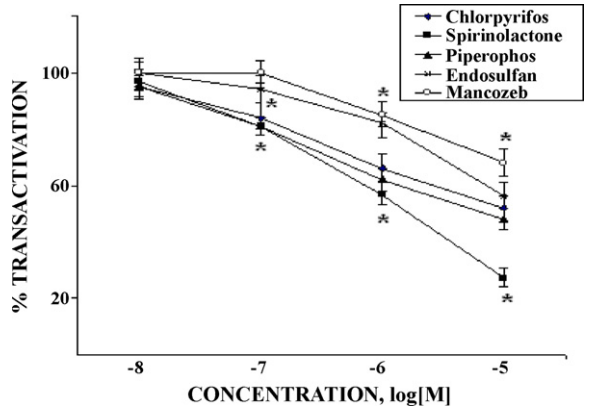

Fig. 2. Determination of anti-androgenic activities of some endocrine disrupting chemicals. Luciferase activities were expressed as percentage of that obtained with 0.4 nM of testosterone alone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates. *Significant inhibition of luciferase activity as compared to vehicle treated cells ($p < 0.05$).

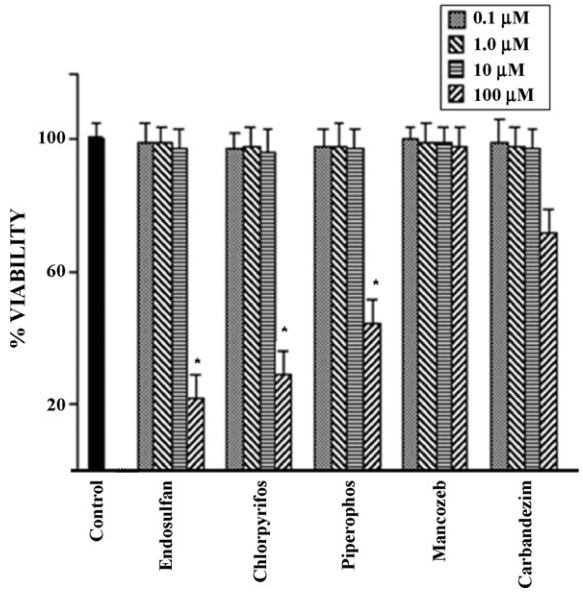

Fig. 3. Viability of isolated Leydig cells treated with varying concentrations of test chemicals. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates. *Significant decrease in viability as compared to control cells ($p < 0.05$).

cells were treated with the chemicals at a concentration of 10 μM. However, endosulfan, chlorpyrifos and piperophos showed significant cytotoxicity at a concentration of 100 μM (Fig. 3) ($p < 0.05$). None of the other two chemicals showed any significant ($p < 0.05$) effect on the viability of cells even at a concentration of 100 μM although carbandezim showed a marginal reduction in cell viability which was statistically non-significant.

In the next set of experiments these chemicals were tested for their effects on the LH induced testosterone production in isolated rat Leydig cells. As shown in Fig. 4, both chlorpyrifos and piperophos showed a significant reduction in the hCG induced testosterone production at a concentration of 10 μM ($p < 0.05$). Mean testosterone produced when the cells are treated with hCG alone was 148 ng/10^6 cells, while the cells treated with 10 μM chlorpyri-

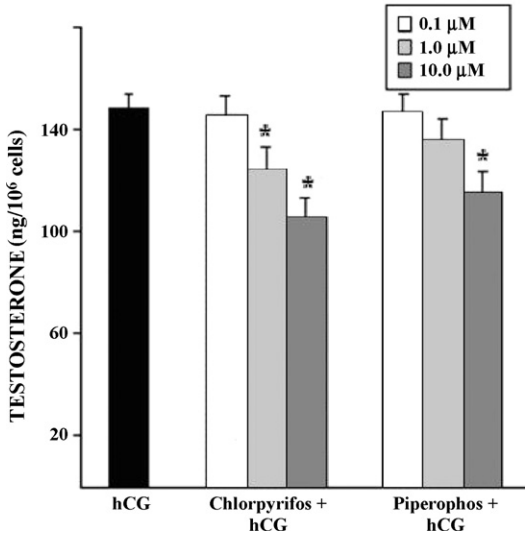

Fig. 4. Effect of various concentrations of test chemicals on testosterone production by Leydig cells in the presence of hCG. The values represent the mean ± S.D. of three similar experiments performed in triplicates. *Significantly different as compared to only hCG treated control cells ($p < 0.05$).

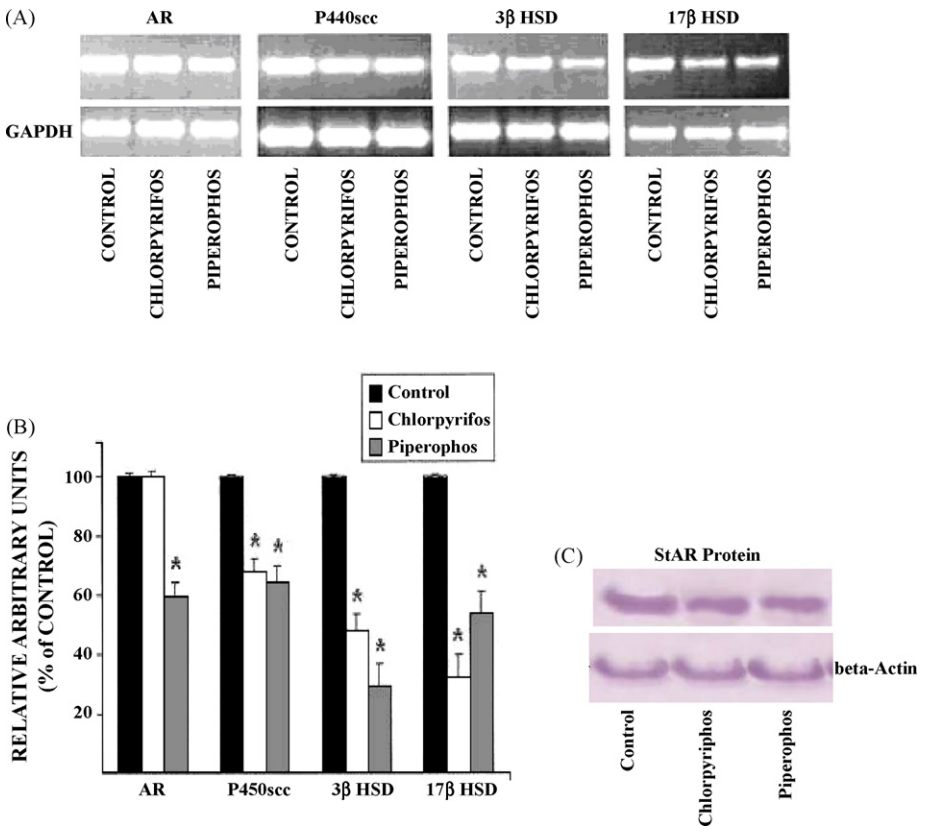

Fig. 5. Effect of piperophos and chlorpyrifos on the expression of Leydig cell specific genes. (A) Representative RT-PCR analysis of androgen receptor, cytochrome P450scc, 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase genes. (B) The relative intensity of the RT-PCR products for three separate experiments performed in duplicates. (C) Representative immunoblot showing the levels of StAR protein. *Significantly different as compared to vehicle treated control cells ($p < 0.05$).

fos and piperophos in the presence of 100 ng/ml hCG showed a decrease in testosterone production to 100 and 115 ng/10^6 cells, respectively. This accounts to a decrease of 33 and 23% by chlorpyrifos and piperophos, respectively. However, the other pesticides, endosulfan, mancozeb and carbandezim, did not show any testosterone inhibitory activity in this assay (data not shown).

To further investigate the effects of chlorpyrifos and piperophos on the steroidogenesis machinery within the Leydig cells, their effects on various steroidogenic gene expression patterns were analyzed. RT-PCR analysis of some of the crucial steroidogenic enzymes, P450scc, 3β-HSD and 17β-HSD showed that both chlorpyrifos and piperophos resulted in a significant down regulation in the expression of all the three enzymes (Fig. 5A and B). When tested for the effect of these chemicals on AR expression, although chlorpyrifos did not show any effect but piperophos significantly down regulated its expression (Fig. 5A and B). Steroidogenic acute regulatory (StAR) protein, a crucial factor for steroidogenesis, was then evaluated in response to these chemicals. As shown in Fig. 5C, the immunoblot analysis with StAR antibody suggests that both piperophos and chlorpyriphos demonstrated remarkable down regulation in the expression of this crucial steroidogenic protein.

3.4. Effects of chemicals on intracellular cAMP production mediated by hLH receptor

When the CHO-K1 cells, transfected with hLH receptor and CRE-Luc constructs, were treated with increasing concentrations of chlorpyrifos and piperophos, chlorpyrifos showed a significant reduction of hCG induced cAMP production at 1 μM concentration as estimated by the reduction of luciferase transactivation (Fig. 6) ($p < 0.05$). Further, at a concentration of 10 μM it showed approximately 30–32% decrease in luciferase induction. However, to our surprise piperophos did not show any significant change when compared with the vehicle treated cells. This further proved that chlorpyrifos not only interacts with AR as antagonists but it also disrupts LH/hCG induced cAMP production.

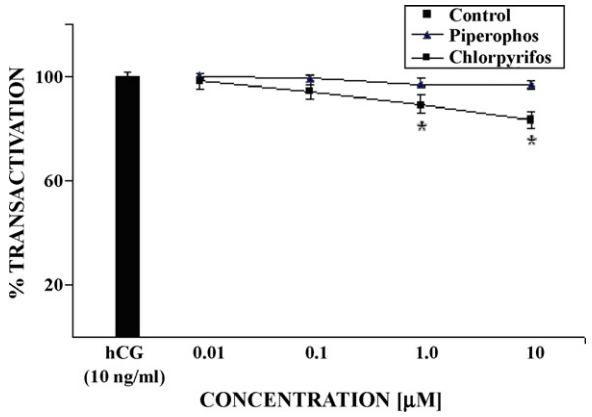

Fig. 6. Effect of chlorpyriphos and piperophos on intracellular cAMP production. The cells treated with only hCG was taken as control whose response was considered as 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates. *Significantly different as compared to only hCG treated cells ($p < 0.05$).

4. Discussion

Many endocrine disrupting compounds identified so far are persistent organochlorine pesticides (e.g. DDT, methoxychlor and dieldrin). However, the endocrine activity of numerous commonly used compounds like pharmaceutics, products in industrial wastes,

pesticides, water treatment plant effluents has not yet been studied in detail so far [2,5,19,25,26]. Out of various EDCs that have been identified till date, majority of them focused on the identification of (anti)estrogenic chemicals from these sources as a result of which the interaction of these chemicals with many other steroid hormones like androgen, progesterone, glucocorticoids and thyroid were understudied. Hence some user friendly assay systems are needed for the screening of potentially active endocrine disruptors affecting the latter set of hormones using various in vitro and in vivo model systems. With an aim to screen and characterize some (anti)androgenic endocrine disrupting chemicals, in the present study initially a cell-based assay was developed. The basic principle of a robust AR-mediated assay is the utilization of an AR transfected cell line that does not express endogenous 5α-reductase, 17β-HSD and 3β-HSD enzymes that are responsible for metabolizing test steroids or environmental EDCs [18]. Transactivation assays with AR and androgen-responsive reporter genes have been performed using various mammalian cell lines like PC-3, COS, CHO, CV-1, MCF-7 and HepG2, HEK293, MDA-MB-453 cells [2,4,6,7,9,18,27–29]. Of these various transactivation assays many of them utilized the recombinant cell lines stably or transiently expressing AR and androgen-responsive reporter genes both of which are suitable for screening androgenic and anti-androgenic compounds albeit the former one with greater efficiency. In the present study we developed a stable cell line stably over-expressing the hAR and MMTV-luc reporter constructs using NIH3T3 cells. This system is well suited for screening various (anti)androgenic chemicals due to its high sensitivity and simplicity. The calculated EC_{50} value of testosterone in this assay was 0.4 nM which is in the range as reported by earlier authors [27,30]. Using this assay initially we tested some well established androgen agonists and antagonists. Danazol, DHT and nandrolone showed androgen agonism with EC_{50} values of 0.75, 0.19 and 0.3 nM, respectively which are in accordance to the earlier reports while some chemicals like spirinolactone, piperophos, chlorpyrifos, endosulfan and mancozeb inhibited the testosterone induced luciferase activity. Some of these chemicals like spirinolactone, chlorpyrifos and endosulfan had been shown to be anti-androgenic using yeast based reporter gene assay [5,31,32] and mammalian cell based bioassay [2,33].

Our study aimed firstly to screen different classes of pesticides and pharmaceuticals for their endocrine disrupting activities using a transactivation assay and then analyze their effects on steriodogenesis in rat Leydig cell model. Among the range of chemicals screened, the pesticides showed anti-androgenic activities with piperophos showing a strong antagonistic activity with an IC_{50} value of 1.4 μM. Endosulfan, chlorpyrifos and mancozeb also exhibited significant anti-androgenic activities ($p < 0.05$) with IC_{50} values of 4.9, 9.3 and 10.6 μM, respectively. Some of these chemicals were shown to be androgen antagonists earlier also employing other cell lines or assay systems [2,32,34]. The variations in the IC_{50} values between different assays could be attributed to the presence of various co-activators in cell lines. It is conceivable that variations in the expression pattern and levels of co-activators of the AR may contribute to the differences as observed in actions of various anti-androgenic compounds in different cell lines [2,4,35].

Many pesticides and fungicides, apart from their anti-androgenic nature had been shown to affect the steriodogenesis pathway leading to the decrease in the testosterone production both in vivo (rat and fish models) and in vitro (Leydig cell cultures). Dieldrin [15], octylphenol [36], atrazine [37], vinclozolin [38], ketoconazole and related imidazole anti-fungal chemicals [39,40] were some of the pesticides/fungicides which were shown to reduce the testosterone production in Leydig cells. Following a similar approach, we studied the interference in steroidogenesis by some common pesticides, endosulfan, chlorpyrifos, piperophos, mancozeb and carbandezim in the primary cultures of Rat Leydig cells. In our study, both chlorpyrifos and piperophos showed a decrease in the testosterone production in Leydig cell cultures in the presence of hCG. This was in accordance with the earlier report where chlorpyrifos was reported to show anti-androgen activity by Hershberger assay in rats [41]. However, none of the other three chemicals showed any remarkable effect on testosterone production by isolated Leydig cells although they inhibited the testosterone induced transactivation. This aspect needs further in detailed study to draw a conclusive evidence for their modes of actions considering the importance of various pathways by which these EDCs might act. Recent reports demonstrate that xenobiotics-dependent direct up/downregulation of steroidogenesis could be attributed to several factors: (i) their action through arylhydrocarbon receptor (AhR) [42]; (ii) direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis (like steroidogenic acute regulatory protein) [43,44] and (iii) increased stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes and steroid receptors [18,45]. In this regard, the decrease in the testosterone synthesis by chlorpyrifos and piperophos in our study could be attributed to the reduction in the expression of the key steriodogenic enzymes – P450scc, 3β and 17β-HSD and even in the alteration of steroid metabolizing enzymes as has been reported earlier [46,47]. Since many of the steroid metabolizing enzymes are also expressed constitutively in accessory reproductive organs like prostate, it raises the possibility that the exposure of these compounds may interfere with steroid hormone metabolism, ultimately disturbing the steroid balance and availability [48]. StAR gene expression is an important rate limiting step in steriodogenesis. This protein helps in the transport of cholesterol from cytoplasm into mitochondria during steroid biosynthesis [49,50]. When chlorpyrifos and piperophos treated cells were investigated for the StAR protein expression, both chlorpyrifos and piperophos showed a significant decrease in the StAR protein.

LH hormone acts via G-protein coupled receptor leading to the increase in cAMP. This increase in cAMP induces the cholesterol transport into the mitochondria suggesting an increase in StAR gene expression in a cAMP responsive manner [51–53]. In our study, chlorpyrifos showed a 30–32% decrease in the intracellular cAMP level in hCG stimulated CHO-K1 cells transiently transfected with hLH receptor and CRE-luc reporter construct. However, piperophos did not show any significant down regulation of cAMP level in this assay although it showed a potent anti-androgen activity in transactivation assay. In summary, probably the reduction in cAMP resulted in the decrease in StAR and P450scc expressions at least in chlorpyrifos treated Leydig cells finally leading to the decrease in testosterone biosynthesis. Although the cascade leading to the decreased steroid biosynthesis by these chemicals are well understood, but their interference with the cAMP decrease and down stream cross-talks needs to be further investigated especially for piperophos. This is of prime importance since these alterations in androgen metabolism may pose serious threat to physiological system especially when they have various modes of actions.

In conclusion, (anti)androgenic EDCs constitute an important problem that has to be addressed as they pose a serious threat to alter male reproductive abilities as well as may result in serious disease like cancers. The assays using NIH3T3-AR-Luc cell lines are sensitive with minimum cross-reactivity with other steroids and generate reproducible results. Some of the EDCs like chlorpyrifos and piperophos, as tested here, showed an inhibitory effect on testosterone biosynthesis in Leydig cells by altering the expression of some of the crucial steroidogenic enzymes. To the best of our knowledge, this is the first ever report to show the anti-androgenic actions of two common organophosphate insecticides, chlorpyrifos and piperophos, in isolated Leydig cells. However, further in vivo and in vitro studies are needed for better understanding of their

modes of endocrine disruption and levels of reproductive toxicity. All these data put together will help to designate these chemicals as endocrine disruptors.

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References

[1] E.T. Keller, W.B. Ershler, C. Chang, The androgen receptor: a mediator of diverse responses, Front. Biosci. 1 (1996) 59–71.
[2] P. Roy, H. Salminen, P. Koskimies, J. Simola, A. Smeds, P. Saukko, I.T. Huhtaniemi, Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay, J. Steroid Biochem. Mol. Biol. 88 (2004) 157–166.
[3] W.R. Kelce, E.M. Wilson, Environmental anti-androgens: developmental effects, molecular mechanisms and clinical implications, J. Mol. Med. 75 (2001) 198–207.
[4] B.M.G. Blankvoort, E.M. de Groene, A.P. van Meeteren-Kreikamp, R.F. Witkamp, R.J.T. Rodenburg, J.M.M.J.G. Aarts, Development of an androgen reporter gene assay (AR-LUX) utilizing a human cell line with an endogenously regulated androgen receptor, Anal. Biochem. 298 (2001) 93–102.
[5] S. Chatterjee, C.B. Majumder, P. Roy, Development of a yeast-based assay to determine the (anti)androgenic contaminants from pulp and paper mill effluents in India, Environ. Toxicol. Pharmacol. 24 (2) (2007) 114–121.
[6] T.J. Schrader, G.M. Cooke, Examination of selected food additives and organochlorine food contaminants for androgenic activity in vitro, Toxicol. Sci. 53 (2000) 278–288.
[7] V.S. Wilson, K. Bobseine, C.R. Lambright, L.E. Gray Jr., A novel cell line, MDA-kb2, that stably express an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists, Toxicol. Sci. 66 (2002) 69–81.
[8] W. Korner, A.M. Vinggaard, B. Terouanne, R. Ma, C. Wieloch, M. Schlumpf, C. Sultan, A.M. Soto, Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals, Environ. Health Perspect. 112 (6) (2004) 695–702.
[9] P. Roy, S. Franks, M. Read, I.T. Huhtaniemi, Determination of androgen bioactivity in human serum samples using a recombinant cell based in vitro bioassay, J. Steroid Biochem. Mol. Biol. 101 (2006) 68–77.
[10] J. Chen, M.R. Sowers, F.M. Moran, D.S. McConnell, N.A. Gee, G.A. Greendale, C. Whitehead, S.E. Kasim-Karakas, B.L. Lasley, Circulating bioactive androgens in midlife women, J. Clin. Endocrinol. Metab. 91 (11) (2006) 4387–4394.
[11] R.W. Moore, C.R. Jefcoate, R.E. Peterson, 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits steroidogenesis in the rat testis by inhibiting the mobilization of cholesterol to cytochrome P450scc, Toxicol. Appl. Pharmacol. 109 (1991) 85–97.
[12] G.T. Ankley, K.M. Jensen, E.J. Durhan, E.A. Makynen, B.C. Butterworth, M.D. Kahl, D.L. Villeneuve, A. Linnum, L.E. Gray, M. Cardon, V.S. Wilson, Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*), Toxicol. Sci. 86 (2) (2005) 300–308.
[13] C.R. Blystone, C.S. Lambright, J. Furr, V.S. Wilson, L.E. Gray, Iprodione delays male rat pubertal development, reduces serum testosterone levels, and decreases ex vivo testicular testosterone production, Toxicol. Lett. 174 (2007) 74–81.
[14] C.R. Blystone, C.S. Lambright, K.L. Howdeshell, J. Furr, R.M. Sternberg, B.C. Butterworth, E.J. Durhan, E.A. Makynen, G.T. Ankley, V.S. Wilson, Sensitivity of fetal rat testicular steroidogenesis to maternal prochloraz exposure and the underlying mechanism of inhibition, Toxicol. Sci. 97 (2) (2007) 512–519.
[15] P.A. Fowler, D.R. Abramovich, N.E. Haites, P. Cash, N.P. Groome, A. Al-Qahtani, T.J. Murray, R.G. Lea, Human fetal testis Leydig cell disruption by exposure to the pesticide dieldrin at low concentrations, Hum. Reprod. 22 (11) (2007) 2919–2927.
[16] K.L. Howdeshell, V.S. Wilson, A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague Dawley rat in a cumulative, dose additive manner, Toxicol. Sci. 105 (1) (2008) 153–165.
[17] E.J. Perkins, N.G. Reyero, D.L. Villeneuve, D. Martinovic, S.M. Brasfield, L.S. Blake, J.D. Brodin, N.D. Denslow, G.T. Ankley, Perturbation of gene expression and steroidogenesis with in vitro exposure of fathead minnow ovaries to ketoconazole, Mar Environ Res. 66 (1) (2008) 113–115.
[18] J. Chen, K.C. Ahn, N.A. Gee, M.I. Ahmed, A.J. Duleba, L. Zhao, S.J. Gee, B.D. Hammock, B.L. Lasley, Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 149 (3) (2008) 1173–1179.
[19] V. Kumar, C. Balomajumder, P. Roy, Disruption of LH-induced testosterone biosynthesis in testicular Leydig cells by triclosan: probable mechanism of action, Toxicology 250 (2008) 124–131.
[20] X.C. Jia, E. Perlas, J.G. Su, F. Moran, B.L. Lasley, T. Ny, A.J. Hsueh, Luminescence luteinizing hormone/choriogonadotropin (LH/CG) bioassay: measurement of serum bioactive LH/CG during early pregnancy in human and macaque, Biol. Reprod. 49 (1993) 1310–1316.
[21] G. Viswanath, S. Chatterjee, P. Roy, Assessment of luteinizing hormone receptor function in an endometrial cancer cell line, Ishikawa cells in response to human chorionic gonadotrophin (hCG), Mol. Cell. Endocrinol. 272 (2007) 14–21.
[22] C. Parthasarathy, K. Balasubramanian, Assessment of in vitro effects of metyrapone on Leydig cell steroidogenesis, Steroids 73 (3) (2008) 328–338.
[23] K. Shiraishi, M. Ascoli, Lutropin/choriogonadotropin stimulate the proliferation of primary cultures of rat Leydig cells through a pathway that involves activation of the extracellularly regulated kinase 1/2 cascade, Endocrinology 148 (7) (2007) 3214–3225.
[24] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1–2) (1983) 55–63.
[25] V. Kumar, A. Chakraborty, G. Viswanath, P. Roy, Androgenic endocrine disruptors in wastewater treatment plant effluents in India: their influence on reproductive processes and systemic toxicity in male rats, Toxicol. Appl. Pharmacol. 226 (2008) 60–73.
[26] S. Chatterjee, V. Kumar, C.B. Majumder, P. Roy, Screening of some anti-progestin endocrine disruptors using a recombinant yeast based in vitro bioassay, Toxicol. in Vitro 22 (2008) 788–798.
[27] B. Terouanne, B. Tahiri, V. Georget, C. Belon, N. Poujol, C. Avances, F. Orio Jr., P. Balaguer, C. Sultan, A stable prostatic bioluminescent cell line to investigate androgen and antiandrogen effects, Mol. Cell. Endocrinol. 160 (2000) 39–49.
[28] L.C. Xu, H. Sun, J.F. Chen, Q. Bian, J. Qian, L. Song, X.R. Wang, Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro, Toxicology 216 (2005) 196–203.
[29] H. Sun, X.L. Xu, L.C. Xu, L. Song, X. Hong, J.F. Chen, L.B. Cui, X.R. Wong, Antiandrogenic activity of pyrethroid pesticides and their metabolite in reporter gene assay, Chemosphere 66 (2007) 474–479.
[30] J.A. Kemppainen, E. Langley, C.I. Wong, K. Bobseine, W.R. Kelce, E.M. Wilson, Distinguishing androgen receptor agonists and antagonists: direct mechanism of activation by medroxyprogesterone acetate and dihydrotestosterone, Mol. Endocrinol. 13 (1999) 440–454.
[31] H.L. Lee, Y.S. Lee, H.B. Kwon, K. Lee, Novel yeast bioassay system for detection of androgenic and antiandrogenic compounds, Toxicol. in Vitro 17 (2) (2003) 237–244.
[32] S.H. Jeong, B.R. Kim, H.O. Ku, J.H. Choi, Studies on the assessment of endocrine disruption effects of animal feed additives and environmental contaminants in animal feeds, Annual Report on NVBQS 43-127.
[33] G.D. Charles, H.L. Kan, M.R. Schisler, B.B. Gollapudi, M.S. Marty, A comparison of in vitro and in vivo EDSTAC test battery results for detecting antiandrogenic activity, Toxicol. Appl. Pharmacol. 202 (2005) 108–120.
[34] H. Kojima, E. Katsura, S. Takeuchi, K. Niiyama, K. Kobayashi, Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells, Environ. Health Perspect. 112 (5) (2004) 524–531.
[35] S. Yeh, H.C. Chang, H. Miyamoto, H. Takatera, M. Rahman, H.Y. Kang, T.H. Thin, H.K. Lin, C. Chang, Differential induction of the androgen receptor transcriptional activity by selective androgen receptor coactivators, Keio J. Med. 48 (1999) 87–92.
[36] E.P. Murono, E.C. Derk, J.H. deLeon, Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats, Reprod. Toxicol. 13 (1999) 451–462.
[37] A.S. Friedmann, Atrazine inhibition of testosterone production in rat males following peripubertal exposure, Reprod. Toxicol. 16 (2002) 275–279.
[38] E.P. Murono, R.C. Derk, The effects of the reported active metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1 trichloroethane, on testosterone formation by cultured Leydig cells from young adult rats, Reprod. Toxicol. 19 (1) (2004) 135–146.
[39] T. Schurmeyer, E. Nieschlag, Effect of ketoconazole and other imidazole fungicides on testosterone biosynthesis, Acta Endocrinol. 105 (1984) 275–280.
[40] P.B. Kan, M.A. Hirst, D. Feldman, Inhibition of steroidogenic cytochrome P-450 enzymes in rat testis by ketoconazole and related imidazole anti-fungal, J. Steroid Biochem. 23 (1985) 1023–1029.
[41] H.G. Kang, S.H. Jeong, J.H. Cho, D.G. Kim, J.M. Park, M.H. Cho, Chlorpyrifos-methyl shows anti-androgenic activity without estrogenic activity in rats, Toxicology 199 (2004) 219–230.
[42] D. Indarto, M. Izawa, Steroid hormones and endocrine disruptors: recent advances in receptor-mediated actions, Yonago Acta Med. 44 (2001) 1–6.
[43] H.G. Kang, S.H. Jeong, J.H. Cho, D.G. Kim, J.M. Park, M.H. Cho, Evaluation of estrogenic and androgenic activity of butylated hydroxyanisole in immature female and castrated rats, Toxicology 213 (2005) 147–156.
[44] S. Rice, H.D. Mason, S.A. Whitehead, Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa–luteal cells, J. Steroid Biochem. Mol. Biol. 101 (2006) 216–225.
[45] M.Y. Chan, H. Huang, L.L. Leung, 2,3,7,8-Tetrachlorodibenzo-para-dioxin increases aromatase (CYP19) mRNA stability in MCF-7 cells, Mol. Cell. Endocrinol. 317 (1–2) (2010) 8–13.

[46] K.A. Usmani, T.M. Cho, R.L. Rose, E. Hodgson, Inhibition of the human liver microsomal and human cytochrome P450 1°2 and 3°4 metabolism of estradiol by development-related and other chemicals, Drug Metab. Disposit. 34 (2006) 1606–1614.
[47] K.A. Usmani, R.L. Rose, E. Hodgson, Inhibition and activation of the human liver microsomal and human cytochrome P450 3°4 metabolism of testosterone by development-related chemicals, Drug Metab. Disposit. 31 (2003) 384–391.
[48] G.S. Prins, Endocrine disruptors and prostate cancer risk, Endocr.-Relat. Cancer 15 (2008) 649–656.
[49] D.M. Stocco, The role of the StAR protein in steroidogenesis: challenges for the future, J. Endocrinol. 164 (3) (2000) 247–253.
[50] D.M. Stocco, StAR protein and the regulation of steroid hormone biosynthesis, Annu. Rev. Physiol. 63 (2001) 193–213.
[51] B.J. Clark, S.C. Soo, K.M. Caron, Y. Ikeda, K.L. Parker, D.M. Stocco, Hormonal and developmental regulation of the steroidogenic acute regulatory (StAR) protein, Mol. Endocrinol. 9 (10) (1995) 1346–1355.
[52] T. Sugawara, D. Lin, J.A. Halt, K.O. Martin, N.B. Javitt, W.L. Miller, J.F. Strauss, Structure of the human steroidogenic acute regulatory protein (StAR) gene:StAR stimulates mitochondrial cholesterol 27-hydroxylase activity, Biochemistry 34 (1995) 12506–12512.
[53] K.M. Caron, Y. Ikeda, S.C. Soo, D.M. Stocco, K.L. Parker, B.J. Clark, Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory (StAR) protein, Mol. Endocrinol. 11 (1997) 136–147.