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DOSE-AND-TIME DEPENDENT EFFECT OF 2,3,7,8-TETRACHLORODIBENZO- P-DIOXIN (TCDD) ON PROGESTERONE SECRETION BY PORCINE LUTEAL CELLS CULTURED IN VITRO

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In the current study, to characterize TCDD action during luteal phase of the ovarian cycle, the direct effect of TCDD was investigated *in vitro* using a system of monolayer cell culture.
Luteal cells isolated from mid-developing corpora lutea were cultured with four different doses of TCDD (0.1, 1.0, 10.0 and 100 nM). The dose of 0.1nM TCDD had no effect on progesterone (P4) secretion by luteal cells while the doses of 10nM and 100nM in the same, statistically significant manner decreased P4 secretion ($p<0.05$). The inhibitory effect of TCDD was dependent not only on doses by also on experimental conditions. In cells treated every day for 72 hrs of culture with 0.1nM TCDD, P4 secretion was 71% of basal secretion. 100nM TCDD added only at the beginning of the culture and nor repeated when medium was changed every 24 hrs decreased P4 secretion to 81.8% of basal secretion. The most inhibitory effect was observed in experiments in which 100nM TCDD was added at the beginning of the culture and medium was not changed for 72 hrs. Secretion of P4 was only 33.9% of that by control cultures.
In order to show the time-dependent response to TCDD in terms of P4 secretion, luteal cells were cultured for 24,48, 72 hrs with 0.1 and 100nM TCDD. 85%, 75% and 72% of basal progesterone secretion was noted after 24, 48 and 72h respectively in 0.1nM TCDD-treated cells. In 100nM TCDD treated cells the decrease of progesterone secretion was 57%, 67% and 82% of basal secretion after 24, 48 and 72 hrs of culture.
These experiments suggest that TCDD by suppressing progesterone secretion by corpora lutea can cause adverse reproductive effects such as early pregnancy failure. Endocrine disrupters that interfere with progesterone production can act as abortifacients.

Key words: *TCDD, luteal cells, progesterone secretion, in vitro*

INTRODUCTION

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congener of a large class of toxic pollutants, collectively

known as halogenated dioxins (1). The toxic effects of TCDD have been elucidated for various tissues in a number of species, including humans (2, 3). The data are especially scarce regarding the effect of TCDD on the female reproductive tract. Steroid hormones play an essential role in the control of ovarian cyclicity and the patterns of episodic release are remarkably conserved across species with respect to the preovulatory rise in oestrogen followed by progesterone secretion during the luteal phase. Enan *et al.* (7), showed a decrease in progesterone production by luteinized granulosa cells after 24h exposure for 10 nM of TCDD. In another study byMoran *et al.* (8), TCDD was shown to reduce oestradiol production by luteinized granulosa cells without an effect on progesterone production. After ovulation the cells of the follicle change into luteal cells which form corpus luteum. Both granulosa and the cells of theca interna are involved in the formation of luteal tissue. The results of our earlier study (Gregoraszczuk, 1983) indicated that the cells isolated from corpus luteum formed *in vivo* are not identical with the granulosa cells which were luteinized in the culture. Corpus luteum is the compartment of the ovary with very strictly limited life span. The regular sexual cycle is dependent on the precise regulation of the life span of corpus luteum. One of the endocrine functions of corpora lutea is the production of progesterone. The direct action of TCDD on steroidogenesis of luteal cells is unknown and the evidence is really scarce. Although some of the effects of TCDD are mediated via the hypothalamic-pituitary axis, direct effects on the ovary have been observed (9). Taking into consideration data of (4, 5) showing TCDD decreased number and size of corpora lutea, decreased plasma progesterone and oestrogen concentrations (4, 5) it seems very important to characterize TCDD action during luteal phase of the cycle. The direct effect of TCDD was investigated in vitro using a system of monolayer cell culture.

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).

Animals and cell isolation

Ovaries were obtained from Large White sows from a local slaughterhouse immediately after slaughter, placed in ice-cold PBS and transported to the laboratory. The phase of the oestrous cycle was determined according to the established morphological criteria (10). Dissected corpora

lutea from each animal were enzymatically dissociated according to the technique of Gregoraszczuk (11). Luteal cells were obtained from pools of freshly existed mature corpora lutea (8—10 days after ovulation) from three animals in order to produce the luteal pool used in any given replicate to minimise the variation possibly existing among corpora lutea in the same ovaries and between ovaries in the some animals and among animals.

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% CO_2 in the air. At least three different experiments (n = 3), each, in triplicate have been done.

Experimental procedure

Experiment 1

In order to show the dose response to TCDD in terms of progesterone secretion, luteal cells were cultured with four different doses : 0.1, 1.0, 10.0 and 100nM TCDD. The cells were culture for 48 hr and the medium was frozen (−20°C) for a further progesterone analysis.

Experiment 2

The concentration of TCDD used in these experiments was establised from dose response curves obtained during experiment 1 (*Fig. 1*).

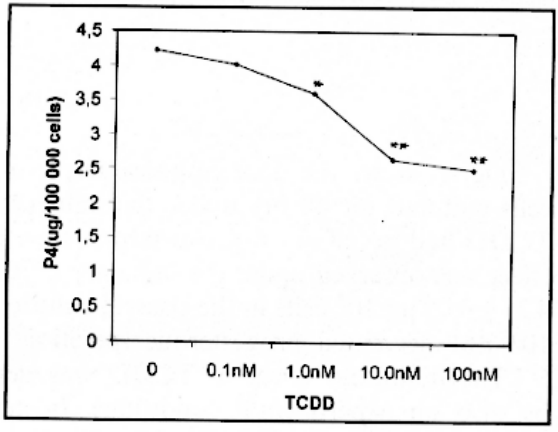

Fig. 1. Dose response curve of progesterone secretion under the influence of 0, 0.1, 1.0, 10.0 and 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). * $p < 0.05$; ** $p < 0.01$

This part of experiment cells was divided for 3 group:

I. The dose of 0.1 nM was added each time when the medium was changed. For steroid analysis medium was collected after 72 hrs.

II. The dose of 100 nM was added only at the beginning of the culture. The media were changed every 24 hrs and collected after 72 hrs for further progesterone analysis.

III. The dose of 100 nM was added at the beginning of the culture, and medium was not changed till the end (72 hrs) of the culture to show the influence of the permanent treatment with this drug.

Experiment 3

In order to shw the time-dependent response to TCDD in terms of progesterone secretion, luteal cells were cultured with 0.1 and 100 nM TCDD. For steroid analysis media were collected for 24, 48, 72 hrs. TCDD was added when the culture were set up and each time when the medium was changed. The time of cultured was chosen on the basis of data of Gregoraszczuk and Wojtusiak, (12).

Progesterone analysis

Progesterone was determined radioimmunologically using Spectra kits (Orion, Diagnica, Finland), supplied by Polatom (Świerk, Poland). The limit of assay sensitivity was 94 pg/ml. The coefficients of variation within vnd between 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 (5ß-dihydroprogesterone, 20ß-hydroxyprogestrone, corticosterone, testosterone, oestrone) showed less then 1% cross-reaction.

Statistical analysis

All data points are expressed as mean $\pm$ SEM. from at least three different experiments (n = 3) each in triplicates. Significant differents between the concentrations of progesterone in the control and TCDD treated cells were compared using Student t'-test (*Figs, 1, 2*). Duncan's new multiple range test was used to evaluate differences in progesterone concentration between control and different doses TCDD and different period of culture (*Fig. 3*).

RESULTS

Fig. 1 shows the dose response curve of progesterone secretion by luteal cells cultured for 48 hrs under the influence of TCDD. The dose of 0.1 nM TCDD had no effect on progesterone secretion by luteal cells. The inhibitory effect was observed under the influence of 0.1 nM (3.6 ± 0.1 µg/10^5 cells versus 4.21 ± 0.05 µg/10^5 cells in the control culture; $p < 0.05$). The doses of 10nM and 100 nM decreased progesterone secretion ($p < 0.01$) in the same manner.

The inhibitory effect of TCDD was dependent not only on doses used by also on experimental conditions. In cells treated every day for 72 hrs of culture with 0.1 nM TCDD the progesterone secretion was 71% of basal secretion ($p < 0.01$). 100 nM TCDD added only at beginning of the culture and not repeated during medium change every 24 hrs decreased progesterone secretion to 81.8% of basal secretion ($p < 0.05$). The most inhibitory effect was observed in experiments in which 100 nM TCDD was added at the beginning of the cultzre and medium was not changed for 72 hrs. The secretion of progesterone was 33.9% of control secretion ($p < 0.001$). (*Fig. 2*)

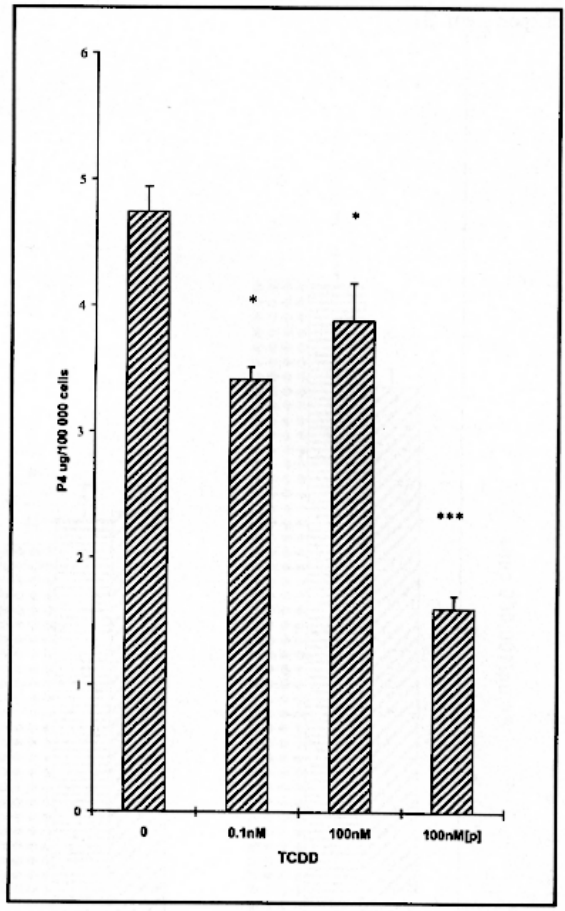

Fig. 2. The influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on progesterone secretion in different culture condition. 1) 0.1 nM — The dose of 0.1 nM was added each time when the medium was changed. For steroid analysis medium was collected for 72 hrs; 2) 100 nM — the dose of 100 nM was added only at the beginning of the culture. The media were changed every 24 hrs and collected for 72 hrs for furhter progesterone analysis; 3) 100 nM [p] — the dose of 100 nM was added at the beginning of the culture, and medium was not changed till the end (72 hrs) of the culture to show the influence of the permanent treatment of this drug. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In long term culture, the statistically significant increase of progesterone secretion after 48 and 72 hrs of culture in basal condition was observed (*Fig. 3*; $p<0.05$). 0.1 nM TCDD added each time when the medium was changed decreased to 85%, 75% and 72% of basal progesterone secretion respectively after 24, 48 and 72h of culture ($p<0.05$). The dose of 100 nM added only at the beginning of the culture and not repeated when media were changed the most inhibitory effect was observed after 24 h of culture (57% of basal secretion; $p<0.001$). The reversibility of the toxic effect of TCDD was observed during long term culture (67% of basal progesterone secretion after 48 h of culture; $p<0.01$ and 82% of basal secretion after 72 hrs of culture; $p<0.05$) (*Fig. 3*)

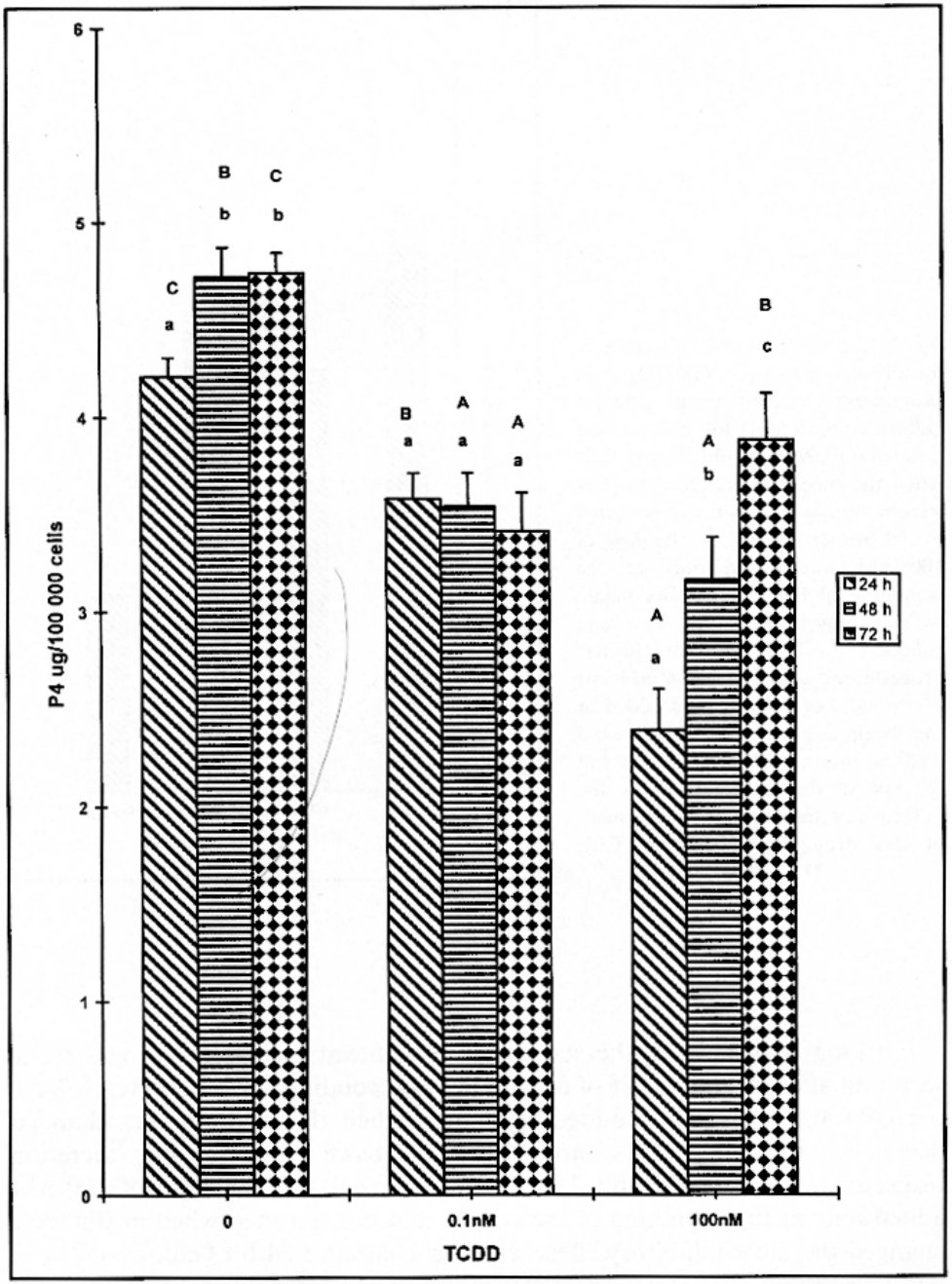

Fig. 3. Time- dependent effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on progesterone secretion by luteal cells collected from mid-developing corpora lutea. Small letters refer to comparison among hours of cultures within treatments. Capital letters refer to comparison among control and TCDD stimulated cells within hours of culture.

DISCUSSION

The high incidence of embryonic loss observed in the pig in early pregnancy has in the past decade resulted in numerous studies designed to elucidate mechanisms contributing to the prenatal mortality. The embryo transfer experiments in pigs (13) showed that developmental stages of the uterus and the transferred blastocyst must be in synchrony for successful implantation and survival of embryos. Although some of the effects of TCDD are mediated via the hypothalamic-pituitary axis, direct effect on the ovary have also been observed (14). The toxic effect of TCDD have been elucidated for male reproduction tissue and for follicular cells taking into consideration the number of growing follicles. Surprisingly the data are especially scarce regarding the effect of TCDD on luteal cell function. Corpus luteum is the compartment of the ovary with very strictly limited life span. The regular sexual cycle is dependent on the precise regulation of the life span of corpus luteum.

In the present study, we have shown a marked decrease of progesterone secretion by porcine luteal cells collected during mid-luteal phase subjected to TCDD in culture. It is in agreement with earlier in vivo data showing decreased plasma progesterone level, number and size of corpora lutea was observed by Johnson *et al.* (4), in rat and by Barsotti *et al.* (5), in the resus monkey. In a recent study, Enan *et al.* (7) have shown that an in vitro treatment with TCDD suppressed progesterone secretion in human luteinized granulosa cells. This effect of TCDD was both dose and time dependent. In the present study by using a system of purified porcine luteal cells, we were able to address the direct effect of TCDD on single-cell level. The first interesting finding was a fact that the inhibitory effect of TCDD was dependent not only on doses used by also on experimental conditions. TCDD in minimal dose of 0.1 nM added every day for 72 hrs of culture decrease progestrone secretion to 71% of basal secretion while in a dose of 100 nM added only at the beginning of the culture and not repeated during medium change decreased progesterone secretion to 81.8% of basal secretion. The most inhibitory effect was observed in experiments in which 100 nM TCDD was added at the beginning of the culture and medium was not changed for 72 hrs. The secretion of progesterone was 33.9% of control secretion.

The second interesting finding was the reversibility of the toxic effect of TCDD observed during long term culture (67% of basal progesterone secretion after 48 h of culture; $p < 0.01$ and 82% of basal secretion after 72 hrs of culture; $p < 0.05$) suggesting that TCDD by acting at the level of the cells, is capable altering processes in the steroidogenic pathway. This conclusion is supported by data of Heimler *et al.*, 1998 showing that that the inhibition of E2 secretion by TCDD can be prevented by supplementation with androgen precursor in human granulosa cells.

Collectively, the present preliminary results have shown a direct role of TCDD at the level of the luteal cells, and may thus further explain TCDD's antifertility effects (15, 16). Further studies are needed to elucidate the mechanism of action of TCDD in luteal cells.

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