# Effect of DDT on Testosterone Production by Modulator Aromatase (CYP 19) in R2C

Kyung Jin Lee, Jong Bin Lee and Hye Gwang Jeong[1]*

*Department of Biological Sciences, College of Natural Sciences, Chonnam National University, Gwangju 500-757, Korea, [1]Department of Pharmacy, College of Pharmacy, Chosun University, Kwangju, 501-759, Korea*

**Abstract - Various pesticides known or suspected to interfere with steroid hormone function were screened for effects in leydig cells on catalytic activity and mRNA expression of aromatase. Dichlorodiphenyltrichloroethane (DDT) is a widespread environmental pollutant. In this study, we investigated the effect of DDT on testosterone production through aromatase activity and its molecular mechanism in testicular leydig cell, R2C by using radioimmunoassay (RIA). As the results, the potent leydig cell activator LH increased testosterone production compared to the control. DDT exposure significantly decreased testosterone production in R2C cell. In addition, DDT was found to increase aromatase gene expression and activity in R2C cell in a dose dependent manner. In order to assess whether the suppressive effects of DDT on LH-inducible testosterone (T) production might be influenced by the ER, ICI 182.780 was used, and it was found that these inhibitory effects of DDT were antagonized by ICI 182.780, implying that the estrogen receptor (ER) mediates the suppressive effects of DDT. Furthermore, the inducible effects of DDT on aromatase gene expression might be influenced by the ER, ICI 182.780 was used, and it was found that these enhancing effects of DDT were antagonized by ICI 182.780, implying that the ER mediates the inducible effects of DDT. Our results indicated that DDT inhibition of luteinizing hormone (LH)-inducible T production in R2C cell is mediated through aromatase. However, the precise mechanisms by which DDT enhance in R2C cell remains unknown. The current study suggests the possibility that DDT might act as a modulator aromatase gene transcription.**

**Key words : DDT, testosterone, aromatase, ER, R2C**

## INTRODUCTION

There is increasing evidence that certain environmental contaminants have the potential to disrupt endocrine processes, which may result in reproductive problems, certain cancers and other toxicities related to (sexual) differentiation, growth, and development. A metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT) is a widespread environmental pollutant. Earlier studies have shown that exposure to DDT at early developmental stage results in altered sexual differentiation in male rats. Affected animals display a number of signs of feminization, including reduced anogenital distance and increased incidence of nipple retention (Kelce *et al.* 1995; You *et al.* 1998). Since 1, 1-bis (4-chlorophenyl)-2, 2-dichloroethene (DDE) is able to bind to the androgen receptor and block the actions of testosterone, its effects

* Corresponding author: Hye Gwang Jeong, Tel. +82-62-230-6639, Fax. +82-62-230-6639, E-mail. hgjeong@chosun.ac.kr

on reproductive development have been attributed primarily to an androgen receptor antagonism (Kelce *et al.* 1995; Kelce *et al.* 1997).

Several classes of relatively persistent pesticides, such as organotin compounds, DDT and several metabolites, and a number of imidazole-like fungicides are suspected or have been shown to interfere with steroidogenesis. Particular attention has been given to the enzyme aromatase (CYP19) which catalyzes the final, rate-limiting step in the conversion of androgens to estrogens. It has been postulated that organotin compounds may cause endocrine-disruptive effects such as "imposex" in molluscs by inhibiting aromatase activity (Fent 1996). Various imidazole-like fungicides are known to inhibit aromatase activity in human placental (Vinggaard *et al.* 2000) and rainbow trout ovarian microsomes (De Mones *et al.* 1993). Recently, DDT, which has antiandrogenic properties (Kelce and Wilson 1997), has been reported to increase aromatase protein in rat (You *et al.*, 2001). Aromatase catalyzes the conversion of C19 steroids to estrogens, a reaction that involves the removal of the C19 carbon and aromatization of the A ring of the steroid. The expression of aromatase is controlled by regulatory pathways involving gonadotropins, steroid hormones, and growth factors (Roselli and Resko 1997).

A recent study reported that DDT exposure significantly increased circulating levels of 17β-estradiol ($E_2$) in male rats (O'Connor *et al.* 1999). This finding suggests a possibility that the feminization seen in DDT-exposed male rats may also involve an overproduction of estrogen. In the present study, we investigated the effect of DDT on testoesterone production through aromatase and investigated its molecular mechanism in testicular leydig cell, R2C. The involvement of estrogen receptor (ER) in this process was also investigated using the ER antagonist, ICI 182.780.

## MATERIALS AND METHODS

### 1. Materials

DDT was obtained from the Sigma Chemical Co. (St. Louis, Korea). This compound was dissolved in ethanol, and the final concentration of ethanol in the cell growth medium was 0.1% (v/v). Dulbeccos's Modified Eagle Medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, Korea). [β-$^3$H]Androstenedione was purchased from Amersham Pharmacia Biotech (Boston, MA).

### 2. Cell treatment

R2C cells were obtained from the American Type Culture Collection. Cells in 24-well culture plates containing 1 ml medium per well were exposed to various concentrations of DTT dissolved in dimethyl sulfoxide (DMSO). All treatments were tested in triplicate, For the DMSO at 0.1% had no effect on CYP19 expression or catalytic activity relative to unexposed cells.

### 3. Animal treatment

Immature male Sprague-Dawley rats (100~120 g) were purchased from KFDA (Seoul, Korea). The rats were unbiasedly divided into the vehicle control and the treatment groups ($n$ = 3 per group) and dosed with DDT (0~100 mg $kg^{-1}$) respectively by daily gavage for 3 days. The rats were killed by decapitation 24 h after the last dose. Testis samples were stored at −20°C until hormone and aromatase assay.

### 4. Aromatase assay

The aromatase activity was determined by measuring the [$^3$H]$H_2O$ release upon the conversion of [1β-$^3$H] androstenedione (A) to estrone ($E_1$) (Lephart and Simpson 1991). Before the experiment, the cells were cultured in DMEM with 5% DCS (dextran-coated, charcoal-treated FBS) for 48 h. After the cells were treated with DDT, [1β-$^3$H] androstenedione was added, and then the cells were further incubated for 6 h. The medium (2.0 ml) was extracted with chloroform and then was centrifuged. The aqueous supernatant was mixed with 5% charcoal/ 0.5% dextran and then was incubated for 30 min. Thereafter, the mixture was centrifuged and the supernatant was added to 5 ml of scintillation fluid and assayed for radioactivity. The amount of radioactivity in [$^3$H]$H_2O$ thus measured was standardized based on the protein concentration which was determined using a micro BCA

kit (Pierce Chemical Co., Korea) and expressed as pmol $mg^{-1}$ protein 6 $hr^{-1}$.

### 5. Testosterone (T) and Estradiol ($E_2$) content assay

To ensure that the measured aromatase activity truly reflected the capability of estrogen production, the cells were treated with or without various concentrations of DDT for 24 hr, and then the medium was collected and the T and $E_2$ were assayed in duplicate by using a Coat-A-Count radioimmunoassay kit. The radioactivity of $^{125}I$ was quantified by a gamma-counter (Packard Tri-carb Scintillation Spectrometer, Model 4530).

### 6. RNA preparation and aromatase mRNA analysis by RT-PCR

R2C cells were incubated with DDT, or/and LH and ICI168,780 for 24 hr. Total cellular RNA was isolated by the acidic phenol extraction procedure. cDNA synthesis, semiquantitative RT-PCR for *aromatase* and GAPDH mRNA, and analysis of results were performed as described previously (Levallet *et al.* 1998a). cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number that fell within the exponential range of response for both aromatase (26 cycles) and GAPDH (17 cycles) was used.

### 7. Statistics

All experiments were repeated at least three times. Student's t-test was used to assess the statistical significance of differences. A confidence level of $< 0.05$ was considered significant.

## RESULTS AND DISCUSSION

Because DDT is known to inhibit luteinizing hormone (LH)-induced testosterone production in leydig cell and has been shown to possess estrogenic properties, we decided to investigate the effects of DDT on testosterone production and its effects on aromatase activity in R2C cell. The potent leydig cell activator LH increased T production compared to the control. DDT exposure decreased testoesterone production in R2C cell (Fig. 1). Furthermore, DDT alone affected testosterone reduction in a dose-dependent manner in R2C cell slightly (Fig. 1). The DDT-mediated suppression of testosterone production was not due to a DDT cytotoxic effect. Cell viability was identical for cultures treated with DDT (Table 1). In addition, DDT was found to increase aromatase activity in R2C cell (Fig. 3). A recent study reported that HPTE (2, 2-bis (p-hydroxyphenyl)-1, 1, 1-tricholoro-ethane, DDT metabolite) inhibited production of testosterone in leydig cells, as down-regulation of $P450C_{scc}$, the enzyme that catalyzes the first reaction in the testosterone biosynthesis pathway (Akingbemi *et al.*, 2000). However, the mechanism by which DDT causes these effects is not clear.

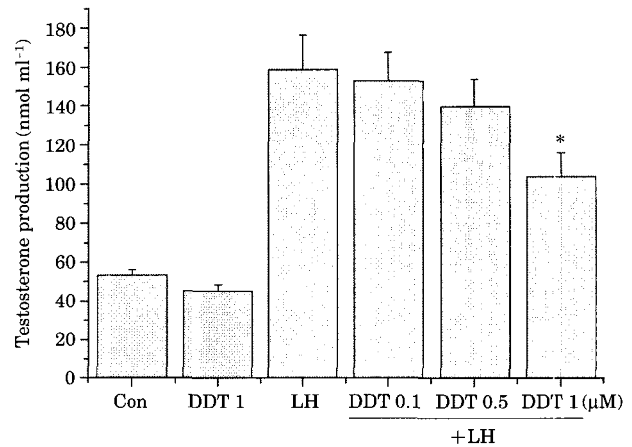


**Fig. 1.** Effects of DDT on testosterone production by R2C cells. After being harvested, R2C cells were coincubated with/without ICI 182,780 in media containing 10 ng $ml^{-1}$ LH. After 24 hr, T concentration was investigated by using RIA. Three experiments were conducted for this determination. *Statistically significant differences with respect to controls (Student test, $P < 0.05$).

**Table 1.** The cytotoxicities of DDT by MTT assay

| Treatment | OD570 nm | % of control |
|---|---|---|
| Control | 0.82±0.03 | 100±4 |
| DDT 0.1 (μM) | 0.86±0.05 | 105±6 |
| DDT 0.5 (μM) | 0.81±0.06 | 98±7 |
| DDT 1 (μM) | 0.78±0.06 | 95±7 |
| ICI 168,780 100 (nM) | 0.81±0.05 | 98±6 |
| LH 10 (nM) | 0.89±0.08 | 109±9 |

MTT activity was measured in the culture medium after 24 hr incubation with DDT Each value was expressed as mean percentage±S.E. of control cultures of three determinations.

Regarding these results, previous studies have report-

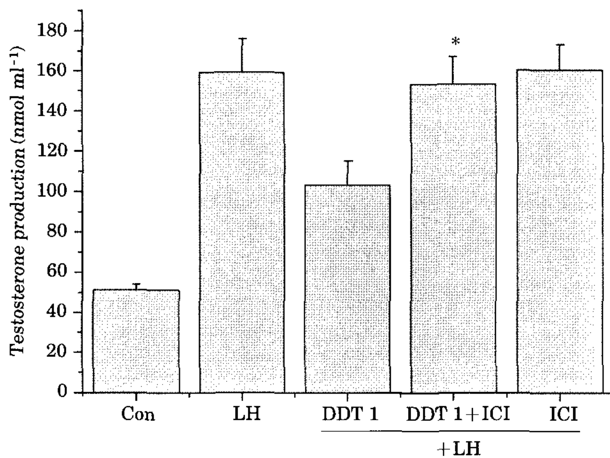


**Fig. 2.** Antagonism of DDT inhibition of testosterone production by antiestrogen. DDT (1 μM) inhibition of testosterone production was prevented when R2C cells were coincubated with ICI 182,780 (ICI) in media containing 10 ng $ml^{-1}$ LH. Three experiments were conducted for this determination. *Statistically significant differences with respect to DDT treatment group (Student test, $P < 0.05$).

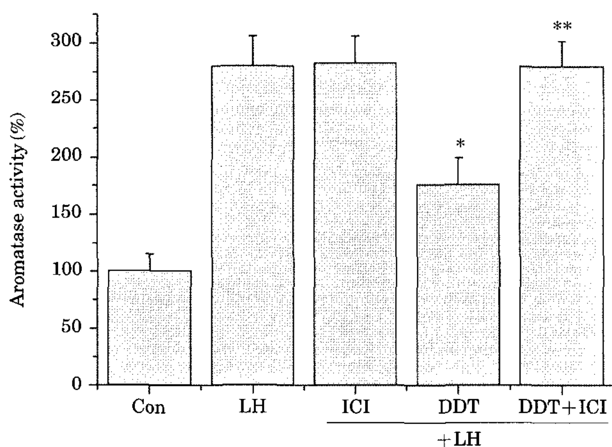


**Fig. 3.** Effects of DDT on aromatase activity in R2C cells. Cells were treated with DDT (1 μM) for 24 hr and then aromatase activities were measured in the spent media by RIA. Three experiments were conducted for this determination. *Statistically significant differences with respect to controls. **Statistically significant differences with respect to DDT treatment group (Student test, $P < 0.05$).

ed that the estrogenic activities of DDT, such as ER binding affinity (Beresford *et al.* 2000). In order to assess whether the suppressive effects of DDT on LH-inducible testosterone production might be influenced by the ER, ICI 182.780, a pure antiestrogen, was used, and it

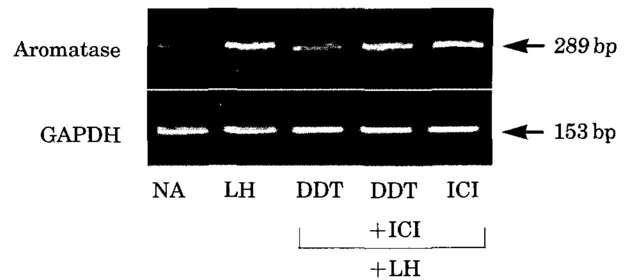


**Fig. 4.** Effects of DDT on aromatase mRNA expression. R2C cells were cultured for 24 h in the presence of media alone, with the indicated concentrations of DDT (1 μM), or with LH (10 ng $ml^{-1}$) and ICI168,780 (100 nM). The ratio of RT-PCR product of aromatase to β-actin was determined.

was found that these inhibitory effects of DDT were antagonized by ICI 182.780, implying that the ER mediates the suppressive effects of DDT (Fig. 2). Furthermore, the inducible effects of DDT on aromatase gene expression and activity might be influenced by the ER, ICI 182.780 was used, and it was found that these enhancing effects of DDT were antagonized by ICI 182.780, implying that the ER mediates the inducible effects of DDT (Figs. 3, 4). Therefore, we believe that decreased LH-inducible testosterone production by DDT is regulated through aromatase. Several previous studies have shown that DDT treatment leads to reduce LH-inducible testosterone production (You *et al.* 2001), and this is confirmed by the present study (Fig. 1).

According to the classical hypothesis, the cellular effects of estrogens are mediated by the intracellular ER, which serve as transcription factors. ER belongs to the superfamily of ligand-activated transcription factors, the nuclear receptors. $E_2$-ER complexes bind to the genomic estrogen response elements. The estrogen-occupied receptor interacts with additional transcription factors and components of the transcription initiation complex to modulate gene transcription.

Aromatase regions homologous to the consensus sequence of the estrogen response elements have been identified in the 5′-flanking regions of the aromatase genes. So, DDT may induce the transcription of the aromatase genes by interacting with these sequences (Fig. 4).

Our results indicated that DDT inhibition of LH-inducible testosterone production in R2C and testis is mediated through aromatase. However, the precise

mechanisms by which DDT enhance in leydig cell remains unknown. The current study suggests the possibility that DDT might act as an modulrator aromatase gene transcription.

## ACKNOWLEDGMENTS

This work was supported by Korea Food Drug Adminstration Grant (KFDA-03132-EDS-410-1).

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Manuscript Received: June 9, 2003
Revision Accepted: August 11, 2003
Responsible Editorial Member: Don Chan Choi
(Yongin Univ.)