

THE EFFECT OF DICHLORODIPHENYLDICHLOROETHYLENE (DDE) ON
STEROIDOGENESIS IN GRANULOSA CELLS

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Abstract

The insecticide dichlorodiphenyltrichloroethane (DDT) and its major metabolite p,p'-dichlorodiphenyldichloroethylene (DDE) are endocrine disrupters. The DDT metabolite p,p'-DDE has been found contaminating human tissues and follicular fluid around the world, due to its persistence and continued use. The focus of this research was to investigate the effects of DDE on progesterone synthesis in a stable porcine granulosa cell line, JC-410, and in primary cultures of porcine granulosa cells. Further objectives of this research were to validate the JC-410 cell line as an *in vitro* model for the study of endocrine disrupters on ovarian steroidogenesis, and to elucidate the mechanism of action of DDE.

Low concentrations of DDE, 0.1-100 ng/ml, did not affect basal progesterone synthesis in the JC-410 cells. However, 10 ng/ml DDE increased 8-Br-cAMP- and cholera toxin (CT)-stimulated progesterone synthesis 0.4-0.7-fold over the levels observed with 8-Br-cAMP or CT alone. This effect was confirmed in primary cultures of porcine granulosa cells. Neither basal nor CT-stimulated cAMP levels were changed by 0.1-100 ng/ml DDE. In the JC-410 cells, 1 and 10 ng/ml DDE increased CT-stimulated cytochrome P450-cholesterol side chain cleavage (P450_{scc}) mRNA levels 0.3- and 0.4-fold over the values observed with CT alone.

High concentrations of DDE, 3000 and 10000 ng/ml, reduced basal progesterone synthesis in the JC-410 cells 0.51- and 0.75-fold, respectively. As well, DDE, 300-10000 ng/ml, blocked the CT-induced stimulation of progesterone synthesis. High concentrations of DDE, 3000 and 10000 ng/ml, decreased basal cAMP generation 0.33- and 0.64-fold, respectively. The addition of DDE 300, 3000, and 10000 ng/ml

decreased CT-stimulated cAMP levels by 0.16, 0.32, and 0.48-fold respectively, as compared to CT alone. Basal expression of the P450scc gene was decreased 0.61- and 0.82-fold by 3000 and 10000 ng/ml DDE, respectively. Addition of DDE, 3000 and 10000 ng/ml, blocked the CT-induced stimulation of P450scc gene expression. Cellular protein levels were increased 0.35- and 0.42-fold, respectively, by 3000 and 10000 ng/ml DDE.

It was concluded that DDE, at environmentally relevant concentrations, modulates granulosa cell steroidogenesis in a dose dependent manner, through two different mechanisms of action. At low concentrations, DDE potentiates the conversion of cholesterol to pregnenolone induced by PKA activators, thus stimulating progesterone production. At high concentrations, DDE inhibits basal and stimulated cAMP generation and P450scc gene expression, thus decreasing progesterone production. Based on these observations, it is possible that DDE could disrupt gonadotropin control of ovarian steroidogenesis, thus representing a threat to human reproductive health and fertility.

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Dedication

*This is dedicated to my parents, Brian and Margaret Crellin,
for their support and wisdom.*

Table of Contents

Permission to Use.....	ii
Abstract	iii
Acknowledgements	v
Dedication	vi
Table of Contents	vii
List of Figures	ix
List of Abbreviations.....	x
1.0 Introduction	1
2.0 Hypothesis.....	3
3.0 Objectives.....	3
4.0 Literature Review	4
4.1 Environmental Endocrine Disrupters- Why Do We Care?.....	4
4.2 History of the Use and Banning of DDT	5
4.3 DDT Metabolites- Persistence and Bioaccumulation	7
4.4 Human Exposure to DDT and its Persistent Metabolites	8
4.5 Estrogenic Effects of DDE	10
4.6 Anti-androgenic Effects of DDE	12
4.7 DDE Receptor Binding.....	14
4.8 Female Reproductive Toxicology.....	15
4.9 Granulosa Cells.....	17
4.10 Gonadotropin Control of Steroidogenesis	18
4.11 Ovarian Steroidogenesis	19
4.12 Female Health Effects of DDE	21
5.0 The Experimental Model.....	23
5.1 JC-410 Cells.....	24
6.0 Materials and Methods	27
6.1 Reagents.....	27
6.1.1 Tissue Culture Reagents.....	27
6.1.2 Radioimmunoassay and Protein Assay Reagents	27
6.1.3 Reagents for cDNA preparation.....	28
6.1.4 Reagents for Northern Hybridization.....	28
6.2 Granulosa Cell Culture	29
6.2.1 Granulosa Cell Treatment	29
6.3 Quantification of Progesterone, cAMP, Cellular Protein and DNA.....	30
6.3.1 Progesterone Radioimmunoassay	30
6.3.2 cAMP Radioimmunoassay.....	30
6.3.3 Protein and DNA content Analysis	31
6.4 Northern Blot Analyses	31
6.4.1 Isolation, Electrophoresis and Transfer of RNA.....	31
6.4.2 cDNA Probe Labeling.....	32
6.4.3 Membrane Hybridization and Autoradiograph analysis	33
6.5 Statistical analysis.....	34
7.0 Results	35

7.1 Potentiating Effect of DDE on PKA Activator-Stimulated Progesterone Synthesis	35
7.2 Effect of 10 ng/ml DDE on CT-stimulated cAMP production	39
7.3 Northern Blot Analysis of the Potentiating Effect of DDE on P450scc and 3 β -HSD gene expression.....	39
7.4 Inhibitory Effect of DDE on Basal and PKA Activator-Stimulated Progesterone Synthesis	42
7.5 Inhibitory Effect of DDE on Basal and PKA Activator-Stimulated cAMP production	42
7.6 Stimulatory Effect of DDE on Protein Levels	47
7.7 Northern Blot Analysis of the Inhibitory Effect of DDE on P450scc gene expression	47
8.0 Discussion	50
9.0 Conclusions	63
10.0 Bibliography.....	64
11.0 Appendix	76

List of Figures

Figure 1: Gonadotropin Stimulation of Steroidogenesis.....	19
Figure 2: Steroidogenic Pathway in Ovary	20
Figure 3: Effect of DDE on 8-Br-cAMP-stimulated progesterone accumulation in JC-410 cells.....	36
Figure 4: Effect of DDE on CT-stimulated progesterone accumulation in JC-410 cells.	37
Figure 5: Effect of DDE and CT on progesterone accumulation in primary porcine granulosa cells.	38
Figure 6: Effect of DDE on CT-stimulated cAMP production in the JC-410 cells.	40
Figure 7: Effect of DDE on the expression of P450scc and 3 β -HSD genes in JC-410 cells.....	41
Figure 8: Effect of high concentration DDE on basal progesterone synthesis in the JC-410 cells.....	43
Figure 9: Effect of high concentration DDE on CT-stimulated progesterone synthesis in the JC-410 cells.	44
Figure 10: Effect of high concentration DDE on basal cAMP production in the JC-410 cells.....	45
Figure 11: Effect of high concentration DDE on CT-stimulated cAMP production in the JC-410 cells.	46
Figure 12: Effect of high concentration DDE on protein levels in the JC-410 cells.	48
Figure 13: Effect of high concentration DDE on the expression of P450scc gene in JC-410 cells.....	49

List of Abbreviations

3 β -HSD	3 β -hydroxysteroid dehydrogenase
8-Br-cAMP	8-bromoadenosine 3':5'-cyclic adenosine monophosphate
AR	Androgen Receptor
Androstenedione	4-androstene-3, 17-dione
AhR	Aryl hydrocarbon receptor
cAMP	3':5'-cyclic adenosine monophosphate
CT	Cholera Toxin
DDA	bis[<i>p</i> -chlorophenylacetic] acid
DDD	1,1-dichloro-2,2-bis[<i>p</i> -chlorophenyl] ethane
DDE	1,1-dichloro-2,2-bis[<i>p</i> - chlorophenyl]ethylene
DDT	1,1,1-trichloro-2,2-bis[<i>p</i> - chlorophenyl]ethane
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylenediamine-tetra acetate
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
FK	Forskolin
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-phosphate

	dehydrogenase
GnRH	Gonadotropin Releasing Hormone
IBMX	3-isobutyl-1-methyl-xanthine
Iodinated Progesterone	11- α -hydroxyprogesterone 11- α D-glucuronide- ¹²⁵ I-iodotiramine
LH	Luteinizing Hormone
Levonorgestrel (LNG)	13 β -ethyl-17 α -ethynyl-17 β -hydroxygon-4-en-3-one
M199	Medium 199
mRNA	Messenger ribonucleic acid
NBCS	New born calf serum
P450arom	Cytochrome P450 aromatase
P450scc	Cytochrome P450 cholesterol side chain cleavage
PKA	Protein kinase A enzyme
PPM	Parts per million
PPB	Parts per billion
PR	Progesterone Receptor
Pregnenolone	5-pregnen-3 β -ol-20-one
Progesterone	4-pregnene-3,20-dione
RIA	Radioimmunoassay
RNA	Ribonucleic acid
Sarkosyl	N-lauroylsarcosine

SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
StAR	Steroidogenic acute regulatory protein

1.0 Introduction

It would be reassuring to suppose that banning the use of a chemical would result in it disappearing from our ecosystem. Unfortunately, this is not always the case. The use of dichlorodiphenyltrichloroethane (DDT) was banned in North America and Western Europe in the 1970's, due to evidence of persistence in soils and aquatic sediments and the potential to bioconcentrate in birds and mammals. However, the use of DDT has continued in developing countries (Ayotte et al., 1995; Wikteliu and Edwards, 1997). The persistence and biomagnification of DDT and its metabolites ensures that residues are carried far from their point of application (Ayotte et al., 1995), thus human exposure may continue even in countries where DDT has been banned. As well, the peculiarities of oceanic currents and global weather patterns have ensured contamination of the previously pristine Arctic ecosystem (Ayotte et al., 1995). This is of particular concern given the dependence of native populations on the arctic aquatic food chain. Indeed, comparison between DDE concentrations in breast milk measured in Inuit women of northern Quebec and women in southern Quebec revealed an average of twice as much DDE in the Inuit population (Ayotte et al., 1995).

The insecticide DDT and its major and most stable metabolite, *p,p'*-dichlorodiphenyldichloroethylene (DDE), have been described as endocrine disrupters (Guillette Jr. et al., 1996; Danzo, 1997; Longnecker et al., 1997; Clark et al., 1998; Sonnenschein and Soto, 1998). Endocrine disrupters are compounds that are capable of causing changes to the endocrine system, resulting in developmental abnormalities.

These physiological changes, including abnormal gonad development and altered steroidogenesis, can have devastating reproductive consequences, as observed in wildlife populations (Guillette Jr. et al., 1994; Guillette Jr. et al., 1995; Guillette Jr. et al., 1996).

Dietary exposure to *p,p'*-DDE has resulted in contamination of human blood, adipose tissue and ovarian follicular fluid (Morgan and Roan, 1971; Jarrell et al., 1993). The presence of *p,p'*-DDE in follicular fluid is of great concern due to the intimate contact between the granulosa cells and the developing oocyte. Toxins can impair fertility by altering follicular growth and hormone biosynthesis or by changing granulosa cell-oocyte interactions (Hirshfield, 1997).

Definitive research investigating the molecular mechanisms by which endocrine disrupters cause reproductive dysfunction is vital to the development of appropriate screening protocols for potential endocrine disrupters (Campbell and Hutchinson, 1998). The objective of this study was to investigate the effects of DDE on mammalian ovarian steroidogenesis. Studies were conducted in JC-410, a stable steroidogenic porcine granulosa cell line (Chedrese et al., 1998; Rodway et al., 1999), and in primary cultures of porcine granulosa cells.

2.0 Hypothesis

DDE, a frequent ovarian contaminant, can affect granulosa cell steroidogenesis by altering steroidogenic gene expression.

3.0 Objectives

1. To validate a stable granulosa cell model for the study of the molecular effects of environmental endocrine disrupters;
2. To ascertain if DDE has an effect on ovarian steroidogenesis, and if so to describe the extent of this effect(s), as well as the relevant dose-response information;
3. To elucidate the molecular mechanism of action by which DDE affects granulosa cell steroidogenesis.

4.0 Literature Review

4.1 *Environmental Endocrine Disrupters- Why Do We Care?*

The European Union has defined an “endocrine disrupter” as “an exogenous agent that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function”(Gillesby and Zacharewski, 1998). The endocrine system has an important regulatory role in the body, and can influence factors controlling development, growth and homeostasis. It is readily apparent upon examination of this definition of an endocrine disrupter that it allows for a multitude of different effects and mechanisms of action. An endocrine disrupter could affect hormone synthesis, degradation, or transport, alter receptor binding and activity, or modulate gene transcription. The diversity of potential mechanisms of action for endocrine disrupters represents a considerable challenge for regulatory agencies attempting to assess the safety of chemicals intended for commercial use.

Environmental exposure to endocrine disrupters has resulted in effects in wildlife including impaired reproductive tract development, feminization or demasculinization of males, masculinization of females, and altered circulating levels of sex steroids, leading to an overall decrease in fertility (Guillette Jr. et al., 1995). It has been hypothesized that exposure to endocrine disrupting chemicals may be responsible for a number of changes observed in the human population. There has been an increase in the incidence of congenital malformations of the male reproductive tract, such as cryptorchidism and hypospadias. Worldwide attention was directed to reports describing decreased sperm

count of men in the past 50 years. Most disturbingly, an increased incidence of testicular, prostate and breast cancer has been observed (Carlsen et al., 1995).

Endocrine disrupters must be investigated because their destructive potential is enormous. They are very common in our environment, and we know very little about the extent of their effects, or the mechanisms of actions that produce these effects. The few definitive examples we have of endocrine disrupters provoked enough concern that research into endocrine disrupters has become a major funding priority for grant agencies around the world.

4.2 History of the Use and Banning of DDT

The insecticidal properties of DDT were discovered in time to be of great value in the Second World War. This pesticide was used to control malaria vectors, and to stop the spread of a typhus epidemic. Following the end of the war, production of DDT was increased to levels exceeding military requirements, and was made available for purchase by the American public (Whorton, 1975; Dunlap, 1981). Shortly thereafter, its use was rampant throughout the United States. It was used in massive spray campaigns to combat Dutch elm disease, and to attempt the eradication of the gypsy moth. Film clips from the time show school children at play being sprayed with clouds of DDT, demonstrating what was considered to be the obvious safety of it to humans.

The chemical DDT is unique in that it was made available to the public without undergoing any sort of long term, chronic toxicity testing(Whorton, 1975). The toxicity tests done by the military prior to the use of DDT on troops in the Second World war were primarily acute in nature, designed solely to determine what the damage to the troops would be at the required concentrations. The military use of DDT after only

these preliminary tests was a calculated risk, justifiable in terms of wartime necessity. Civilian use of it for mosquito control did not possess a similar urgency, and complete toxicity testing should have been finished prior to public availability. However, reports from troops exposed to DDT during the war indicated no adverse effects, and the official government position was that DDT was safe for human exposure.

Indeed, it was not human health that eventually raised concerns regarding the toxicity of DDT. Rather, it was the large numbers of birds found dead after spraying that started to attract the attention of scientists (Whorton, 1975; Dunlap, 1981). This did not happen quickly. It was accepted by most that some loss of wildlife was the necessary price to be paid for progress. However, within a few years it became evident that entire avian populations were being wiped out, including species such as the peregrine falcon and the bald eagle. Legal attempts to restrict the use of DDT were hampered by a lack of laboratory evidence or a postulated mechanism of action (Whorton, 1975; Dunlap, 1981).

It was not until 1960 that a study was released describing the ability of DDT to bioconcentrate as it moved up the food chain. This finding changed the way scientists considered pesticide safety (Dunlap, 1981). As well, it altered thinking regarding the safety of persistent pesticides. Entomologists and wildlife biologists had assumed that it was possible to find a safe level of 'discriminate' use that would cause no permanent damage to the ecosystem. Given this information on bioconcentration, it became possible that there was no safe level of use for DDT.

The debate over the safety of DDT was conducted in scientific journals and professional circles. Not until 1962, with the publication of Rachel Carson's book Silent Spring, did the public take note (Carson, 1962). Silent Spring ignited a political

controversy in which scientists were forced to take part. Still, more evidence was required before any change in government policy would be considered. It was not until 1972, when evidence had accumulated detailing the contamination of breast milk with DDT, and the potential alteration of sex steroid levels in humans that it was finally banned in the United States (Dunlap, 1981).

4.3 DDT Metabolites- Persistence and Bioaccumulation

The biotransformation and excretion of DDT (1,1,1-trichloro-2,2-bis[*p*-chlorophenyl] ethane) proceeds by one of two possible pathways in both animals and humans. The chemical DDT may undergo dehydrochlorination in a glutathione-dependent reaction, resulting in the unsaturated DDE (1,1-dichloro-2,2-bis[*p*-chlorophenyl] ethylene), or a chlorine atom may be substituted in place of a hydrogen molecule, forming the saturated DDD (1,1-dichloro-2,2-bis[*p*-chlorophenyl] ethane). If DDD is produced, it is then easily degraded to the readily excretable DDA (bis[*p*-chlorophenylacetic] acid) and is removed from the body. However, if DDE is the result of the initial metabolism of DDT, no further degradation takes place. The metabolite DDE is not broken down to DDA, and thus remains in the body (Morgan and Roan, 1971).

Commercial DDT consists of two different isomers, *o,p'*-DDT, and *p,p'*-DDT, as defined by the positioning of chlorine on the phenyl rings. The DDT metabolite DDE exists in two forms within the body, *p,p'*-DDE, and *o,p'*-DDE (Clark et al., 1998). It is the *p,p'*-DDE isomer that is the most stable, and thus it is the most persistent metabolite of DDT. For this reason, *p,p'*-DDE is often selected as an indicator of chronic exposure. It is interesting that although *p,p'*-DDE is the most commonly found DDT metabolite,

most of it present in people today is from dietary exposure, rather than from the biotransformation of DDT (Morgan and Roan, 1971).

The metabolite DDE has a large fat:water partition coefficient, and so is very lipid soluble. The high lipid solubility of DDE, the very stable nature of the molecule, and the extremely slow rate of biotransformation in the body result in bioaccumulation. As a result of these properties, DDE has also been shown to biomagnify, meaning that concentrations of it in an organism increase the higher up the organism is on the food chain. Thus, the human occupation of the top niche in the food chain ensures higher levels in humans than in the surrounding environment. Once it enters the body, it is stored in fat deposits. Levels remain virtually unchanged unless some great demand upon the body, such as pregnancy or starvation, results in mobilization of fat stores.

4.4 Human Exposure to DDT and its Persistent Metabolites

Though the use of DDT has been banned in Canada, USA and many European countries, it is still in use in many other parts of the world. The most persistent DDT metabolite, *p,p'*-DDE, has been detected in human serum, adipose tissue and follicular fluid around the world. In Zimbabwe, *p,p'*-DDE was detected in human breast milk from 100% of the sampled population (Chikuni et al., 1997). The ratio of *p,p'*-DDT/*p,p'*-DDE was used to indicate recent contamination with DDT, usually found in areas with vector control programs in use (Chikuni et al., 1997). In Mexico, DDT is still used systematically in campaigns against malaria (Rivero-Rodriguez et al., 1997). An exposure assessment for the workers applying DDT in one such campaign found a geometric mean concentration of *p,p'*-DDE in adipose tissue to be 67.41 µg/g (Rivero-Rodriguez et al., 1997). Another study conducted in Mexico found high levels of DDE

in serum lipids, ranging from 10.24 – 4661.4 ppb, with a mean of 562.48+ 676.18 ppb (Lopez-Carrillo et al., 1997). In Kazakhstan, school children were found to have serum DDE levels ranging from 183-4927 ppb, with a mean of 1078 ppb (Mazhitova et al., 1998). A random sampling of the general population of Singapore found widespread contamination of serum with both DDT and DDE (isomer not specified) (Luo et al., 1997). In this study, the geometric mean of DDT and DDE in serum was found to be 1.9 ppb (0.2-8.9 ppb), and 10.8 ppb (1.5-88.1 ppb) (Luo et al., 1997).

As previously stated, the stability and lipophilic nature of *p,p'*-DDE result in it being an extremely persistent compound. For this reason, *p,p'*-DDE may be detected in areas in which DDT has not been applied for over 20 years, such as in Quebec, where a survey of *p,p'*-DDE concentrations in breast milk found an average of 0.34 mg/kg lipids (Dewailly et al., 1996). This mean concentration is lower than averages reported in countries still actively using DDT, however levels are high enough to still represent a cause for concern. As well, *p,p'*-DDE levels ranging between 0.61 (0.47) – 1.07 (0.84) ppb have been detected in follicular fluid of women receiving IVF treatment in Canada (Jarrell et al., 1993).

Concern has arisen regarding the presence of DDT metabolites such as *p,p'*-DDE in the Arctic ecosystem. Although there are few local sources of DDT, the peculiarities of oceanic currents and global weather patterns have ensured contamination of this previously pristine ecosystem (Ayotte et al., 1995). Given the dependence of native populations on the arctic aquatic food chain, there is an obvious potential for exposure to organochlorine residues such as *p,p'*-DDE. Comparison between DDE concentrations in breast milk measured in Inuit women of northern Quebec and women in southern Quebec revealed an average of twice as much DDE in the Inuit population (Ayotte et al.,

1995). This observation raises obvious concerns regarding the long-term health effects of these high levels of exposure in the Inuit population.

4.5 Estrogenic Effects of DDE

The chemical DDE has often been described as being estrogenic in nature. The term “estrogenic” specifies compounds that mimic the activity of naturally occurring estrogens. The first assays to determine the estrogenicity of a compound involved measuring changes in estrogen responsive tissue, or alterations in the developmental timetable. For example, changes in uterine wet weight are reliable indicators of exposure to estrogens. More recent methods examine proliferation of estrogen responsive cell lines, or study receptor- binding affinities.

Early whole animal studies demonstrated that although *o,p'*-DDT and *p,p'*-DDT increased uterine wet weight in rats, thus possessing estrogenic activity, *p,p'*-DDE had little or no activity in this system (Welsh et al., 1969). As well, *p,p'*-DDE did not affect estradiol uptake by the uterus (Welsh et al., 1969). However, pretreatment with carbon tetrachloride, a chemical used to potentiate the action of drugs and estrogens as it was believed to inhibit their metabolism, indicated that the estrogenic effect of DDT was likely due to a metabolite (Welsh et al., 1969).

Another study demonstrated that while *o,p'*-DDT had an estrogenic effect on the activity of uterine enzymes, *p,p'*-DDT had only minimal effects (Singhal et al., 1970). As well, the supposedly estrogenic *o,p'*-DDE had no effect on estrous cycles or endocrine gland weights in female rats, although *o,p'*-DDT resulted in a significant alteration (Gellert and LeRoy Heinrichs, 1975). In a study examining the effect of contaminants on the synthesis of vitellogenin, an estrogen responsive protein in fish,

p,p'-DDE was found to have no effect (Andersen et al., 1999). However, in a study on sexual differentiation in salamanders, *p,p'*-DDE acted as an estrogen on the mullerian ducts of females (Clark et al., 1998).

More recently, molecular techniques have been used to address the question of whether or not DDE is estrogenic. Receptor binding studies have been conducted to determine the extent of the interaction of DDE with the estrogen receptor. The isomer *o,p'*-DDE had 0.00004% of the potency of estradiol; and *o,p'*-DDT had 0.00011% (Coldham et al., 1997). In a study comparing short term estrogenicity tests, *o,p'*-DDT had a binding affinity to a recombinant human estrogen receptor (hER) approximately 1:350th that of 17- β estradiol (Andersen et al., 1999). The binding affinity for *p,p'*-DDE in the same system was only 1:10000th that of 17- β estradiol (Andersen et al., 1999). Chen et al., 1997, conducted a similar receptor binding study and reported that *o,p'*-DDE bound to and activated the hER, but *p,p'*-DDE did not (Chen et al., 1997).

A study designed to compare the reliability of several test systems found that in a transient reporter gene assay using MCF-7 cells, *p,p'*-DDE acted as full agonist in one laboratory and a partial agonist in two other laboratories (Andersen et al., 1999). However, in the same study, *p,p'*-DDE was found to be inactive in a yeast reporter gene assay (Andersen et al., 1999). Experiments conducted with the E-screen assay, which measures the proliferation of an estrogen responsive cell line, found that both isomers of DDT and DDE provoked an estrogenic response (Soto et al., 1995). It has been observed that *o,p'*-DDD and *p,p'*-DDD had estrogenic activity in a combination of *in vitro* assays, although *p,p'*-DDE appeared to have no effect (Klotz et al., 1996). This is interesting, since it is possible that the estrogenicity of the DDD metabolites of DDT

could be responsible for the much earlier observation made by Welsh et al., 1969, which indicated that the estrogenic effects of DDT were caused by a metabolite.

In summary, DDE can be best described as a weak estrogen, with activity dependent on the isomer being studied. It appears to possess some small capacity to bind to the estrogen receptor, though *in vivo* studies reveal no definitive effects.

4.6 Anti-androgenic Effects of DDE

A very important observation about DDE came from a study examining the binding affinity of DDE for the androgen receptor, where it was reported that *p,p'*-DDE is a potent androgen receptor antagonist (Kelce et al., 1995). As well, it was also observed that *p,p'*-DDE inhibits androgen-induced transcriptional activity (Kelce et al., 1995). Experiments performed on rats demonstrated that *p,p'*-DDE inhibits androgen action in developing, pubertal and adult male rats (Kelce et al., 1995).

Previously, it had been suggested that activation of the estrogen receptor was responsible for the feminizing or demasculinizing effects reported following exposure to DDE. However, it now appears likely that these phenotypic alterations are a result of the potent anti-androgenic properties of DDE, rather than its weak estrogenic action. As a result of these observations on the strong anti-androgenic activity of *p,p'*-DDE, cases involving contamination by *p,p'*-DDE were examined in a new way. It was suspected as a causal agent for developmental changes observed in alligators of Lake Apopka, which had been contaminated by a pesticide spill resulting in large amounts of *p,p'*-DDE present in the ecosystem (Guillette Jr. et al., 1996). These alligators exhibited smaller penis size, lower plasma androgen concentrations, and lack of responsiveness of the

penis to plasma androgens (Guillette Jr. et al., 1996). These effects seemed consistent with an anti-androgenic effect of *p,p'*-DDE (Guillette Jr. et al., 1996).

Confirmation that *p,p'*-DDE mediates its effects via the androgen receptor was received with evidence that *p,p'*-DDE acts as an anti-androgen *in vivo* by altering the expression of androgen-dependent genes (Kelce et al., 1997). Further confirmation was received with the demonstration that *p,p'*-DDE caused a 100% inhibition of binding of [³H]5 α -DHT to the androgen receptor, and that *p,p'*-DDE was capable of decreasing the binding of [³H]5 α -DHT to the androgen binding protein, resulting in only 80% binding as compared to DHT alone (Danzo, 1997).

Subsequent whole animal studies found a strain-specific sensitivity to the action of *p,p'*-DDE (You et al., 1998). Long-Evans hooded rats were more sensitive to *p,p'*-DDE than were Sprague-Dawley rats (You et al., 1998). Pregnant dams exposed to 100 mg/kg/day of *p,p'*-DDE produced offspring with reduced male anogenital distance, increased retention of male thoracic nipple and alterations in expression of the androgen receptor in one or both strains (You et al., 1998). Animals receiving a lower dose of *p,p'*-DDE produced a weaker response (You et al., 1998). This study determined that developmental effects of *p,p'*-DDE were minimal below 10 mg/kg/day to dams (You et al., 1998).

Mortality due to prostate and testicular cancer has increased dramatically worldwide in the last 50 years. The global prevalence of *p,p'*-DDE as an environmental contaminant, and its potent ability to bind to the androgen receptor, suggested the hypothesis that there was a link between environmental exposure to DDT derivatives and mortality from cancers of the male reproductive tract (Cocco and Benichou, 1998). This association was examined in an epidemiological study. No evidence was gathered

from this study that supported the hypothesis that DDE exposure and mortality from cancer of the male reproductive tract were linked, although a possible weak inverse correlation was observed (Cocco and Benichou, 1998). This observation is of medical interest, as it is reasonable to speculate that the anti-androgenic effect of *p,p'*-DDE might have a protective effect against an androgen-dependent cancer such as prostate cancer (Cocco and Benichou, 1998).

4.7 DDE Receptor Binding

In an attempt to define the mechanism of action of DDE, many receptor-binding studies have been conducted. The results of these experiments indicate species-specific binding to several different receptor types. Low affinity binding of *p,p'*-DDE and *o,p'*-DDE to rat, rabbit, alligator and human estrogen receptor has been observed, and once bound to the receptor, DDE causes transcriptional activation of genes (Kelce et al., 1995; Vonier et al., 1996; Chen et al., 1997; Danzo, 1997; Gaido et al., 1997). As well, both *o,p'*- and *p,p'*-DDE have been shown to bind to the human, duck, hen and rabbit progesterone receptor and block transcriptional activation (Lundholm, 1988; Gaido et al., 1997; Klotz et al., 1997). However, neither isomer of DDE competed for binding to the alligator progesterone receptor (Vonier et al., 1996). The binding of dexamethasone, a glucocorticoid receptor ligand, to the glucocorticoid receptor of chicken was inhibited by *p,p'*-DDE (Lundholm, 1991), but dexamethasone-induced human glucocorticoid receptor activation did not change (Kelce et al., 1995). While these receptor-binding studies are informative, they do not provide enough information to indicate potential mechanisms of action of DDE.

4.8 Female Reproductive Toxicology

To date, considerable attention has been focused on the effects of endocrine disrupters on male fertility. Equal attention has not yet been focused on the consequences of exposure to endocrine disrupters on female fertility. A discussion of female reproductive toxicology serves to demonstrate why research in this area is required.

Female reproductive toxicology is very unlike male reproductive toxicology, as a result of some major physiological differences. In the male, the stem cells from which sperm cells eventually differentiate are constantly dividing. There is no 'stock' for sperm cells, as there is in the female. Thus, unless a toxin causes a developmental alteration to the testis, exposure to a toxin may disrupt spermatogenesis for a time, but it is likely to resume once sufficient time has passed for a new wave of sperm to differentiate from the stem cells. A female, however, does not begin to produce new follicles at puberty. In fact, she was born with all the primordial follicles that she will ever have. The toxicological implication of these functional and developmental differences is that a toxic insult, at any point in a woman's life, could have consequences ranging from disturbances in menstrual cycling, to permanent sterility if the primordial follicles were targeted.

The pre-ovulatory or Graafian follicle is basically a fluid filled bubble near the surface of the ovary and is considered to be the basic functional unit of the ovary (Hsueh et al., 1984). Folliculogenesis, or follicle formation, begins with the migration of germ cells to the embryonic gonad. These primordial germ cells divide rapidly for a time, then enter meiosis. However, meiosis is not completed, and the germ cells are arrested at the diplotene stage of the first meiotic prophase. Following this arrest, somatic cells

surround them and form discrete complexes called primordial follicles. A primordial follicle consists of an oocyte surrounded by a distinct layer of granulosa cells.

Primordial follicles represent the developmental stock from which all mature oocytes originate. The developmental hiatus these follicles enter *in utero* may last only until puberty, or it may never be broken. Throughout the fertile life of the woman, follicles gradually re-awaken and begin to grow, resulting in a steady stream of developing follicles and ovulations.

When the arrested primordial follicles are activated, the granulosa cells begin to proliferate and the oocyte increases in size. As well, stromal cells immediately outside the basement membrane surrounding the granulosa cells differentiate to form the cells of the theca. At this stage, the follicle is referred to as a primary follicle. Cellular growth and oocyte maturation continues, resulting in the formation of a central, fluid-filled cavity called the antrum. This is known as a Graafian, or pre-ovulatory follicle.

The long-term consequences of exposure to reproductive toxicants depend on what stage of follicular development is targeted. For example, a toxicant affecting existing Graafian follicles will have no appreciable effect on fertility, as less developed follicles can be rapidly recruited to replace those destroyed by the toxin. An insult to primary follicles will render the animal infertile for a time, but this effect will be transitory. The primordial follicles will develop into primary follicles, and the developmental stream will continue unbroken. Only the destruction of the entire resting pool of primordial follicles will cause the permanent cessation of menstrual cycling (Hirshfield, 1997).

This of course assumes that the toxicant in question acts in a very direct manner and totally destroys specific cell types. Endocrine disrupters do not generally act in such an obvious fashion. Their effect is likely to be much more subtle, but still damaging. It

has been observed that the presence of contaminants in follicular fluid results in the oocyte being exposed to toxins during the second division of meiosis. This is considered to be an important time in the determination of the genetic contents of the oocyte (Jarrell et al., 1993). The observed presence of DDE, a known endocrine disrupter, in human ovarian follicular fluid is thus particularly concerning because the developing oocyte is exposed to it and thus may be altered in some fashion.

4.9 Granulosa Cells

The ovarian follicle consists of three components: theca interna cells, granulosa cells, and the oocyte. The oocyte is suspended in the center of the follicle, encapsulated and anchored by granulosa cells forming the oocyte-cumulus complex. Surrounding the granulosa cell layers are the cells of the theca interna. Follicular fluid fills the space between the walls of the follicles, much like water in a balloon.

Granulosa cells have several functions within the ovary. Perhaps most importantly, they are the ovarian source of the two most important reproductive steroids, estradiol and progesterone (Hsueh et al., 1984). Biosynthesis of estrogens requires the interaction of both theca and granulosa cells as granulosa cells lack the requisite enzymes to synthesize androgens from progesterone. Progesterone synthesis is not limited to granulosa cells, and takes place in the theca as well. Thus, theca cells convert progesterone to androgens, which granulosa cells then convert to estrogen (Hsueh et al., 1984).

Following ovulation, the follicle undergoes a drastic metamorphosis and becomes the corpus luteum. This process, called luteinization, involves the differentiation of both the theca and granulosa cell layers, resulting in the so-called large and small cells of the

corpus luteum. It also initiates a shift in follicular steroidogenesis, resulting in progesterone being the dominant end product of the steroidogenic pathway, rather than estradiol.

4.10 Gonadotropin Control of Steroidogenesis

The term gonadotropin refers to two hormones secreted by the anterior pituitary, leutinizing hormone (LH) and follicle stimulating hormone (FSH). Gonadotropin secretion by the anterior pituitary is under control of the hypothalamic hormone gonadotropin releasing hormone (GnRH), which is secreted in a pulsatile fashion. Gonadotropins are required for follicular growth, ovulation, luteinization, and steroidogenesis(Goodman, 1994).

The only ovarian cells known to have FSH receptors are granulosa cells. In response to FSH, granulosa cells secrete estradiol. Estradiol increases the sensitivity of granulosa cells to FSH, thus greatly amplifying the effect of FSH. The combination of FSH and estradiol results in granulosa cell proliferation. If additional FSH is supplied, several follicles will begin to grow and be capable of ovulation. Levels of FSH are therefore considered critical to the selection of a dominant follicle. It is thought that the dominant follicle is simply most efficient at making use of low levels of FSH.

The gonadotropins FSH and LH bind to specific cell-surface receptors. Once bound, they activate a receptor bound G protein, which then stimulates adenylyl cyclase activity. Adenylyl cyclase is a membrane bound enzyme that uses ATP to synthesis cAMP, a common second messenger molecule that is also used by gonadotropins. The resulting increase in intracellular cAMP levels activates protein kinase A (PKA), which is an enzyme that phosphorylates critical proteins, leading to the transcription of

steroidogenic enzymes (Figure 1). Compounds that are referred to as PKA pathway activators, such as cholera toxin (CT) and 8-Bromo cAMP (8-Br-cAMP), stimulate PKA activity and thus mimic the effect of gonadotropins.

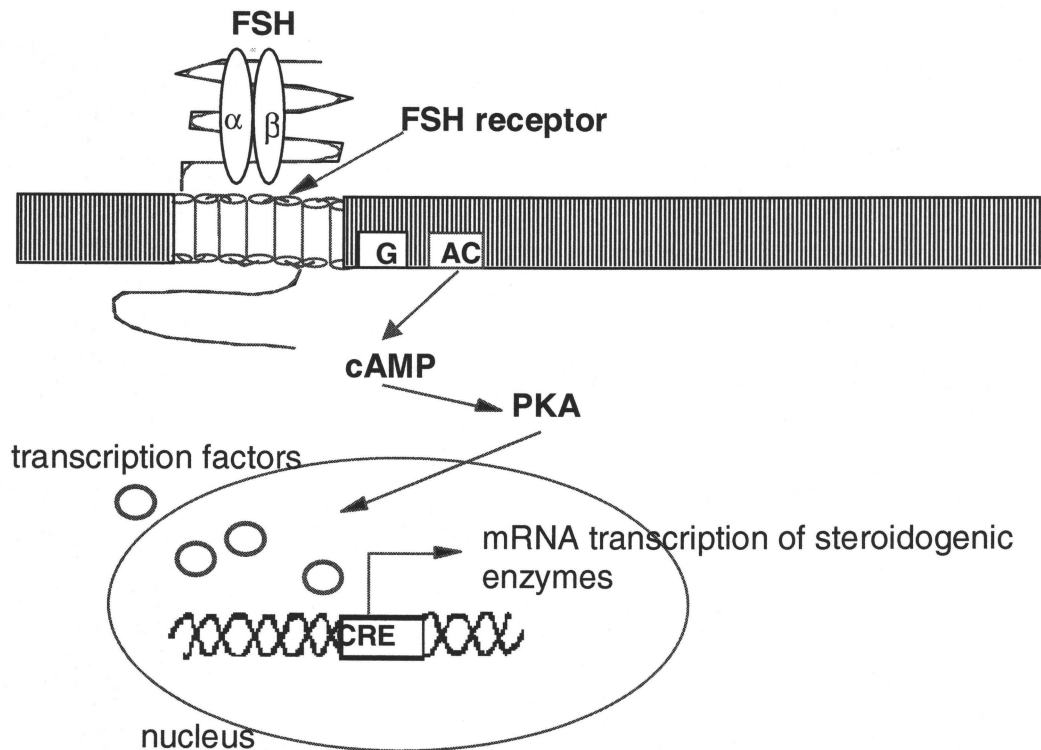


Figure 1: Gonadotropin Stimulation of Steroidogenesis

Where G is G protein, and AC is adenylate cyclase

4.11 Ovarian Steroidogenesis

The initial stages of steroidogenesis, whether ovarian or adrenal, occur within the mitochondria. The basic building block of steroid hormone synthesis is cholesterol (Stocco and Clark, 1996). In granulosa cells, most of the cholesterol required for steroidogenesis is synthesized de novo from acetate, because they are not vascularized and thus have no access to the cholesterol in the bloodstream (Maitra et al., 1995; Di Croce et al., 1999). Control of the transport of cholesterol into the mitochondria appears to be the major regulatory step in granulosa cell steroidogenesis. The protein considered

responsible for this transport is the steroid acute regulatory protein (StAR) (Stocco and Clark, 1996).

Once cholesterol enters the mitochondria, a few key enzymes regulate the production of progesterone in granulosa cells. As indicated in Figure 2, P450 cholesterol side chain cleavage (P450_{scc}) is found within the mitochondria and converts cholesterol into pregnenolone, the precursor of progesterone. The enzyme responsible for converting pregnenolone to progesterone is 3 β -hydroxy-5-ene steroid dehydrogenase (3 β -HSD). Synthesis of P450_{scc} and 3 β -HSD are both regulated in granulosa cells (Stocco and Clark, 1996). The enzyme aromatase then converts the androgens produced by the cells of the theca into estrogen.

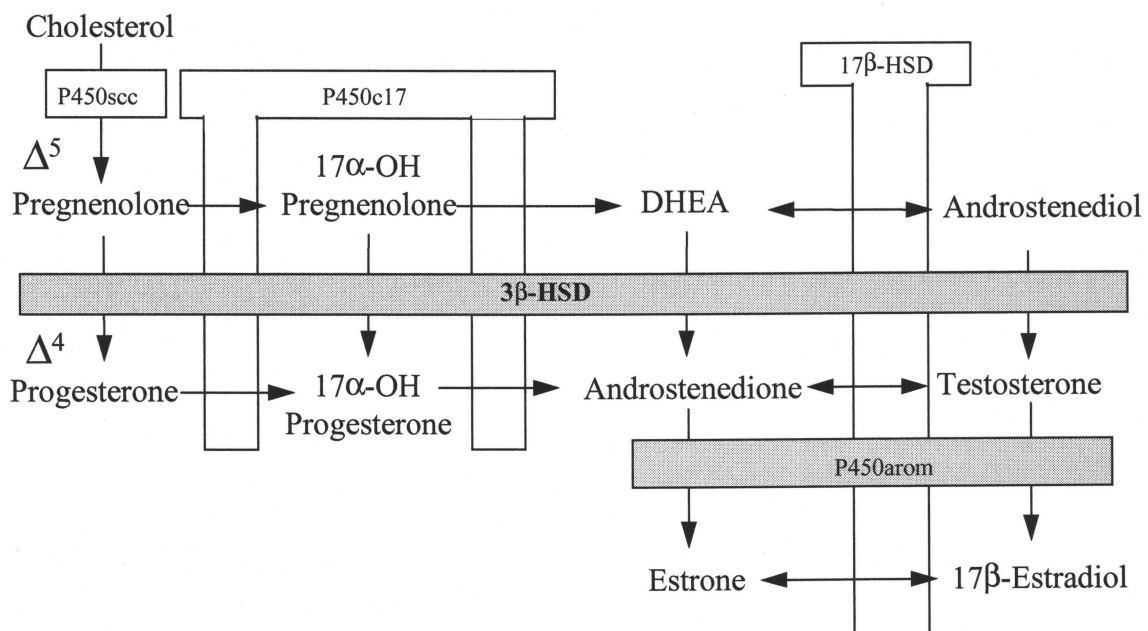


Figure 2: Steroidogenic Pathway in Ovary

4.12 Female Health Effects of DDE

There is evidence that DDT and DDE are capable of affecting ovarian function. Alligators hatched from eggs laid in an environment highly polluted with DDT and its metabolites, including DDE, had increased plasma estradiol levels and ovaries with prominent polyovulatory follicles and multinucleate oocytes. *In vitro* cultures of ovaries from these exposed alligators produced lower basal estradiol compared with ovaries from control animals. However, LH stimulated estradiol production to a much greater magnitude than observed in ovaries from control alligators.

The effect of DDT, and its major metabolite DDE, on female reproduction has been of concern recently because residues have been found in the follicular fluid of women with lower conception rates. Levels between 0.61 (+0.47) and 1.07(+0.84) ppb of DDE were found in the follicular fluid of women undergoing in vitro fertilization (Jarrell et al., 1993). Although these levels of DDE appeared to have no effect on oocyte cleavage rate, or the time of the first cleavage, the causes of infertility are unknown in these women.

In recent years, there has been considerable controversy over research finding a link between DDE levels and breast cancer. It has been hypothesized that human exposure to DDE is responsible for the increased breast cancer incidence rates (Davis et al., 1993; Wolff and Toniolo, 1995; Hunter et al., 1997). The incidence of breast cancer has risen approximately 1% every year since 1940, and established risk factors are not considered sufficient to explain the increase (Hunter et al., 1997). Evidence exists indicating that environmental or lifestyle factors may be responsible for the observed increase in breast cancer rates. This evidence includes the observations that breast cancer rates vary across the world, and that a woman moving from a low risk to a high

risk area soon develops the increased risk for breast cancer associated with the high risk area (Hunter et al., 1997). Therefore, sufficient evidence exists to consider DDE as a potential agent responsible for promoting breast cancer incidence.

The research conducted to test this hypothesis has proven to be largely inconclusive. In three early studies, conducted on a small scale, there was an association between DDE levels and breast cancer (Falck et al., 1992; Wolff et al., 1993; Dewailly et al., 1994). However, in more recent studies with larger statistical power, no significant relationship between DDE levels and breast cancer was demonstrated (Krieger et al., 1994; Hunter et al., 1997; Lopez-Carrillo et al., 1997; van't Veer et al., 1997). In fact, a study conducted by van't Veer et al., 1997 found a small protective effect of DDE against breast cancer. It is of note that these studies examined current body burdens of DDE in relation to breast cancer, without considering previous exposure. It has been speculated that *in utero* or pubertal exposure to endocrine disruptors such as DDE increases the risk of these chemicals promoting breast cancer (Ardies and Dees, 1998). To date, there have not been any studies that examined the relationship between *in utero* or pubertal exposure to DDE and breast cancer incidence. Although deserving of further research, the evidence at this time does not conclusively indicate that DDE causes breast cancer.

Another proposed effect of DDE on human health is an association with a decreased duration of lactation. In 1987, Rogan et al. conducted a study and found *p,p'*-DDE levels to be inversely correlated with duration of lactation (Rogan et al., 1987). However, when this study was later repeated, this correlation was only evident with women who had previously lactated (Gladden and Rogan, 1995). The authors of the study did a simulation on their data, accounting for the effects of breast-feeding duration

and *p,p'*-DDE excretion via lactation, and still identified an independent effect of *p,p'*-DDE on duration of lactation (Gladen and Rogan, 1995). Overall, these observations are suggestive of an effect of *p,p'*-DDE on the duration of lactation, but are inconclusive at this time.

A startling observation that has been made recently is that girls currently appear to be entering puberty at a younger age than in previous generations (Herman-Giddens et al., 1997). A popular explanation is that nutrition has improved, allowing girls to reach the critical body mass and fat accumulation sooner, and thus mature faster. However, an alternative hypothesis has been proposed. Exposure *in utero* to endocrine disrupters has been hypothesized to affect puberty in girls (Herman-Giddens et al., 1997; Rogan, 1998). Preliminary findings of a study still in progress find that girls exposed to higher levels of DDE *in utero* mature earlier (Rogan, 1998). Although preliminary and unadjusted, these findings are of great interest, and if confirmed may finally provide tangible evidence of the human health effects of developmental exposure to DDE.

5.0 The Experimental Model

Due to a variety of reasons, there is a push in reproductive toxicology to move away from whole animal, *in vivo* studies. Public pressure has played an important role, urging the development of test systems that do not require the extensive use of animals. As well, *in vivo* studies are expensive and very time consuming. *In vitro* test systems are less expensive to use, can examine a large number of chemicals very rapidly, and do not require the use of animals (Schwetz, 1993). Although it is generally recognized that the use of *in vivo* studies will always be required for regulatory purposes, it is felt that *in vitro* data provides valuable information of the site and mechanism of action of toxicants

(Schwetz, 1993). This information serves to increase the degree of confidence in the findings of *in vivo* studies (Schwetz, 1993).

Though many endocrine disrupters interact directly with a receptor, it is known that other potential mechanisms of action exist that do not involve binding to hormone receptors. For example, an exoestrogen could interact with an estrogen responsive element (ERE), and produce an estrogenic response without ever binding to the estrogen receptor (Gillesby and Zacharewski, 1998). To continue with the example of the exoestrogen, it could also act via a completely different receptor system that has its effects mediated by the same signaling cascade as the estrogen receptor (Gillesby and Zacharewski, 1998). As well, modulation of steroid binding proteins by an endocrine disrupter can effect the amount of active hormone present. The myriad of potential mechanisms of endocrine disrupters presents a unique challenge in the design of screening protocols. It has been proposed that further mechanistic investigation of endocrine disrupters is necessary for the development of valid screening assays (Campbell and Hutchinson, 1998).

5.1 JC-410 Cells

Our laboratory has recently established an immortalized steroidogenic porcine granulosa cell line, called JC-410 (Chedrese et al., 1998; Rodway et al., 1999). The JC-410 cell line produces basal levels of progesterone and is functionally responsive to hormonal steroids and to protein kinase A (PKA) pathway activators such as cholera toxin (CT), forskolin (FK), and a cell permeant analogue of the second messenger cAMP, 8-bromo-cAMP (8-Br-cAMP) (Chedrese et al., 1998). Although these activators do not actually affect the activity of protein kinase A enzyme, but stimulate the pathway

at points previous to the PKA enzyme, they will be referred to as PKA activators here after for convenience. These cells will also produce estrogen if supplied with an androgen substrate, as would be expected of granulosa cells (Chedrese et al., 1998). Androgens, estrogens, and progestins have effects in primary porcine granulosa cell cultures, and in the JC-410 cell line.

This cell line represents a homogeneous source of material, which does not have the variability inherent in primary cell culture. This variability is a result of the circumstances necessary to the establishment of primary culture. Each time a primary culture is established, the ovaries are taken from several animals whose exact age and stage of development may differ considerably from culture to culture. As well, the animals may be exposed to a variety of environmental factors of which we are unaware. Essentially, each time a primary culture is established it is a different population of cells, which may or may not respond, in an experimental setting, in the same way as the culture before. The use of a stable cell line circumvents these limitations, and is also much more convenient to culture and maintain.

Currently, the use of granulosa cells for *in vitro* reproductive toxicology research has focused on primary granulosa cell culture and luteinized granulosa cells (Treinen et al., 1990; Treinen et al., 1993; Enan et al., 1996). The present investigation represents the first time toxicological experiments have been conducted on a stable steroidogenic granulosa cell line. The JC-410 cell line provides a number of different endpoints for study, such as progesterone synthesis and cAMP generation, both basal and in response to stimulation, cellular protein levels, and the expression of the steroidogenic genes characteristic of granulosa cells.

One of the limitations of this model is the lower level of basal progesterone synthesis as compared to primary cells. As well, it has been suggested that a model consisting of a homogenous population of cells is less valid than the use of a more heterogeneous population of cells, as it removes the natural variation present in a population. This is certainly a valid criticism, however the nature of scientific research requires that compromises in designing a model be made in the name of statistics. Overall, this cell line is an excellent model with which to study the effects, as well as the mechanism of action, of the endocrine disrupter DDE on steroid hormone production in granulosa cells.

6.0 Materials and Methods

6.1 Reagents

6.1.1 Tissue Culture Reagents

Tissue culture reagents including medium 199 (M199), penicillin, trypsin, streptomycin, amphotericin B (Fungizone[®]), and new born calf serum (NBCS), were purchased from Gibco (Burlington, Ontario, Canada). Bovine insulin was purchased from Sigma Chemical Co. (St. Louis, MO). Plastic culture plates were purchased from Falcon (Lincoln Park, NJ). Materials used in experiments such as Cholera toxin (CT), Forskolin, and 8-bromoadenosine 3':5'-cyclic monophosphate (8-Br-cAMP) were purchased from Sigma Chemical Co. (St. Louis, MO). The DDE used in the experiments resulting in Figures 3-9 was part of a chlorinated pesticide kit from PolyScience, (IL), and consisted of approximately 96% p,p'-DDE and 4% o,p'-DDE. The p,p'-DDE and o,p'-DDE used in the experiments resulting in Figures 10-15 were purchased from ChemService, (West Chester, PA).

6.1.2 Radioimmunoassay and Protein Assay Reagents

Materials for cAMP iodination and RIA including 2'-O-monosuccinyladenosine 3':5'-cyclic monophosphate tyrosyl methyl ester (cAMP derivative), N-chloro-p-toluene-sulfonamide sodium salt (Chloramine T), sodium metabisulfite, sodium iodide, bovine serum albumin (BSA), and 3-isobutyl-1-methyl-xanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). Na ¹²⁵I (100 mCi/ml) was purchased from New

England Nuclear (Boston, MA). cAMP antibody was a gift from University of Virginia, School of Medicine (Charlottesville, VA). Glass culture tubes, 12x75 mm, were purchased from VWR Canlab (Mississauga, ON).

Iodinated progesterone (11- α -hydroxyprogesterone 11- β D-glucuronide-¹²⁵I-iodotiramine) was purchased from Amersham (Arlington Heights, IL); now Amersham-Pharmacia Biotech, Piscataway, NJ). Progesterone antibody was purchased from Department of Physiology, University of Saskatchewan (Saskatoon, SK).

The Bio-Rad DC Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

6.1.3 Reagents for cDNA preparation

E. Coli strain JM105 competent cells were purchased from Amersham-Pharmacia Biotech, (Piscataway, NJ). The components of LB broth, Bacto-Agar, Tryptone-peptone, and yeast extract, were purchased from Difco Laboratories, (Detroit, MI). Wizard[®] miniprep DNA purification kit was purchased from Promega, (Madison, WI). Gel Eclipse [™] DNA Purification Kit was purchased from Gordon Technologies (Mississauga, ON). Restriction enzymes were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ) and Canadian Life Technologies, (Burlington, ON).

6.1.4 Reagents for Northern Hybridization

Agarose was purchased from Canadian Life Technologies, (Burlington, ON). Hybond-N nylon membranes, random primers (5'-PO₃, Na⁺ salt) and klenow polymerase were purchased from Amersham (Arlington Heights, IL); now Amersham-Pharmacia Biotech, Piscataway, NJ). ³²P was purchased from New England Nuclear (Boston, MA). Bovine serum albumin, and ingredients in the RPRB buffer were

purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-10 was purchased from Amersham-Pharmacia Biotech (Baie d' Urfé, PQ). X-ray film was purchased from University of Saskatchewan, audio-visual department (Saskatoon, SK).

6.2 Granulosa Cell Culture

Granulosa cells for primary culture were obtained from small-sized follicles (1-3 mm) in ovaries of prepubertal gilts collected at the local slaughterhouse and cultured as previously described (Chedrese et al., 1998). The JC-410 cell line is a stable cell line originated from a spontaneously immortalized primary culture of porcine granulosa cells (Chedrese et al., 1998; Rodway et al., 1999). Cells were maintained in a CO₂ incubator (Forma Scientific Inc. Marietta, Ohio) at 37° C in a water-saturated atmosphere of 95% air and 5% CO₂. Cells were grown in phenol red-free Medium 199 supplemented with 5% newborn calf serum, 5 µg/ml insulin, 100 IU/ml penicillin, and 100 µg/ml streptomycin (culture medium). In experiments which measured progesterone and cAMP as end points, 24 (1.5 x 2.0 cm) well culture plates were used. In experiments from which RNA was extracted, cells were cultured in 10 x 2.0 cm plates.

6.2.1 Granulosa Cell Treatment

Once 70-80% confluency was attained, generally within 48 hours, culture medium was replaced with serum-free culture medium containing the treatments and incubated for 24 hours. Treatments were delivered in 5 µl to wells containing 1 ml of media. Control groups in experiments resulting in Figures 3-7 received vehicle, consisting of ethanol and benzene, up to a maximum of 0.01% benzene and 0.49% ethanol. Control groups in experiments resulting in Figures 8-13 received vehicle consisting of ethanol up to a maximum of 0.5% ethanol, since the *o,p*- and *p,p*-DDE used for these experiments

was purchased as a powder and resuspended in ethanol. This percentage is volume/volume and has not been adjusted for the specific gravity of alcohol.

For experiments resulting in Figures 8-13, the two isoforms of DDE were mixed so that the DDE applied to the cells contained 96% *p,p*-DDE and 4% *o,p*-DDE, the same relative proportion of the two isoforms as that occurring in the DDE used in Figures 3-7.

6.3 Quantification of Progesterone, cAMP, Cellular Protein and DNA

6.3.1 Progesterone Radioimmunoassay

Progesterone content in 100 μ l of culture media was determined by radioimmunoassay (RIA), as previously described (Desbuquois and Aurbach, 1971; Orczyk et al., 1974). The inter- and intra-assay coefficient of variation for the progesterone assay was below 10%. The minimum detectable amount of progesterone was 6.25 pg. Levels of radioactivity were determined using a model 1277 GammaMaster Gamma Counter (Turku, Finland). Progesterone synthesis was normalized between experiments by dividing the progesterone levels measured in each treatment group by the coinciding protein content.

6.3.2 cAMP Radioimmunoassay

The generation of cAMP was tested in cells cultured for 24 hours with DDE, then exposed to control media or CT in the presence of the phosphodiesterase inhibitor IBMX (113 μ M) for 30 minutes. Media was then discarded and cAMP was extracted with 200 μ l absolute alcohol. The extract was dried and resuspended in 1 ml of 0.05M sodium acetate buffer pH 6.2. An acetylation reaction with acetic anhydride and tri-ethyl amine at a ratio of 2:5 was then performed on 50 μ l of sample. A cAMP derivative, 2'-O-

monosuccinyladenosine 3': 5'-cyclic monophosphate tyrosyl methyl ester, was labeled with ^{125}I as previously described (Steiner et al., 1972). cAMP content was then determined by radioimmunoassay (RIA), as previously described (Steiner et al., 1972). The inter- and intra-assay coefficient of variation for the cAMP assay was below 15%. The minimum detectable amount of cAMP was 3.25 fmol. Levels of radioactivity were determined using a model 1277 GammaMaster Gamma Counter (Turku, Finland). cAMP production was normalized between experiments by dividing the cAMP levels measured in each treatment group by the coinciding protein content.

6.3.3 Protein and DNA content Analysis

After completion of the experiment, media was removed and the cells were washed twice with 0.9 % phosphate-buffer salt solution. 250 μl of a 0.1 % sodium dodecyl sulphate (SDS) solution was added to each well to solubilize cellular protein. Total cellular protein content in 200 μl of SDS was determined using the Bio-Rad DC Protein Assay Kit.

Trypsin-dispersed cells were collected and DNA was estimated by a flourometric assay using the bisbenzimidazole fluorescent dye.

6.4 *Northern Blot Analyses*

6.4.1 Isolation, Electrophoresis and Transfer of RNA

Cells were collected by adding 2 ml of a solution containing 3.52 M guanidinium isothiocyanate, 0.022 M sodium citrate (pH 7), 0.44% N-lauroylsarcosine (sarkosyl) and 0.1 M 2 β -mercaptoethanol, with a final pH 5.5. Total RNA was isolated by acid phenol/chloroform extraction according to methods described by Chomczynski and

Sacchi (1987) (Chomczynski and Sacchi, 1987). Samples of total RNA were denatured, size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (Hybond-N) by diffusion blotting. The agarose- formaldehyde gel contained 0.5 g of agarose, 5 mls 10 X MOPS buffer (Appendix A) and 2.6 ml formaldehyde (37%) in a final volume of 50 mls. Electrophoresis of RNA was conducted with 10 µg of RNA. Samples were diluted in distilled H₂O, 15 µl of RNA loading buffer (Appendix B), and 1 µl of 1 µg/µl ethidium bromide. Samples were incubated at 65⁰C for 10 minutes and then loaded onto the agarose-formaldehyde gel. Electrophoresis was conducted at 100 volts (constant) for 1.5 hours in 1 X MOPS buffer. The separated RNA bands were then transferred from the gel to a nylon membrane by capillary elution using 20 X SSC buffer (Appendix C). RNA was crosslinked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

6.4.2 cDNA Probe Labeling

cDNAs for human type II 3β-hydroxy-5-ene steroid dehydrogenase (3β-HSD); (Luu The et al., 1989), porcine cytochrome P450 cholesterol side chain cleavage (P450scc) (Mulheron et al., 1989), porcine steroid acute regulatory protein (StAR) (Pilon et al., 1997), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985), were used as probes. Briefly, a plasmid vector containing the desired cDNA was used to transform JM105 competent cells. The transformed cells were grown to amplify the plasmid, then the plasmid was purified using the wizard[®] miniprep DNA purification kit. The purified plasmid was digested with appropriate restriction enzymes, and separated by gel electrophoresis on a 0.8% agarose gel in 1 x TAE buffer (Appendix D), with 2 µl of 10 µg/µl ethidium bromide. Samples were diluted with

loading buffer (Appendix E) and distilled H₂O. The cDNA was then purified from the gel using the Gel Eclipse™ DNA Purification Kit.

Heating at 65°C for 10 minutes denatured the cDNA in use. After denaturing, 2 µl random primers, 1 µl BSA, and 2.5 X RPRB buffer (Appendix F), 5 ml of ³²P and 2 ml of klenow polymerase were added to the cDNA. This mixture was then incubated at 37°C for 1 hour. The labeled probe was then separated from the free ³²P using a G-10 sephadex column. cDNAs were labeled by random primer synthesis (Feinberg and Vogelstein, 1983) with [α ³²P] dCTP (>3000 Ci/mmol; New England Nuclear, Boston, MA, USA) to a specific activity of 1.5-3.0 x 10⁹ dpm/mg DNA.

6.4.3 Membrane Hybridization and Autoradiograph analysis

Prior to hybridization, the membranes were incubated in a prehybridization solution containing 7 % SDS, 1 mM EDTA (pH 8), 0.263 M Na₂HPO₄ and 1 % of bovine serum albumin, or a QuikHyb solution obtained from Stratagene (La Jolla, CA). The ³²P labeled probe was then added to the prehybridization solution and the membranes were incubated for 1-16 hours, depending on the prehybridization solution used. The membranes were washed with a solution containing 0.1 % SSC and 0.1 % (w/v) SDS. X-ray films were exposed to the hybridized blotted membranes for 1 hour to 5 days. A Kodak Electrophoresis Documentation and Analysis System 120 was used for gel photography and densitometric analysis of autoradiographs. Quantitative values of mRNA levels were determined by calculating a ratio of the densitometric value of the signal produced by the steroidogenic enzyme probe to the densitometric value of the signal produced by the GAPDH probe.

6.5 Statistical analysis

Data are presented as the mean \pm SEM of four independent experiments. Data were analyzed using a 1-way or 2-way analysis of variance. When a significant F value was present, Fisher's Least Significant Difference test was used for individual comparison of means (Steel and Torrie, 1980). Significant differences indicated by different letters were established as $P < 0.05$.

7.0 Results

7.1 *Potentiating Effect of DDE on PKA Activator-Stimulated Progesterone*

Synthesis

The effect of DDE on 8-Br-cAMP-stimulated progesterone accumulation in the JC-410 cells is depicted in Figure 3. No changes in progesterone accumulation were observed with 10 ng/ml DDE (Figure 3). The PKA-activator, 1mM 8-Br-cAMP, induced a 0.7-fold increase in progesterone accumulation. Addition of 10 ng/ml DDE, potentiated 8-Br-cAMP-stimulated progesterone accumulation 0.4-fold over the levels observed with 8-Br-cAMP alone. Although an elevation was observed, there was no significant stimulation of progesterone accumulation with 30 ng/ml CT (Figure 4). Addition of 10 ng/ml DDE potentiated the effect of CT on progesterone accumulation 0.7-fold over the levels observed with CT alone (Figure 4). Figure 5 depicts the effect of DDE, in the presence or absence of 30 ng/ml CT in cultures of primary porcine granulosa cells. Progesterone levels were not altered by DDE alone at any of the concentrations used in this study. Progesterone accumulation was elevated 0.4-fold when CT was added to the cultures. Addition of 0.1, 1, 10 and 100 ng/ml DDE potentiated CT-stimulated progesterone accumulation 0.4, 1.9, 1.2 and 1.7 -fold, respectively, over the levels observed with CT alone.

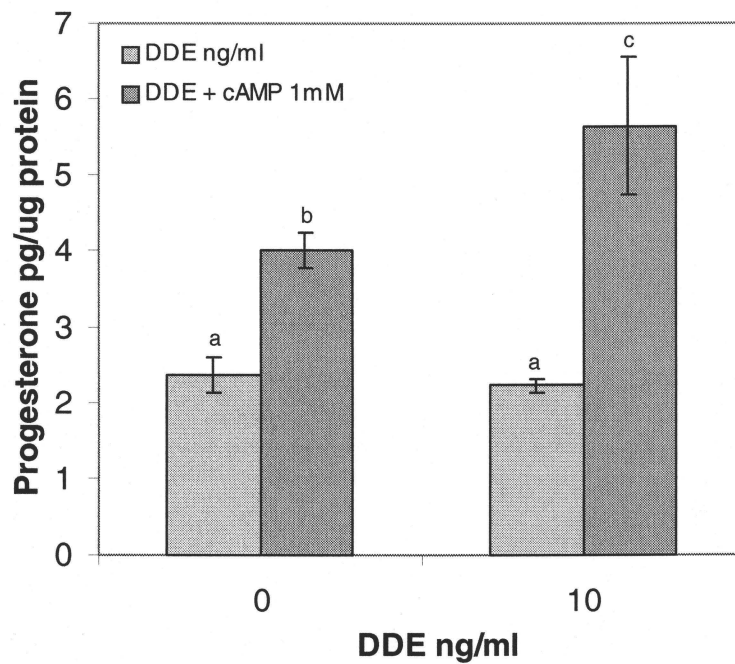


Figure 3: Effect of DDE on 8-Br-cAMP-stimulated progesterone accumulation in JC-410 cells.

Cells were cultured with 10 ng/ml DDE, in the presence or absence of 1mM 8-Br-cAMP (cAMP) for 24 hours. Each bar represents the mean \pm SEM of four independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

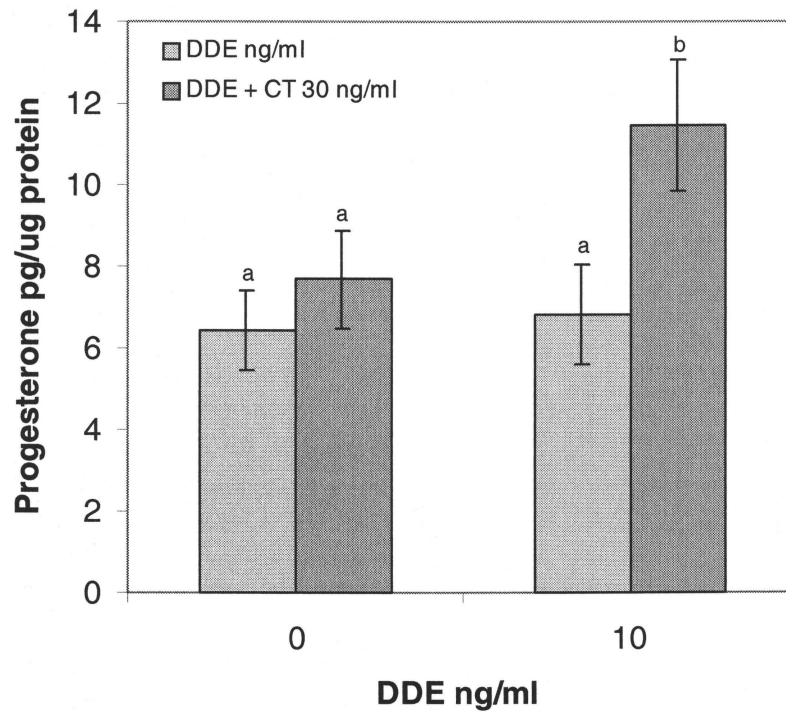


Figure 4: Effect of DDE on CT-stimulated progesterone accumulation in JC-410 cells.

Cells were cultured with 10 ng/ml DDE, in the presence or absence of 30 ng/ml CT for 24 hours. Each error bar represents the mean \pm SEM of four independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

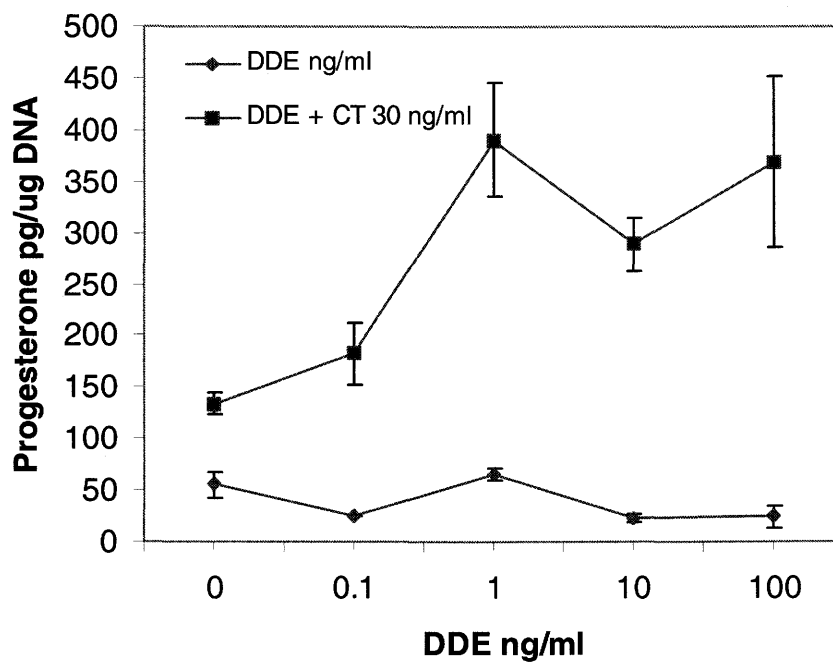


Figure 5: Effect of DDE and CT on progesterone accumulation in primary porcine granulosa cells.

Cells were cultured with increasing concentrations of DDE in the presence or absence of 30 ng/ml CT for 24 hours. Figure is a single representative experiment. Each point represents the mean \pm SEM of four wells.

7.2 Effect of 10 ng/ml DDE on CT-stimulated cAMP production

Figure 6 depicts the effect of DDE on CT-stimulated cAMP production in the JC-410 cells. Addition of 10 ng/ml DDE had no effect on basal cAMP production. Intracellular levels of cAMP were stimulated 6-fold by the addition of 30 ng/ml CT to the cultures. No difference in the CT-stimulated cAMP levels was observed by the addition of DDE.

7.3 Northern Blot Analysis of the Potentiating Effect of DDE on P450scc and 3 β -HSD gene expression

The effect of DDE on the expression of the P450scc and 3 β -HSD genes in the JC-410 cells is depicted in Figure 7. Expression of the P450scc and 3 β -HSD genes was not altered by DDE alone. The presence of 30 ng/ml CT increased mRNA levels of P450scc and 3 β -HSD 26- and 4-fold, respectively. Addition of 1 and 10 ng/ml DDE potentiated the CT-stimulated increase in P450scc mRNA levels 0.3- and 0.4-fold, respectively, over the levels observed with CT alone. The CT-stimulated 3 β -HSD mRNA levels were not changed by DDE. Similar results were observed in 3 repetitions of the experiment.

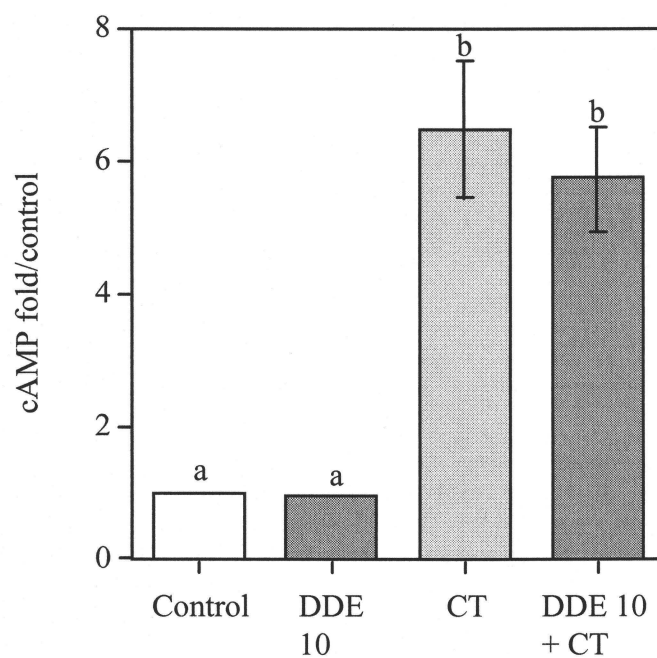
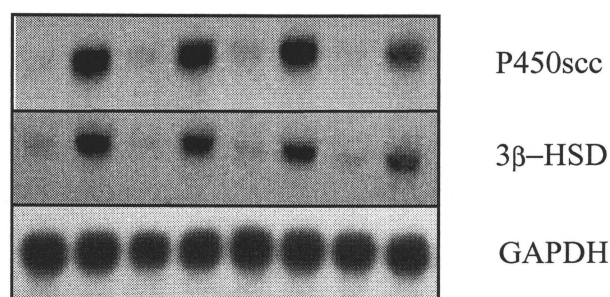


Figure 6: Effect of DDE on CT-stimulated cAMP production in the JC-410 cells.

Cells were cultured with 10 ng/ml DDE for 24 hours, then 30 ng/ml CT was added for 30 minutes. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

A



B

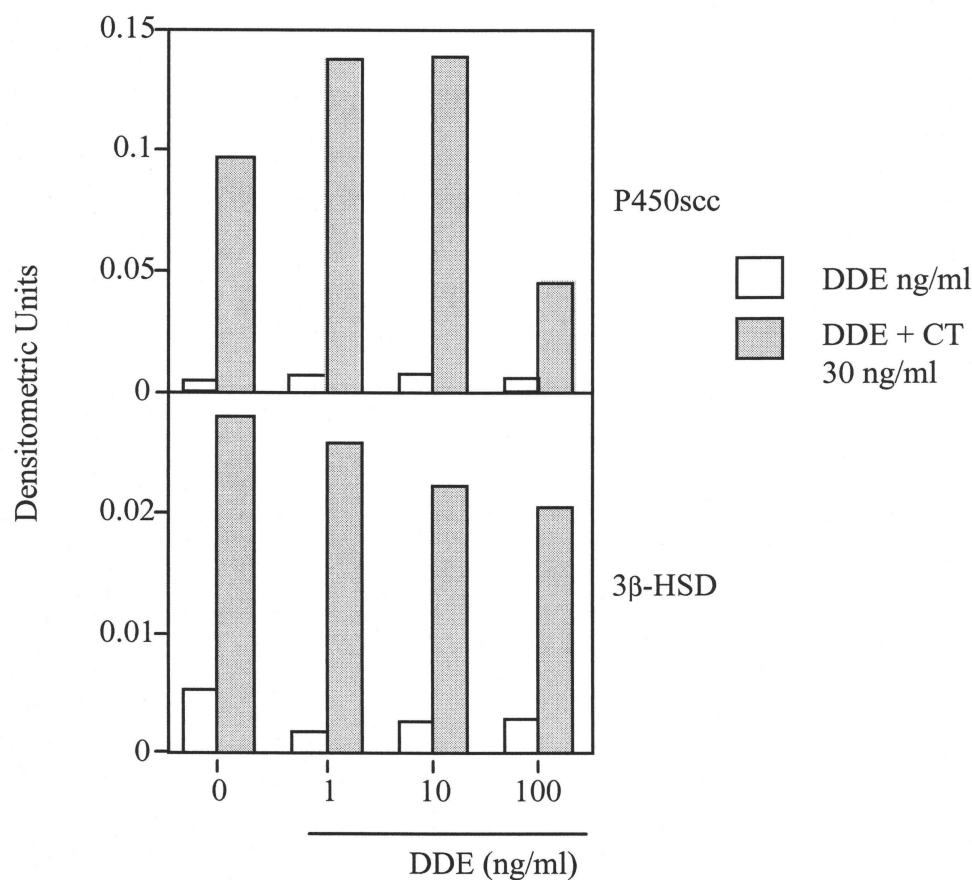


Figure 7: Effect of DDE on the expression of P450scc and 3β-HSD genes in JC-410 cells.

Cells were cultured with 0.1-100 ng/ml DDE in the presence or absence of 30 ng/ml CT for 24 hours. Total RNA was extracted and analyzed by Northern blot. Panel A is an autoradiograph of a representative experiment. Panel B is a graph of densitometric analysis of the autoradiographs from the Northern hybridization described in Panel A. Values graphed are the ratio of P450scc or 3β-HSD over GAPDH.

7.4 Inhibitory Effect of DDE on Basal and PKA Activator-Stimulated Progesterone Synthesis

Figure 8 and Figure 9 depict the effect of higher concentrations of DDE than those used in previous experiments on basal and CT-stimulated progesterone synthesis in the JC-410 cells. Addition of 3000 and 10000 ng/ml DDE decreased basal progesterone synthesis 0.51 and 0.75-fold, respectively (Figure 8). Progesterone synthesis was stimulated 0.7-fold by the addition of 100 ng/ml CT to the cultures (Figure 9). The addition of DDE 300, 1000, 3000, and 10000 decreased CT-stimulated progesterone levels by 0.32, 0.54, 0.66, and 0.84-fold respectively, as compared to CT alone (Figure 9).

7.5 Inhibitory Effect of DDE on Basal and PKA Activator-Stimulated cAMP production

As part of the mechanistic investigation, the effect of high concentration DDE on basal and CT-stimulated cAMP production was studied, as depicted in Figure 10 and Figure 11. Addition of 3000 and 10000 ng/ml DDE decreased basal cAMP production 0.33 and 0.64-fold, respectively (Figure 10). Intracellular levels of cAMP were stimulated 6.2-fold by the addition of 100 ng/ml CT to the cultures. The addition of DDE 300, 3000, and 10000 decreased CT-stimulated cAMP levels by 0.16, 0.32, and 0.48-fold respectively, as compared to CT alone (Figure 11).

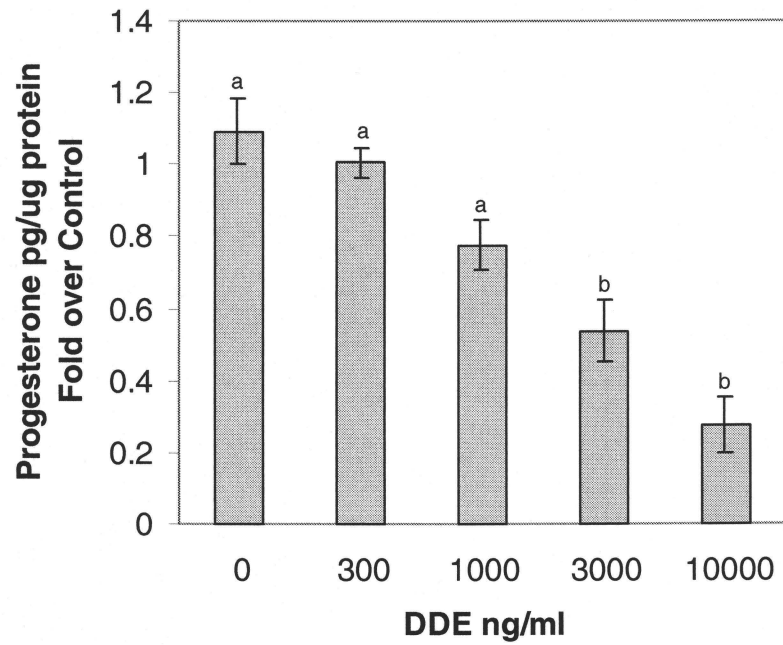


Figure 8: Effect of high concentration DDE on basal progesterone synthesis in the JC-410 cells.

Cells were cultured with DDE for 24 hours. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

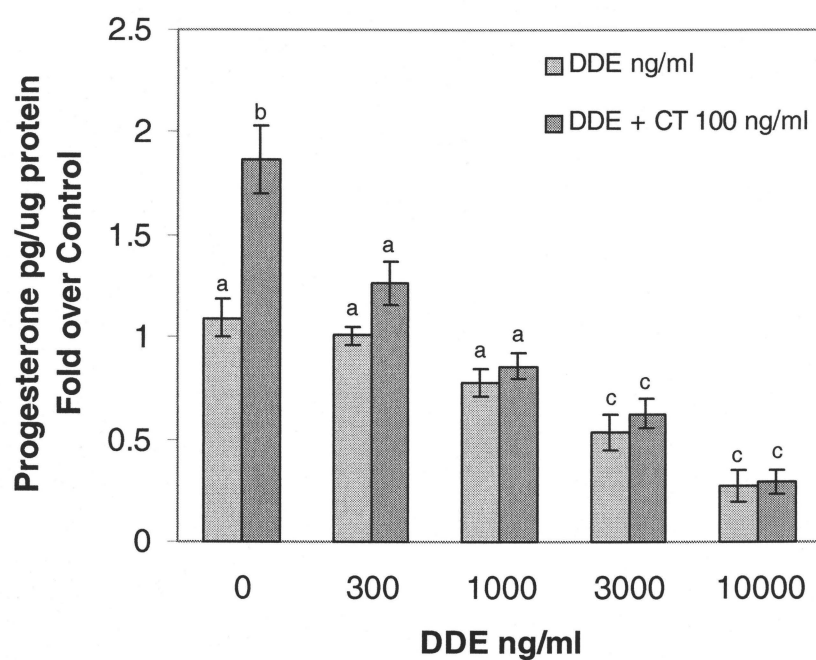


Figure 9: Effect of high concentration DDE on CT-stimulated progesterone synthesis in the JC-410 cells.

Cells were cultured with DDE and CT 100 ng/ml for 24 hours. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

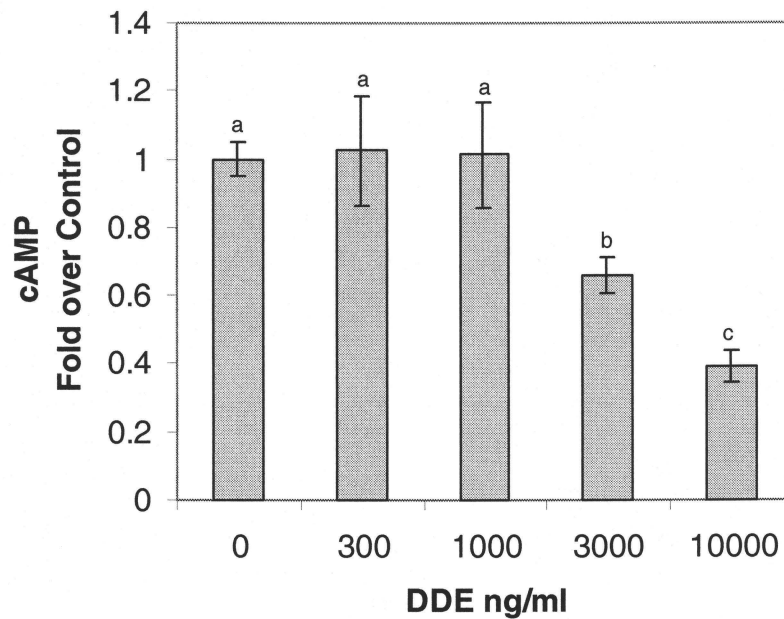


Figure 10: Effect of high concentration DDE on basal cAMP production in the JC-410 cells.

Cells were cultured with DDE for 24 hours. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

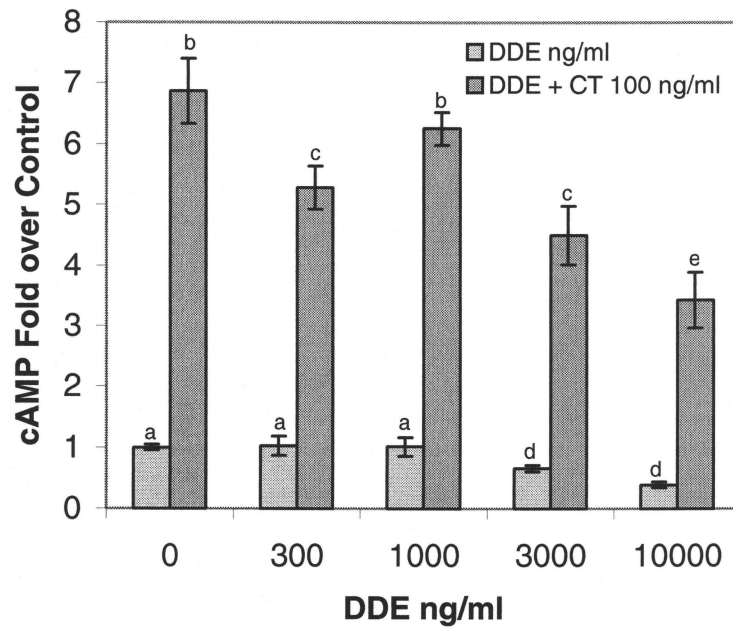


Figure 11: Effect of high concentration DDE on CT-stimulated cAMP production in the JC-410 cells.

Cells were cultured with DDE for 24 hours, then 100 ng/ml CT was added for 30 minutes. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

7.6 Stimulatory Effect of DDE on Protein Levels

Cellular protein levels were quantified as an indicator of cellular density. Figure 12 depicts the effect of high concentrations of DDE on basal and CT-stimulated protein levels in the JC-410 cells. Addition of 3000 and 10000 ng/ml DDE increased basal protein levels 0.35 and 0.42-fold, respectively. The addition of 100 ng/ml CT to the cultures did not alter protein levels.

7.7 Northern Blot Analysis of the Inhibitory Effect of DDE on P450scc gene expression

As part of the mechanistic investigation, the effect of DDE on the expression of the P450scc genes in the JC-410 cells is depicted in Figure 13. Expression of the P450scc gene was decreased 0.61- and 0.82-fold by 3000 and 10000 ng/ml DDE, respectively. The presence of 100 ng/ml CT increased mRNA levels of P450scc 0.47-fold. Addition of 3000 and 10000 ng/ml DDE inhibited the CT-stimulated increase in P450scc mRNA levels 0.31- and 0.67-fold, respectively, as compared to CT alone. Similar results were observed in 3 repetitions of the experiment.

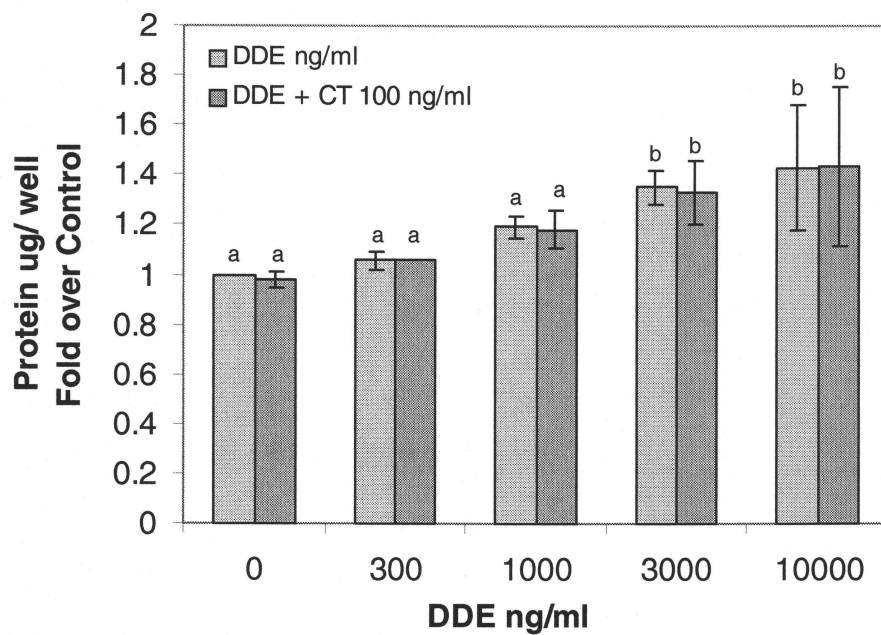


Figure 12: Effect of high concentration DDE on protein levels in the JC-410 cells.

Cells were cultured with DDE \pm 100 ng/ml CT for 24 hours. Each bar represents the mean \pm SEM of three independent replications. Different letters indicate significant differences from control groups. Data was analyzed by a 2-way ANOVA, $P < 0.05$.

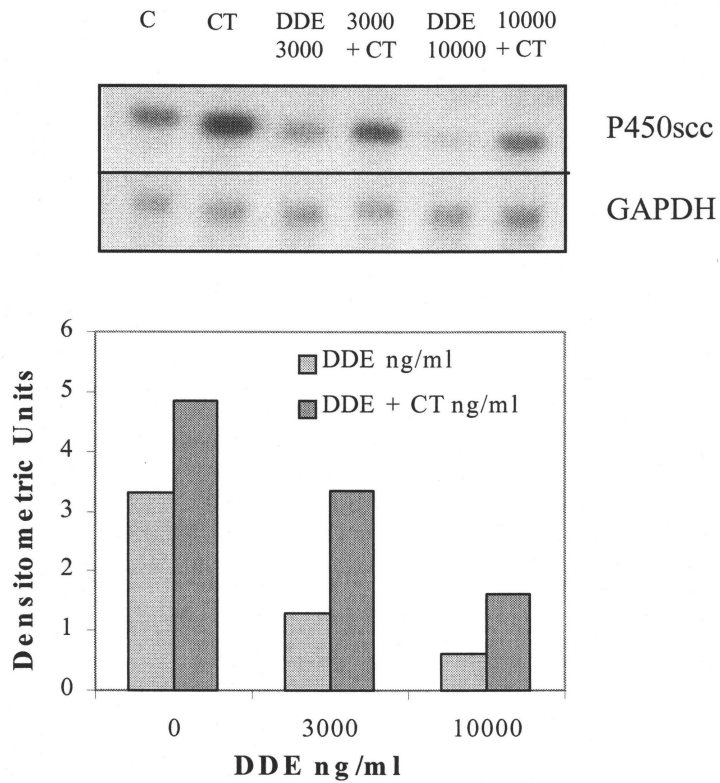


Figure 13: Effect of high concentration DDE on the expression of P450scc gene in JC-410 cells.

Cells were cultured with 3000 and 10000 ng/ml DDE in the presence or absence of 100 ng/ml CT for 24 hours. Total RNA was extracted and analyzed by Northern blot. Panel A is an autoradiograph of a representative experiment. Panel B is a graph of densitometric analysis of the autoradiographs from the Northern hybridization described in Panel A. Values graphed are the ratio of P450scc over GAPDH.

8.0 Discussion

The use of *in vitro* toxicity models has become increasingly popular in reproductive toxicology. While there is a general agreement that *in vitro* systems will never completely replace *in vivo* experiments, *in vitro* models have been recognized as providing valuable information of the site and mechanism of action of toxic agents, thus increasing confidence in *in vivo* data (Schwetz, 1993). Primary porcine granulosa and human luteinized granulosa cells in culture have been used to test the toxic potential of xenobiotics on reproduction (Haney et al., 1984; Treinen et al., 1990; Treinen et al., 1993; Enan et al., 1996; Xu et al., 1997). Here we report the validation of a stable, spontaneously immortalized granulosa cell line as a model for the study of environmental endocrine disrupters. In the present study we used JC-410 cells and primary cultures of porcine granulosa cells to investigate the effect of DDE on progesterone synthesis. The JC-410 cell line is a stable porcine steroidogenic granulosa cell line that responds to the PKA activators, 8-Br-cAMP and CT, with elevations in progesterone synthesis similar to those seen in primary cultures of granulosa cells (Chedrese et al., 1990; Chedrese et al., 1998; Rodway et al., 1999). The use of this cell line has permitted investigation into the mechanism of action of DDE.

Addition of 10 ng/ml DDE potentiated PKA pathway activator-stimulated progesterone synthesis in the JC-410 cells. In primary cultures of porcine granulosa cells, DDE also potentiated the effect of PKA activators. Moreover, primary cultures appeared to be more sensitive to the potentiating effect of DDE on PKA pathway

activator-induced progesterone synthesis than the JC-410s. Potentiation of PKA pathway activator-stimulated progesterone synthesis in primary cultures was observed at 0.1 ng/ml DDE, while in the JC-410 cells, potentiation was observed at concentrations greater than 1 ng/ml DDE. The potentiating effect of DDE on PKA activator-stimulated progesterone synthesis was not a result of an augmentation of cAMP synthesis. Addition of 10 ng/ml DDE, a dose that maximally potentiated the effect of CT on progesterone synthesis, did not change CT-stimulated cAMP levels. The observed changes in progesterone synthesis following exposure to low concentrations of DDE corresponded to changes in the levels of P450scc mRNA, while no effects were observed on 3 β -HSD mRNA levels.

These observations suggest that DDE, at both low and high concentrations, altered P450scc gene expression, thus affecting an obligatory and regulated step of steroid synthesis in the ovary (Richards, 1994). It must be said that as the values presented in the northern blots are a ratio of P450scc/GAPDH, a change in GAPDH expression could be misinterpreted as altered P450scc gene expression. For example, if DDE decreased GAPDH expression, the value of P450scc/GAPDH would increase. However, this is unlikely to be the cause of the observed altered P450scc gene expression, as GAPDH levels have not been affected following exposure to a wide range of concentrations of DDE.

The addition of 10 ng/ml DDE had no effect on CT-stimulated cAMP synthesis, indicating that low concentrations of DDE are not affecting G-protein activity. Therefore, DDE in low concentrations mediates its effects on progesterone synthesis by modulating expression of the P450scc gene and not by affecting the generation of cAMP. In comparison, high concentrations of DDE affected basal and CT-stimulated

cAMP generation, as well as P450scc mRNA levels. Thus, it appears that the inhibitory effect of high concentration DDE on progesterone synthesis is mediated through cAMP generation. However, high concentrations of DDE only decreased the CT-induced stimulation of cAMP generation, and did not completely block it. The effect on CT-stimulated P450scc levels was much more pronounced, completely blocking the stimulatory effect of CT. Therefore, it is likely that high concentrations of DDE also act directly at the level of P450scc gene expression.

The opposite effects observed following exposure to low and high concentrations of DDE were probably because the potentiating effect was masked by the inhibitory effect, since the inhibitory effect occurs upstream in the signal cascade. Exposure to 3000 and 10000 ng/ml DDE inhibited basal cAMP production, while CT-induced stimulation of cAMP production was blocked by 300-10000 ng/ml DDE. However, the addition of 10 ng/ml DDE had no effect on basal or CT-stimulated cAMP production. Alterations in SCC gene expression corresponded well with changes in progesterone synthesis for all concentrations of DDE studied. Thus, it is likely that the inhibitory effect of high concentration DDE on cAMP production overwhelms the potentiation of progesterone synthesis observed with lower concentrations of DDE.

Further investigations revealed an effect of high concentration DDE on protein levels in the JC-410 cells. Exposure to 3000 and 10000 ng/ml DDE increased protein levels, in a fashion that appears to be independent of the presence or absence of the PKA pathway activator, CT. It has been previously observed that estrogen-responsive cells will increase their proliferation rate following exposure to estrogens (Soto et al., 1995). DDE has been described as a weak estrogen, capable of binding to the estrogen receptor (Coldham et al., 1997; Andersen et al., 1999). The JC-410 cells are estrogen-responsive,

with an increase in progesterone synthesis occurring following exposure to 17- β -estradiol (Rodway et al., 1999). It may be that the increase in protein observed after exposure to 3000 and 10000 ng/ml DDE is a result of increased cellular proliferation induced by the estrogenic activity of DDE. However, quantification of protein levels is a very rough tool for measuring cellular proliferation. Protein levels could be increasing in a fashion completely independent of division rate. Nevertheless, it is an interesting observation that may be explained by the estrogenicity of DDE.

The first set of experiments, those examining the potentiating effect of DDE on PKA activator-stimulated progesterone synthesis, (Figure 3-7) used a concentration of 30 ng/ml CT for stimulation. Although stimulation in progesterone synthesis was observed at this concentration, it was not statistically significant. A large stimulatory effect, however, was observed on both P450scc and 3 β -HSD gene expression at this concentration. For the next set of experiments, those examining the inhibitory effect of DDE on basal and CT-stimulated progesterone and cAMP production (Figure 8-13), the concentration of CT was increased to 100 ng/ml. This concentration increase was sufficient to produce a statistically significant stimulation in progesterone production. However, the increase in P450scc gene expression was not as large as that observed with 30 ng/ml CT.

Although the concentrations of DDE required to inhibit basal and CT-stimulated progesterone synthesis are outside of the range reported present in ovarian follicular fluid, they are still environmentally relevant in countries where DDT is still in use. In Mexico, serum lipid concentrations of DDE from a sampling of the population ranged from 10.24 - 4661.4 ppb, with a mean of 562.48+ 676.18 ppb (Lopez-Carrillo et al., 1997). In Kazakhstan, school children were found to have serum DDE levels ranging

from 183 - 4927 ppb, with a mean of 1078 ppb (Mazhitova et al., 1998). Serum and follicular fluid concentrations of DDE are correlated, although follicular fluid concentrations are on average slightly less than serum concentrations (Jarrell et al., 1993). Statistically significant inhibition of basal progesterone synthesis was first observed at 3000 ng/ml (3000 ppb) DDE, although a trend towards inhibition was apparent at 1000 ng/ml (1000 ppb) DDE. These concentrations are in the upper range of those reported in serum (Lopez-Carrillo et al., 1997; Mazhitova et al., 1998). However, the CT-induced stimulation of progesterone synthesis was blocked by lower concentrations of DDE, 300-10000 ng/ml (300-10000 ppb). This effect is thus perhaps more relevant because it is reasonable to believe people might be exposed to these levels of DDE. As well, experiments investigating the potentiating effect of DDE in primary granulosa cells found that they were more sensitive than the JC-410 cells, since a response was first observed at a lower concentration of DDE. Thus, it may be that lower concentrations of DDE are required to produce a similar response in humans.

The presence of DDE in follicular fluid raises some questions. How does DDE become a constituent of follicular fluid? Follicular fluid is believed to originate primarily from the peripheral plasma, and enters by transudation across the follicle basement lamina (Hafez, 1993). It is then modified by the metabolic activity of follicular cells, thus containing specific steroids and glycoproteins (Hafez, 1993). An equilibrium must then form between follicular fluid and serum, as metabolite composition and concentrations are similar. It is therefore probable that DDE enters the follicular fluid passively complexed to serum lipids, since its concentration in follicular fluid has been found to be similar to that in serum (Jarrell et al., 1993). However, as mentioned previously, DDE binds to a number of receptors expressed in the granulosa

cells. These include the estrogen, progesterone, androgen, and glucocorticoid receptors. It is possible that DDE binds to one of these receptors, and is then selectively taken up into the follicular fluid. This would likely result in higher levels of DDE in follicular fluid than in serum. As the opposite has been demonstrated to occur, it is likely that DDE is present in the follicular fluid due to passive diffusion.

It is interesting to note that an inhibitory effect similar to that of DDE was observed with methoxychlor, a DDT analog and potent endocrine disrupter currently registered for use in Canada. In recent studies in our laboratory, we observed a decrease in basal progesterone production in the JC-410 cells following exposure to 1000 ng/ml methoxychlor. As well, 100 and 1000 ng/ml methoxychlor blocked CT-induced stimulation of progesterone synthesis. However, no changes in basal or CT-stimulated cAMP production were observed following exposure to 1-1000 ng/ml methoxychlor. Methoxychlor also had no effect on basal or CT-stimulated P450_{scc} mRNA levels. It is possible that methoxychlor could affect StAR expression or a regulatory enzyme distal to P450_{scc}. Thus we can conclude that although high concentration DDE and methoxychlor appeared to have the same effect on progesterone synthesis, this effect occurred by differing mechanisms of action.

This is an important observation because it may indicate a potential pattern of effect between organochlorine pesticides. This is in keeping with predictive systems based on structure, called Structure Activity Relationships (SARs). This model holds that compounds sharing structural similarities will behave in similar ways in biological systems. While not completely accurate, this model does hold for certain chemical families, and serves to provide predictive information regarding effect and potency. What is interesting is that although our studies showed a similar effect between DDE

and methoxychlor on progesterone synthesis, the mechanism differed. Considering that DDT analogs such as methoxychlor are still in use, this mechanistic information may be valuable for regulatory purposes.

Collectively, it may be said that DDE affects granulosa cell steroidogenesis, at concentrations present in the environment. Therefore, the next point to be addressed is the relevance of these observations to human health. Extrapolation from an animal model to humans introduces a factor of uncertainty, and this uncertainty is compounded exponentially when trying to extrapolate from an *in vitro* animal cell line model to humans. There are a huge number of factors at work *in vivo* that can never be replicated *in vitro*. These include absorption, biotransformation, protein binding, sequestration, and enzyme induction. For example, a chemical that had an effect in an *in vitro* model could be of absolutely no consequence in an *in vivo* situation because it might be metabolized into an inert compound before it reached its target site.

However, there are good reasons why the results obtained from our model are relevant *in vivo*. Most obvious of these is the observation that DDE is present in human follicular fluid. Ovarian granulosa cells are thus exposed to DDE in much the same way that the granulosa cells used in the reported experiments were. Also of interest is the observation that DDE levels have been correlated with decreased rates of conception in women (Gerhard and Runnebaum, 1992). It has been reported that DDE was present in the follicular fluid of women receiving *in vitro* fertilization therapy (Jarrell et al., 1993). Levels between 0.61 ± 0.47 and 1.07 ± 0.84 ppb (0.61 ± 0.47 and 1.07 ± 0.84 ng/ml) of DDE were found in the follicular fluid of women undergoing in vitro fertilization (Jarrell et al., 1993). In the present study, 10 ng/ml (10 ppb) DDE was required to potentiate PKA activator-stimulated progesterone synthesis in JC-410 cells. This concentration is

in the range of levels reported in human follicular fluid (Jarrell et al., 1993). Therefore, it is reasonable to speculate that concentrations of DDE that appear to have no effect on oocyte viability, may affect fertility by altering granulosa cell steroidogenesis and the endocrine milieu that surrounds the oocyte. Although the causes of infertility were not reported, when considered with the previous observation it is possible that DDE may have contributed to their infertility. Correlation does not necessarily imply causation. However, these observations do provide some evidence that DDE may be affecting human reproductive health, and thus the results of this study are relevant and applicable.

Alligators hatched from eggs laid in an environment highly polluted with DDT and its metabolites, including DDE, had increased plasma estradiol levels and ovaries with prominent polyovulatory follicles and multinucleate oocytes (Guillette Jr. et al., 1995). In vitro cultures of ovaries from these exposed alligators produced lower basal estradiol compared with ovaries from control animals. However, LH stimulated estradiol production to a much greater magnitude than observed in ovaries from control alligators (Guillette Jr. et al., 1995). The effect of LH on steroid synthesis is mediated by an elevation in the levels of cAMP and stimulation of the PKA pathway. We have observed that low concentrations of DDE potentiate the effect of PKA activators on progesterone synthesis. Therefore, it is possible that a common stimulatory mechanism may mediate the effects of low concentration DDE on PKA-activator induced steroid synthesis in the ovary. This provides further evidence that the observations derived from our *in vitro* model are applicable to an *in vivo* situation.

It is known that the concentration of FSH that the ovaries are exposed to is critical to the selection of dominant follicles. It has been suggested that one of the characteristics of the dominant follicle is that it is most efficient at making use of small

amounts of FSH during the follicular stage of the cycle (Goodman, 1994). Increasing FSH levels experimentally results in more than one follicle growing and becoming ready for ovulation. This is a possible outcome of exposure to low concentrations of DDE, such as those observed present in the follicular fluid. DDE may amplify the signal cascade induced by FSH, thus magnifying the effect of FSH. This could conceivably result in multiple ovulations, thus increasing the chances for multiple births. Although the frequency of multiple births has certainly increased, this is considered a result of the increased use of fertility treatments. As yet, there is no evidence that links exposure to low levels of contaminants such as DDE to an increased frequency of multiple births.

Alternatively, higher concentrations of DDE, sufficient to block stimulated progesterone synthesis without affecting basal levels, could reduce the effect of FSH by partially blocking its signal cascade. This could result in a higher percentage of anovulatory cycles, producing a subtle yet detrimental effect on fertility.

Progesterone synthesis increases dramatically in granulosa cells just before ovulation. It is believed that this progesterone surge has a facilitative role in ovulation, allowing proteolytic enzymes to weaken the follicular wall (Tanaka et al., 1993). If progesterone synthesis is blocked, ovulation is prevented. Therefore, levels of DDE high enough to decrease basal progesterone synthesis in granulosa cells could prevent ovulation *in vivo*.

Regulation of steroid hormone production is critical for reproductive health and fertility, but it also has important roles throughout the body. For example, there are progesterone receptors in the brain, and it is believed that the Schwann cells produce progesterone to aid myelination (Koenig et al., 1995). It is believed that progesterone is not only a sex steroid, but also an important neurosteroid (Koenig et al., 1995).

Progesterone synthesis is tightly regulated within the ovary. This regulation is achieved through a combination of factors, including gonadotropins, the PKA pathway, and expression of genes involved in steroidogenesis. Progesterone is involved in ovulation and follicular development, and is required for the maintenance of pregnancy (Curry Jr and Nothnick, 1996). Progesterone also has effects on the uterus.

Previous research has established that DDE can bind to the progesterone receptor of several different species. Experiments conducted by Klotz et al., 1997, have demonstrated that some DDT isomers and metabolites can block progesterone-induced reporter gene activity (Klotz et al., 1997). It was observed that *o,p'*-DDE appeared to act consistently in both yeast and mammalian cells, indicating solely progesterone receptor dependent activity. However, *p,p'*-DDE was more active in mammalian cells than in yeast cells, yet did not compete for binding to the progesterone receptor in mammalian cells (Klotz et al., 1997). The authors of this study interpreted the results to mean that inhibition of progesterone-dependent responses may occur through both progesterone receptor dependant and progesterone receptor-independent pathways (Klotz et al., 1997). Thus *p,p'*-DDE was considered to be more active in mammalian cells than yeast cells due to activation of other pathways specific to mammalian cells (Klotz et al., 1997).

Research within our laboratory has found that exposure to progesterone and levonorgestrel (LNG), an artificial progestin, stimulates progesterone synthesis in the JC-410 cells (Rodway et al., 1999). This information supports the Rothchild hypothesis that progesterone is an autocrine regulator of its own synthesis (Rodway et al., 1999). However, recent studies with the progesterone receptor blocker RU486 have demonstrated that RU486 does not block LNG-stimulated progesterone synthesis in the JC-410 cells. This may therefore indicate that LNG-stimulated progesterone synthesis is

not classically progesterone receptor mediated. Other possible pathways include a cell surface-bound progesterone receptor, the presence of different progesterone receptor isoforms, or a non receptor-mediated pathway.

The classical understanding of a steroid receptor describes the receptor as located intracellularly, usually within the nucleus. Following ligand binding the receptor undergoes a conformational change, and binds to a hormone-specific response element on the DNA. Binding of the receptor-ligand complex then initiates transcription and may result in translation. The DDT metabolite *p,p'*-DDE has been reported to act as an estrogen (Clark et al., 1998), an anti-estrogen (Vonier et al., 1996), an anti-androgen (Danzo, 1997), an anti-progestin (Lundholm, 1988; Klotz et al., 1997), and an anti-glucocorticoid (Lundholm, 1991) depending upon the system studied. We previously reported that the JC-410 cells responded to estrogens, androgens, and progestins with an increase in progesterone synthesis (Rodway et al., 1999; Rodway et al., 1999). Thus, it is likely that estrogen, androgen, and progesterone receptors are present in these cells. The effect of DDE may therefore be mediated via these receptors. However, since there is no endogenous androgen or estrogen production in the JC-410 cells (Rodway et al., 1999) it is unlikely that DDE acts as an anti-estrogen or anti-androgen.

The existence of cell membrane-bound steroid hormone receptors has recently been proven. Membrane bound estrogen receptors have been described, and in human granulosa cells the presence of a membrane bound progesterone receptor has been reported (Peluso and Pappalardo, 1999). As yet, no pathway has been described by which these receptors exert their effects, although it has been suggested that a hormonal signal cascade may be involved (Peluso and Pappalardo, 1999). It is possible that the

PKA pathway could be involved, as second messenger cascades are a common method of translating messages from the cell surface to the target site.

Thus it can be summarized that *p,p'*-DDE has been demonstrated to inhibit progesterone-dependent responses through a non progesterone receptor-mediated pathway; and LNG may possibly stimulate progesterone synthesis through a non-progesterone receptor mediated pathway. Although pure speculation, it is possible that these pathways may be the same, or at least related through modulation of cAMP generation. If this were the case, then it is possible that DDE could interfere with the autocrine regulation of progesterone synthesis in the body.

Another possible mechanism for the action of DDE could be the presence of a receptor specific to DDE. It has been recently demonstrated that methoxychlor, an estrogenic pesticide, continues to exert an estrogenic action even in mice that do not express estrogen receptors (Ghosh et al., 1999). It was suggested that methoxychlor therefore acts via a non-estrogen receptor mediated pathway. It is possible that methoxychlor might bind to a different receptor, the so-called 'methoxychlor receptor' (Ghosh et al., 1999). This is an interesting speculation. A family of receptors exists called orphan receptors. Dioxin, an extremely toxic chemical, enters the body via selective binding to a receptor in this family, the Ah (aryl hydrocarbon) receptor. No physiological ligand has been identified for this receptor. Thus, it is possible that although DDE has been demonstrated to bind to and agonize/antagonize a number of receptors, it may actually be exerting its effect(s) through an as-yet unidentified 'DDE receptor'.

Overall, it may be concluded that the JC-410 cell line is a valuable *in vitro* model, particularly for the study of environmental endocrine disrupters. This cell line can be

used to examine parameters of cell growth, such as proliferation or cytotoxicity. As well, functional endpoints can be assessed, such as basal progesterone production. The use of PKA pathway activators allows for the assessment of more subtle functional endpoints, such as the interpretation of gonadotropin signals. Experiments with primary granulosa cells confirm observations from the JC-410 cell line. As well, use of the JC-410 cell line appears to avoid the variability present with primary cultures.

This research has identified the effect(s) of a persistent endocrine disrupter on granulosa cell steroidogenesis, as well as on granulosa cell growth. Overall, DDE disrupts basal and stimulated progesterone synthesis in a dose dependent manner. The observation that DDE at some concentrations can inhibit stimulation of granulosa cell steroidogenesis without affecting basal steroidogenesis is of particular concern because it is a very subtle effect. It is possible that endocrine disrupters such as DDE could be disrupting gonadotropin control of ovulation, potentially becoming a causal agent in some idiopathic infertilities.

As well, this research has contributed information that begins to elucidate the mechanism of action of this reproductive toxin. The ovarian contaminant DDE alters cAMP generation and affects P450_{scc} gene expression. This mechanistic information is of particular value because structural analogs of DDT continue to be widely used, and thus may have similar effects.

In summary, it may be said that this research has met its objectives, by demonstrating the value of the *in vitro* model used, describing the disruptive effects of a common environmental contaminant on ovarian steroidogenesis, and identifying a plausible mechanism of action for this chemical.

9.0 Conclusions

1. The JC-410 cell line is a valid and practical *in vitro* model for the study of endocrine disrupters.
2. At low concentrations, DDE potentiates the stimulatory effect of PKA activators on progesterone synthesis in granulosa cells, without affecting basal production. This effect appears to be mediated through P450scc gene expression.
3. At high concentrations, DDE inhibits basal and stimulated progesterone synthesis in granulosa cells. This effect appears to be mediated primarily through alterations in cAMP generation, although a direct effect on P450scc gene expression is also present.
4. At high concentrations, DDE appears to have a proliferative effect on granulosa cell growth.
5. Overall, DDE affects the signal cascade induced by gonadotropins.

Gonadotropin control of steroidogenesis is necessary for oocyte maturation and ovulation; therefore DDE may be a causal factor in some idiopathic infertilities.

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11.0 Appendix

- A. 10 X Mops buffer: 0.2 M [3-(N-morpholino) propanesulfonic acid], 10 mM EDTA pH 7 and 50 mM sodium acetate
- B. RNA loading buffer: 0.75 ml formamide, 0.24 ml formaldehyde, 0.15 ml 10 X MOPS buffer, 0.18 ml H₂O, 0.1 ml glycerol and 0.3mg/ml of bromophenol blue (1 %) Final volume is 1.42 mls
- C. 20 X SSC: 3 M NaCl, 0.3 M trisodium citrate, adjusted to pH 7
- D. 50 X TAE: 242g Tris base, 571 ml glacial acetic acid, 100ml 0.5 M EDTA pH 8, volume correct to 1 L with distilled water
- E. DNA loading buffer: 30% glycerol and 0.25% bromophenol blue in distilled water
- F. 2.5 X RPRB buffer: 0.5M Hepes pH 6.6, 12.5 mM MgCl₂, 25 mM β-mercaptoethanol, 125 mM Tris pH 8.0, 50 μM each dNTP (A, G, T)