

Rapid communication

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ARYL HYDROCARBON RECEPTOR (AhR)-LINKED INHIBITION OF LUTEAL CELL PROGESTERONE SECRETION IN 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN TREATED CELLS

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In this study, we tested firstly, the hypothesis that decrease of progesterone secretion by luteal cells under the influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is due to influence on specific enzymatic steps in the biosynthetic pathway of steroidogenesis and secondly, involvement of aryl hydrocarbon receptor (AhR) or estradiol receptor (ER) in this process. Luteal cells isolated from mature porcine corpora lutea were cultured with 25-hydroxycholesterole (25-OH) or pregnenolone (P5) as a substrate. Additionally aminoglutethimide, the inhibitor of P540_{sc} or trilostane the inhibitor of 3 β -HSD was added to basal and stimulated cells. The synergistic action of TCDD with aminoglutethimide in decreasing of progesterone secretion was observed. In pregnenolone treated cells 1.6 fold decrease of progesterone secretion was observed that in both TCDD alone and together with trilostane treated cells. In the second part of experiments to show the involvement of AhR and ER in TCDD action on progesterone secretion α -naphthoflavone, the AhR blockers and 4-hydroxytamoxifen (4-OH-TMX), the inhibitor of ER were used. α -naphthoflavone, the inhibitory effect of TCDD while 4-OH-TMX had no effect on TCDD-treated cells. These experiments suggest TCDD decreased progesterone secretion by luteal cells by reduction of the activity of mitochondrial enzymes, which converts cholesterol into pregnenolone. Moreover points to AhR dependent but not ER-dependent mechanisms in TCDD action in luteal cells.

Key words: *dioxin, Ah receptor, progesterone secretion, luteal cells*

INTRODUCTION

Organochlorine industrial chemicals have been extensively used in the production of plastics, flame retarding, pesticides, drugs, and a host of other commercial products. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds elicit a diverse spectrum of toxic responses. In our previous data (1) it have been shown that TCDD given to the culture medium accumulate in the

tissue, and influence the cell proliferation and steroids secretion. Enan *et al.* (2) showed a decrease in progesterone production by luteinized granulosa cells after 24 h exposure for 10 nM of TCDD. In another study by Moran *et al.* (3) TCDD was shown to reduce estradiol production by luteinized granulosa cells without an effect on progesterone production. From the our previous data it has been shown that that TCDD added to the culture medium alters steroidogenic function in porcine follicular and luteal cells (4, 5, 6) by acting on steroid secretion. The intercellular mechanisms of the action are only beginning to be understood. Largely the relative levels and tissue-specific array of steroidogenic enzymes expressed at the cellular level regulate steroid production. The complexity of steroidogenesis, comprising many interrelated pathways suggests that regulation may occur at several points.

TCDD exerts its effects and alters the hormonal profile of an organism, in part, by binding to a receptor known as the aromatic hydrocarbon receptor (Ahr), which exists in the cytosol complexes with at least three additional proteins (7). The presence a functional Ahr capable of binding DNA, in the rat ovary and in primate ovarian tissue, including human granulosa cells was showed by Chaffin *et al.* (8, 9). The aim of the presented data was to show firstly, the influence of TCDD on specific enzymatic steps in the biosynthetic pathway of steroidogenesis and secondly to investigate involvement of aryl hydrocarbon receptor (AhR) or estradiol receptor (ER) in this process.

MATERIALS AND METHODS

Chemicals

2,3,7,8-TCDD solutions were prepared by dilution of evaporated, concentrated toluene standard (Promochem) in DMSO. The concentrations of TCDD DMSO solutions were adjusted and confirmed by GC-MS/MS analysis. Medium M199, Penicillin, Trypsin, and Calf Serum (Laboratory of Vaccines, Lublin, Poland). 25-hydroxycholesterol, pregnenolone, aminoglutethimide, 4-hydroxytamoxifen, α -naphthoflavone, was purchased from Sigma Chemical Co., St. Louis, MO, USA. 4 α , 5 α ,17 β)-4,5-epoxy-3, 17 hydroxyandrost-2-ene-2-carbonitrile (trilostane) was a gift from Sanofi Pharmaceuticals (Malvern, PA, USA).

Animals and cell isolation

Ovaries were obtained from Large White sows from a local slaughterhouse immediately after slaughter. The phase of the oestrous cycle was determined according to the established morphological criteria (10). Luteal cells were obtained from pools of freshly existed mature corpora lutea (8–10 days after ovulation) from three animals according to the technique of Gregoraszcuk (11). Cells were suspended in medium M199 supplemented with 5% of calf serum at a concentration of 3.5×10^5 cells/ml medium. Cell viability measured using the trypan blue exclusion test was 85%. Cells were grown in multiwell plates (Nunc) in a humidified atmosphere with 5% CO₂ in the air. At least three different experiments (n=3), each, in triplicate have been done.

Experimental procedure

Experiment 1.

It was conducted to show TCDD action on:

a) cholesterol side chain cleavage cytochrome P450_{sc}. TCDD in a dose 3.2 ng/ml was added to the control and 25-hydroxycholesterol (25-OH) treated cells.

Cells were plated into 24 well plates by 18h incubation with M199 supplemented with 5% of calf serum. After that time 25-OH in a dose 10 µg/ml was added to the control (not TCDD-treated) and TCDD treated cells. Six hours later, all cultures were terminated and the media were frozen until further progesterone analysis. Additionally aminoglutethimide (AMG; 20 µg/ml), the inhibitor of P540_{sc} was added to the control and TCDD-treated cells. The dose of 25-OH and aminoglutethimide was established on the basis of the dose response curve performed during preliminary studies (12).

b) 3β-hydroxysteroid dehydrogenase (3β-HSD), enzymes involved in progesterone secretion. TCDD in a dose 3.2 ng/ml was added to the control and pregnenolone (P5) treated cells.

Cells were plated into 24 well plates by 18h incubation with M199 supplemented with 5% of calf serum. After that time P5 in a 10 µg/ml was added to the control (not TCDD-treated) and TCDD treated cells. Six hours later, all cultures were terminated and the media were frozen until further progesterone analysis. Additionally 100 µM trilostane, the inhibitor of 3β-HSD was added to the control and TCDD-treated cells. The dose of trilostane was established on the basis of our earlier study (13).

Experiment 2.

This experiments was performed to show:

a) the involvement of aryl hydrocarbon receptor (AhR) in TCDD action on luteal cells.

Luteal cells were cultured in M199 medium supplemented with 5% of calf serum as a control medium or with the addition of either TCDD (3.2 ng/ml), or TCDD + α-naphthoflavone (AhR-Inh); 10 µM. α-naphthoflavone, is a widely used inhibitor of aryl hydrocarbon receptors (2, 14, 15).

b) the involvement of estradiol receptor (ER) in TCDD action on luteal cells.

Luteal cells were cultured in M199 medium supplemented with 5% of calf serum as a control medium or with the addition of either TCDD (3.2 ng/ml), or TCDD + 4-OH-tamoxifen (4 OH-TMX); 10⁻⁷ M. The dose of 4-OH-TMX was established on the basis of our earlier study (16).

Progesterone analysis

Progesterone was determined radioimmunologically using Spectra kits (Orion, Diagnostics, Finland), supplied by Polatom (Świerk, Poland). The limit of assay sensitivity was 94 pg/ml. The coefficients of variation between and within assays were 5.8% and 2.9% respectively. The mean recoveries were 95.1–103.7%. The cross-reaction with pregnenolone was 2.9%. All other tested steroids (5β-dihydroprogesterone, 20β-hydroxyprogesterone, corticosterone, testosterone, estrone) showed less than 1% cross-reaction.

Statistical analysis

All data points are expressed as means ± SEM, from at least three different experiments (n = 3) each in triplicates. Significant difference between the concentrations of progesterone in the control and experimental cultures were compared by analysis of variance and by using Duncan's new multiple range test.

RESULTS

TCDD added to the culture medium decreased basal (264 ng/ml vs. 475 ng/ml in control culture), 25-OH-stimulated (378 ng/ml vs. 742 ng/ml in 25-OH treated cells) and pregnenolone (P5)-stimulated (3424 ng/ml vs. 5589 ng/ml in P5-treated cells) progesterone secretion by luteal cells. (Fig. 1).

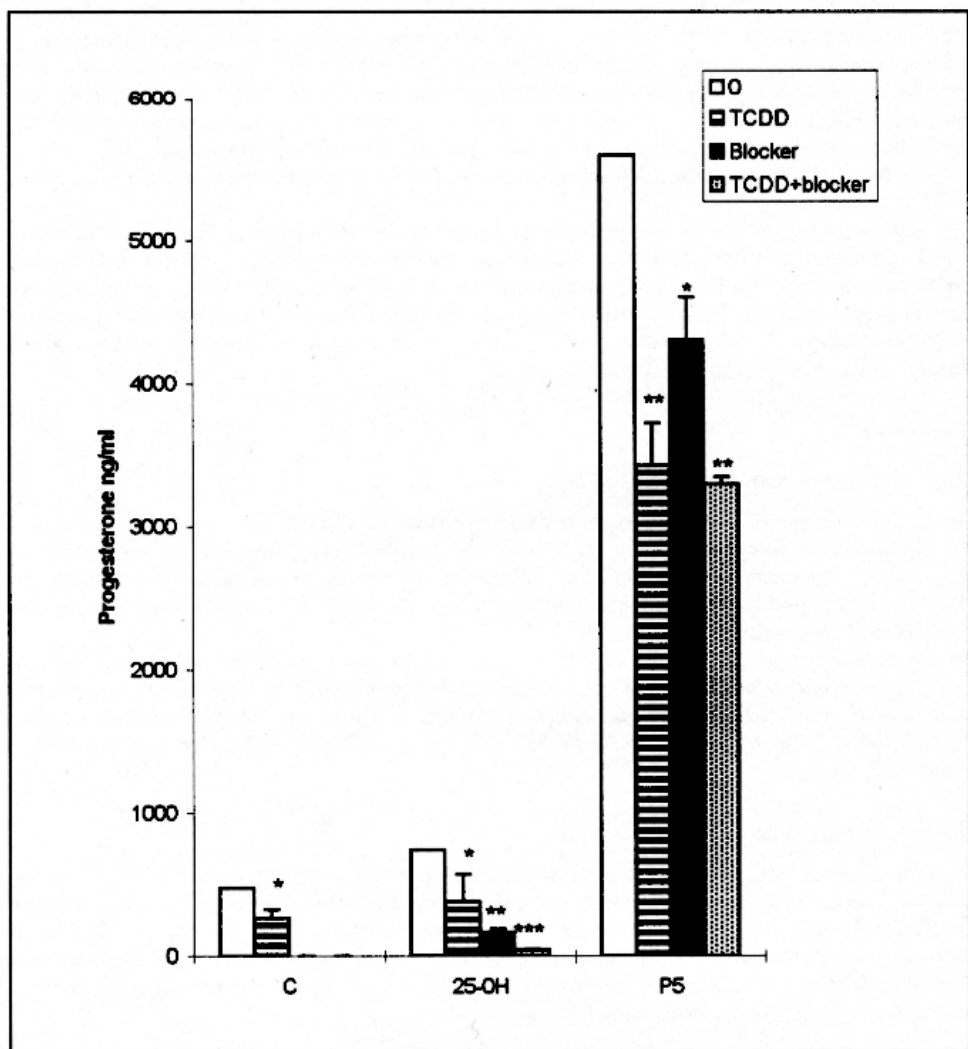


Fig. 1. The influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on control (C), 25-hydroxycholesterol (25-OH) and pregnenolone (P5)-stimulated progesterone secretion by luteal cells. Aminogluthethimide, the inhibitor P450scs was added to the 25-OH-stimulated cells, trilostane, the inhibitor 3β -HSD was added to P5-stimulated cells.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

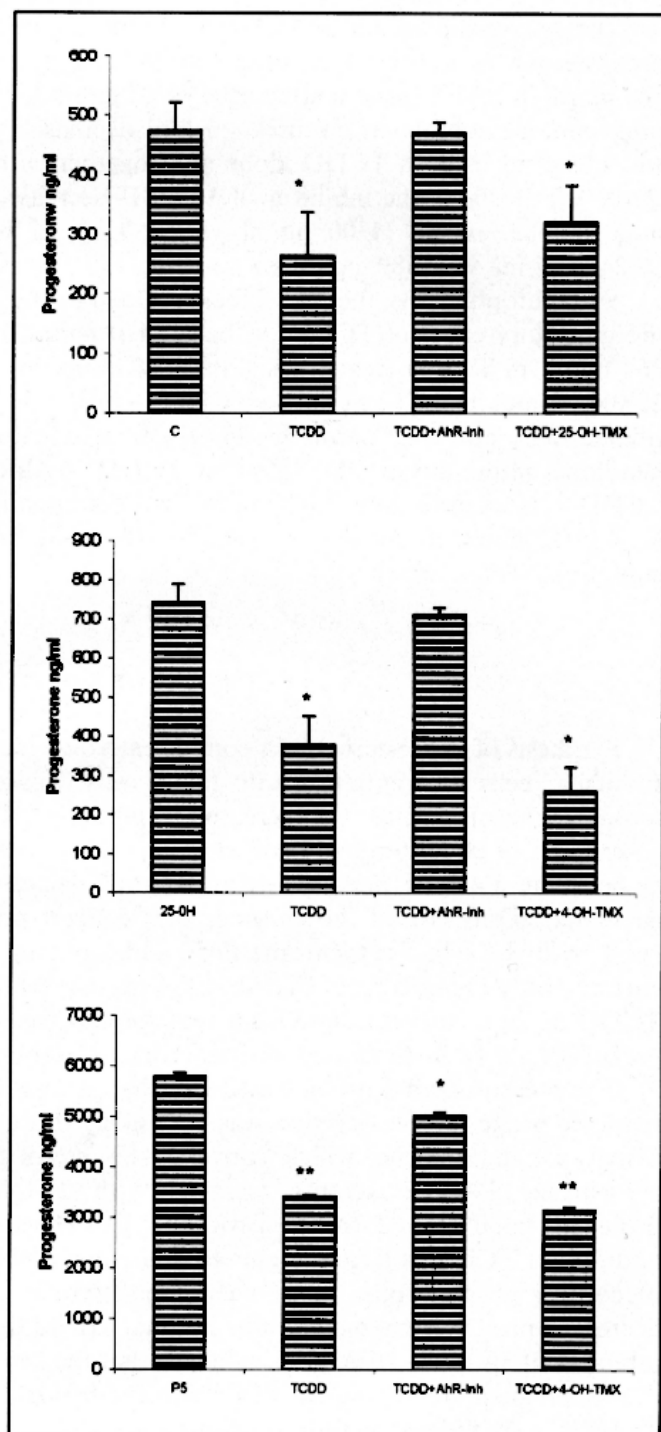


Fig. 2. The influence of α -naphthoflavone, the AhR inhibitor (AhR-Inh) and 4-hydroxy tamoxifen (4-OH-TMX), the inhibitor of ER on a) basal, b) 25-OH-stimulated and c) P5-stimulated progesterone secretion by luteal cells. TCDD -2,3,7,8-tetrachlorodibenzo-p-dioxin; 25OH- 25-hydroxy cholesterol; P5-pregnenolone.

* $p < 0.05$; ** $p < 0.01$;

*** $p < 0.001$.

The synergistic action of TCDD with aminoglutethimide in decreasing of progesterone secretion was observed (44.3 ng/ml in TCDD+AMG vs. 163 ng/ml in AMG alone treated cells vs. 378 ng/ml in TCDD treated cells). In pregnenolone-stimulated cultures 1.6 fold decrease of progesterone secretion was observed in both TCDD alone and together with trilostane treated cells. (Fig. 1). Trilostane, the inhibitor of 3β -HSD decreased progesterone secretion in a smaller extend (4300 ng/ml vs. 5589 ng/ml in control) than TCDD (3424.5 ng/ml vs. 5589 ng/ml in control).

α -naphthoflavone, the AhR blocker added to the culture medium reversed the inhibitory effect of TCDD on basal (470 ng/ml in TCDD + AhR-Inh vs. 264 ng/ml in TCDD treated cells and 474 ng/ml in control culture; Fig. 2a), 25-OH-stimulated (712 ng/ml in TCDD + AhR-Inh vs. 378 ng/ml in TCDD treated cells and 742 ng/ml in 25-OH treated culture; Fig. 2b) and pregnenolone-stimulated (5017 ng/ml in TCDD + AhR-Inh vs. 3422 ng/ml in TCDD treated cells and 5589 ng/ml in P5-stimulated culture; Fig. 2c).

4-OH tamoxifen did not reverse effect of TCDD in all investigated culture conditions. (Figs. 2a-c)

DISCUSSION

Synthesis of progesterone is a complex steroidogenic pathway in the ovary. Follicular cells differentiation into luteal cells capable of producing progesterone is accomplished by increased expression of enzymes necessary for conversion of cholesterol to progesterone, i.e., cholesterol side-chain cleavage cytochrome P450_{scc} and 3β -hydroxysteroid dehydrogenase (3β -HSD), and decreased expression of the enzymes that convert progesterone to estrogens, i.e., 17 α -hydroxylase cytochrome P450 and aromatase cytochrome P450. The current study demonstrates that the exposure of luteal cells to 3.2 ng/ml TCDD in vitro caused a significant reduction of basal, 25-OH-stimulated and pregnenolone (P5)-stimulated progesterone secretion by luteal cells.

The present studies provide evidence that at least of the effect of TCDD in reducing progesterone secretion was very likely through a direct action on the activity of the enzyme, which converts cholesterol to pregnenolone. Luteal cytochrome P450_{scc} activity (assayed with 25-OH as a substrate) was decreased to 50% of control progesterone. This effect was intensifying after the addition of TCDD with aminoglutethimide, the inhibitor of P450_{scc} to 5.9% of control progesterone. In pregnenolone-stimulated cultures TCDD alone decreased progesterone secretion in a higher extend than TCDD trilostane, the inhibitor of 3β -HSD. It suggests evidence that the key lesion occurs prior to or during pregnenolone formation. Inhibition of histochemically tested activity of 3β -HSD was showed in our previous data (17).

The best-characterised mechanism for the action of TCDD involves the recently cloned AhR, a cytosolic receptor that binds TCDD acts as a transcription factor to regulate gene expression. Safe, (18) showed that toxic and biochemical effects associated with exposure to TCDD are mediated via initial binding to the cytosol aryl hydrocarbon (Ah) receptor protein present in target tissues and organs. Chaffin *et al.* (9) detected AhR in whole primates ovaries and isolated corpus luteum. Levels of AhR appear to be lowest in early CL, and increased in mid- and late luteal phase tissues. Studies using α -naphthoflavone, the AhR blocker are currently being conducted to examine the potential involvement of the AhR in TCDD action (2, 14)

The current study demonstrates that the exposure of luteal cells to α -naphthoflavone, the AhR blocker reversed the inhibitory effect of TCDD on basal, 25-OH-stimulated and pregnenolone-stimulated progesterone secretion, indicating the involvement of AhR-mediated mechanism in this process. Taking into consideration data of Umbreit and Gallo, (19) suggesting that TCDD's effects on reproductive functions may occur via modulating estradiol and/or ER depending on the species, tissue and hormonal status of an animal, 4-OH-tamoxifen (TMX), a synthetic ER antagonist that inhibits transcription of target gene binding to ER was used to focused on the effect of TCDD on ER. Caruso *et al.* (20) investigated the 90kDa heat shock protein (HSP90) as a mediator of cross-talk between the Ahr and the ER signal transduction pathways in human breast cancer cells demonstrated that Ah-responsiveness is dependent upon cellular ER content. The present studies showed that 4-OH-tamoxifen did not reverse effect of TCDD in all investigated culture conditions indicating non ER-dependent signalling in TCDD action on luteal cells. This observation is probably due to low expression of ER in luteal cells..

We concluded, that the reduction of progesterone secretion is caused by a reduction in the activity of mitochondria enzymes, which converts cholesterol into pregnenolone and that this reduction in steroid secretion is dependent of the Ah locus but independent of the ER locus. The next study are necessary for investigation cross-talk between the Ahr and the progesterone receptor (PR) signal transduction pathways in luteal tissue

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REFERENCES

1. Grochowalski A, Pieklo R, Gasinska A, Chrzaszcz R. Accumulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in porcine follicles after *in vitro* exposure to TCDD: effects on steroid secretion and cell proliferation. *Cytobios* 2000; 102: 21–31.

2. Enan E, Moran F, Vandervoort CA, Steward DR, Overstreet JW, Lasley BL. Mechanism of toxic action of 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) in cultured human luteinized granulosa cells. *Toxicol* 1996; 10: 497—483.
3. Moran FM, Enan E, Vandervood CA, Seward DR, Conley AJ, Overstreet JW, Lasley BL. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) modulates function of human luteinizing granulosa cells *in vitro*. *Society for The Study of Reproduction*, Portland, OR 1997. (Suppl 1) 56 (Abstract).
4. Gregoraszczuk EL, Zabiely E, Piekło R, Grochowalski A, Wójtowicz AK, Mika M. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on luteal cell function. Tissue culture approach. *Organohalogen Compounds* 1999; 42: 67—71.
5. Piekło R, Grochowalski A, Gregoraszczuk EL. 2,3,7,8-tetrachloro-dibenzo-p-dioxin alters follicular steroidogenesis in time- and cell- specific manner. *Exp. Clin. Endocr & Diab.* 2000; 108: 1—6.
6. Gregoraszczuk EL, Wójtowicz AK, Zabiely E, Grochowalski A. Dose-and-time dependent effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on progesterone secretion by porcine luteal cells cultured *in vitro*. *J Physiol Pharmacol* 2000; 51: 127—135.
7. Hankinson O. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 1995; 35: 307—340.
8. Chaffin CL, Peterson RE, Hutz RJ. In utero and lactation exposure of female Holtzman rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): modulation of the estrogen signal. *Biol Reprod* 1996a; 55: 62—67.
9. Chaffin CL, Heimler I, Rawlins RG, Wimpee BAB, Sommer C, Hutz RJ. Estrogen receptor and aromatic hydrocarbon receptor in the primate ovary. *Endocrine* 1996 b; 5: 315—321.
10. Gregoraszczuk EL. Interrelationship between steroid hormone secretion and morphological changes of porcine corpora lutea at various periods of luteal phase. *Endocrine Regulation* 1992; 26: 189—194.
11. Gregoraszczuk EL. Steroid hormone release in cultures of pig corpus luteum and granulosa cells. Effect of LH, hCG, PRL and estradiol. *Endocr Exp* 1983; 17: 59—68.
12. Gregoraszczuk EL, Piekło R. Thyroid hormone action in porcine luteal cells. Effect of triiodothyronine on mitochondrial cytochrome P450sc. *J Physiol Pharmacol* 1998; 49: 467—475.
13. Gregoraszczuk EL, Kolodziejczyk J, Rzaśa J. Triiodothyronine stimulates 3 β -hydroxysteroid dehydrogenase activity in the porcine corpus luteum. *Endocrine Regulations* 1999; 33: 155—160.
14. Enan E, Liu PC, Matsumura F. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) caused reduction of glucose transporting activities in the plasma membranes of adipose tissue and pancreas from the quinea pig. *J Biol Chem.* 1992; 267: 19785—19791.
15. Gasiewicz TA, Mahon MJ. Isomers of phenanthroline as antagonists. *Society of Toxicology, 31st Ann Meeting* 1992, 12: Abstr 714, Seattle, WA March,
16. Gregoraszczuk EL. Is thyroid hormone a modulator of estrogen receptor in porcine follicular cells? *Endocrine Regulations* 2000; 34: 151—155.
17. Gregoraszczuk EL, Zabiely E, Piekło R, Grochowalski A, Wojtowitz A, Mika M. Effects of 2,3,4,7-tetrachlorodibenzo-p-dioxin (TCDD) on luteal cell function. Tissue culture approach. *Organohalogen Compounds* 1999; 42: 67—71.
18. Safe SH. Development validation and problems with the toxic equivalency factor approach for risk assessment of dioxin and related compounds. *J. Anim. Sci* 1998; 76: 134—140.
19. Umbreit TH, Gallo MA. Physiological implications of estrogen receptor modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Lett* 1988; 42: 5—14.

20. Caruso JA, Laird DW, Batist G. Role of HSP90 in mediating cross-talk between the estrogen receptor and the Ah receptor signal transduction pathways. *Biochem Pharmacol* 1999; 58: 1395—1403.

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