EFFECTS OF DIOXIN (2,3,7,8-TCDD) AND PCDDs/PCDFs CONGENERS MIXTURE ON STEROIDOGENESIS IN HUMAN PLACENTA TISSUE CULTURE

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Objective. The aim was to compare the direct effect of most toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as of naturally occurring congener mixture of polychlorinated dibenzo-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) extracted from fly ash on the placental steroidogenesis. The concentration of all 17 toxic congeners was reported and the toxic equivalent (TEQ) was calculated as a 27.7 μg-TEQ/kg of fly ash.

Methods. Placental cotyledons were harvested immediately after expulsion of placenta. The cells were prepared according to Kliman et al. (1986). To examine TCDD and PCDDs/PCDFs mixture action on cytochrome P450 side change cleavage enzyme (P450 scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) activity the placental cells were cultured either in basal conditions or with the addition of 25-hydroxycholesterol (25-OH) or pregnenolone (P5).

Results. TCDD in all doses used decreased basal P4 secretion, while did not show any effect on 25-hydroxycholesterol (25-OH) and pregnenolone (P5) supplemented cultures. In all variants of culture PCDDs/PCDFs mixture was without effect on basal and substrate supplemented progesterone (P4) secretion suggesting a reduction in the activity of cytochrome $P450_{scc}$ or 3β-HSD. To examine TCDD and PCDDs/PCDFs mixture action on aromatase cytochrom P450 (P450 arom) activity the placental cells were cultured in basal condition or with the addition of dehydroepiandrosterone (DHEA) or testosterone (T). Significant increase of estradiol secretion under the influence of TCDD in DHEA and T supplemented cultures suggests its action on the activity of P450 arom.

Conclusion. The discrepancy found between the action of pure TCDD and dioxin mixture on placental steroids secretion is possibly due to an additional effect of pentachlorodibenzo-p-dioxin (PeCDD) and pentachlorodibenzo-furan (PeCDF) which covered >50 % of the total toxic equivalents (TEQ) present in this mixture.

Key words: TCDD – PCDDs/PCDFs mixture – Human placenta – Hormonal status

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds elicit a diverse spectrum of toxic responses. As these compounds are lipophylic, they are not easily metabolized and thus any environmental exposure of living organisms results in their accumulation in adipose tissue and thus in bioconcentration in the body via food chain. Additionally, they are able to pass through the human placenta (Koppe et al. 1992). Taking into account the fat solubility of these compounds and the maternal origin of 10 to 20

% of fetal fatty acids, PCBs and hexachlorobenzene (HCB) may impair the fetal development (MANCHESTER and JACOBSY 1984).

Human exposure to halogenated aromatic hydrocarbons such as polychlorinated biphenyls is known to be associated with adverse pregnancy outcomes including intrauterine growth retardation (IUGR), congenital structural anomalies and cognitive developmental deficits. Our previous study showed the tendency for increase of PCB 156, PCB 114 and PCB 123 concentration in placentas from abnormal pregnancies. The most toxic congener PCB 126 was found only in the placentas from women smoking cigarettes during pregnancy (GROCHOWALSKI et al. 2000). In turn, maternal smoking may result in placental insufficiency (NIETO et al. 1994). CHEN et al. (1992) and ROGAN et al. (1988) observed the impaired fetal development in women exposed to PCB, dibenzodioxins and dibenzofurans. These substances thus represent a serious risk to human health, especially for fetuses and infants, since their enzymatic and metabolic systems are not yet mature. The exposure to organochlorinated compounds (OC) such as TCDD has been linked to deleterious endocrine effects observed in wildlife, laboratory animals and humans (CAMBELL and HUTCHINSON 1998). OC mediated effects on endocrine systems were primarily focused on their agonist/antagonist activity via the Ah-receptor and estradiol receptor.

Aromatase is one of the proposed enzymes subjected to disruption under the influence of xenobiotics. High levels of this enzyme are expressed in the human placenta, which thus regulates the balance of estrogens in the uterus. An alteration in uterine aromatase function has been shown to permanently affect human embryos (SIMSON et al. 1994). Human JEG-3 and JAR choriocarcinoma cell cultures of cytotrophoblast from malignant placental tissue have been used as *in vitro* models for investigating the effects of xenobiotics in placental toxicity (ALBRECHT and PEPPE 1990).

Human subjects are usually exposed to mixtures of organochlorinated chemicals rather than to individual substances. So far, in several experiments concerning with the endocrine disruption effects or other toxic actions of dioxins and furans only individual congeners were investigated.

Taking into account that in the environment PCDDs and PCDFs are present in the mixed forms of all 17 toxic congeners as well as non-toxic congeners, we decided to conduct an experiment with the appropriate standard DMSO solution obtained from the extraction and purification of a real fly ash, which is spread-out into the atmosphere from thermal, industrial processes. In our opinion there is no doubt that the mixed PCDDs and PCDFs congeners may show synergic and/or antagonist action in humans as well as in animals. Therefore the study should be focused on real PCDDs/PCDFs mixtures which may be found in the human environment, to evaluate their potential action. This will help us to understand their action in a natural mixture rather than that of an individual congener, which never occurs in nature. In this investigation the effect of pure TCDD was compared with that of dioxin mixture for specific endpoints of endocrine toxicity.

Materials and Methods

Reagents. Medium M199, PBS, penicillin, trypsin, and calf serum were purchased from the Laboratory of Vaccines (Lublin, Poland). Testosterone, dehydroepiandrosterone, 25- hydroxycholesterol, pregnenolone, Antibiotic antimitotic solution (100x), DNAse I, Red Blood Cell Lysing Buffer Hybri-Max – were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,3,7,8-TCDD solutions were prepared by the dilution of evaporated, concentrated toluene standard (Promochem) in DMSO. PCDDs and PCDFs natural congener mixture in DMSO was prepared by toluene Soxhlet extraction of 10 g of fly ash sample collected from the hospital waste incinerator and Alumina column cleaned-up according to GROCHOWALSKI (1998). This mixture contained 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8 pentachlorodibenzo-p-dioxin (PeCDD), hexachlorodibenzo-p-dioxin (1,2,3,4,7,8-HxCDD and 1,2,3,6,7,8-HxCDD),1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin(HpCDD), octachlorodibenzo-p-dioxin (OCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), pentachlorodibenzofuran (1,2,3,7,8-PeCDF and 2,3,4,7,8-PeCDF), hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF and 1,2,3,7,8,9-HxCDF), heptachlorodibenzofuran (1,2,3,4,6,7,8-HpCDF and 1,2,3,4,7,8,9-

HpCDF) and octachlorodibenzofuran (OCDF). The concentration of all 17 toxic congeners was reported and TEQ was calculated as a 27,7 µg-TEQ/kg of fly ash. After extract clean-up using standard procedure on Alumina, the solvent was exchanged to DMSO to obtain the stock solution of a concentration of 10 ng-TEQ/ml. Working solutions were prepared by a dilution of the stock solution with DMSO to obtain appropriate PCDDs/PCDFs concentration just before adding to the culture medium.

Cell Culture. Placental tissue was collected in Krakow, Poland where the clinical information on pregnancy outcomes was gathered. Collection of placentas and gathering of clinical histories followed previous established protocols. Placental cotyledons were harvested immediately after expulsion of placenta, placed in ice-cold PBS and transported to the laboratory. The cells were prepared by a method of KLIMAN et al. (1986) with our own modification. Cell suspensions were obtained by digesting pieces of placental tissue (20 g/ 100 ml) in calcium- and magnesium free PBS containing 0.125 % trypsin and 15 IU/ml DNAse I pH 7.4 at 37 ^{0}C for 30 min. The flask was set at an angle, and tissue fragments were allowed to settle for 1 min. Four aliquots (20 ml each) of the supernatant were layered over 1.5-2 ml calf serum in 50 ml polystyrene conical centrifuge tubes and centrifuged. The resultant pellets were resuspended in 2 ml M199 medium. The remaining placental tissue was subjected to the digestion two more times with the addition of fresh trypsin-DNase solution. The three resultant cell suspensions were pooled. After centrifugation cell pellet was gently mixed for 1 min with Red Blood Cell Lysing Buffer Hybri-Max. If lysis of red blood cells was incomplete the procedures was repeated 2-3 times. Finally the cells were spun and resuspended in M-199 medium supplemented with 10 % calf serum, and plated at 1-1,5´10^5 cells per well in 48 well plastic cell-culture plates (Falcon, Lincoln Park, NJ). The cell viability, using the trypan blue exclusion test, was 85 %. The cultures were maintained at 37 ^{o}C in humidified atmosphere of 5 % CO_2.

Experimental procedure. The activity of cholesterol side chain cleavage cytochrome P450scc was measured by conversion of hydroxylated cholesterol derivative (25-hydroxycholesterol; 25-OH) to progesterone. TCDD was added in doses of 10, 20, 40, 60 and 80 pgTEF/ml and dioxin mixture – 10, 20, 40, 60 and 80 pgTEQ/ml to the control or 25-OH treated cells. 48 hours later media were collected and frozen for progesterone estimation. The dose of 10 µg/ ml 25-OH was used according to Gregoraszczuk and Piekło (1998).

The activity of 3β-hydroxysteroid dehydrogenase was measured by conversion of pregnenolone (P5; 10 µg/ ml) to progesterone. TCDD and dioxin mixture was added in doses of 10, 20, 40, 60 and 80 pgTEF or TEQ/ml to the control or P5 treated cells. 48 hours later media were collected and frozen for progesterone estimation.

The activity of $P450_{arom}$ activity was measured by conversion of testosterone (T; 10^{-7} M) or dehydroepiandrosterone (DHA; 1 ng/ml) to estradiol. TCDD and dioxin mixture was added in doses of 10, 20, 40, 60 and 80 pgTEF or TEQ/ml to the control and DHEA or T treated cells. 48 hours later media were collected and frozen for estradiol estimation.

The net synthesis and secretion of estradiol to the culture medium was used as the indicator of aromatase activity.

Steroid analysis. P4, T and E2 were determined radioimmunologically using Spectra kits (Orion, Diagnica, Finland), supplied by Polatom (Swierk, Poland).The limit of P4 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 (5ß-dihydroprogesterone, 20ß-hydroxyprogesterone, corticosterone, testosterone, estrone) showed less then 1 % cross-reaction. The limit of T assay's sensitivity was 5 pg/ml and the coefficients of variation within and between assays were 5.4 % and 5.3 %, respectively. The mean recoveries were 84.2 to 121.7 %. Cross-reactivity with 5a-dihydrotestosterone was 4.5 %. All other tested steroids (methyltestosterone, androstendione, progesterone, 17β-estradiol) showed less than 0.5 % cross-reactivity. The limit of E2 assay was 5 pg and the coefficients of variation between and within assays were 10.28 % and 2.9 %, respectively. The mean recoveries were 85.6 to 108.9 %. The cross-reactivity with ethinyl estradiol was 1.4 %. All other tested steroids (estrone, estriol, progesterone, testosterone, and corticosterone) showed less than 1 % cross-reactivity.

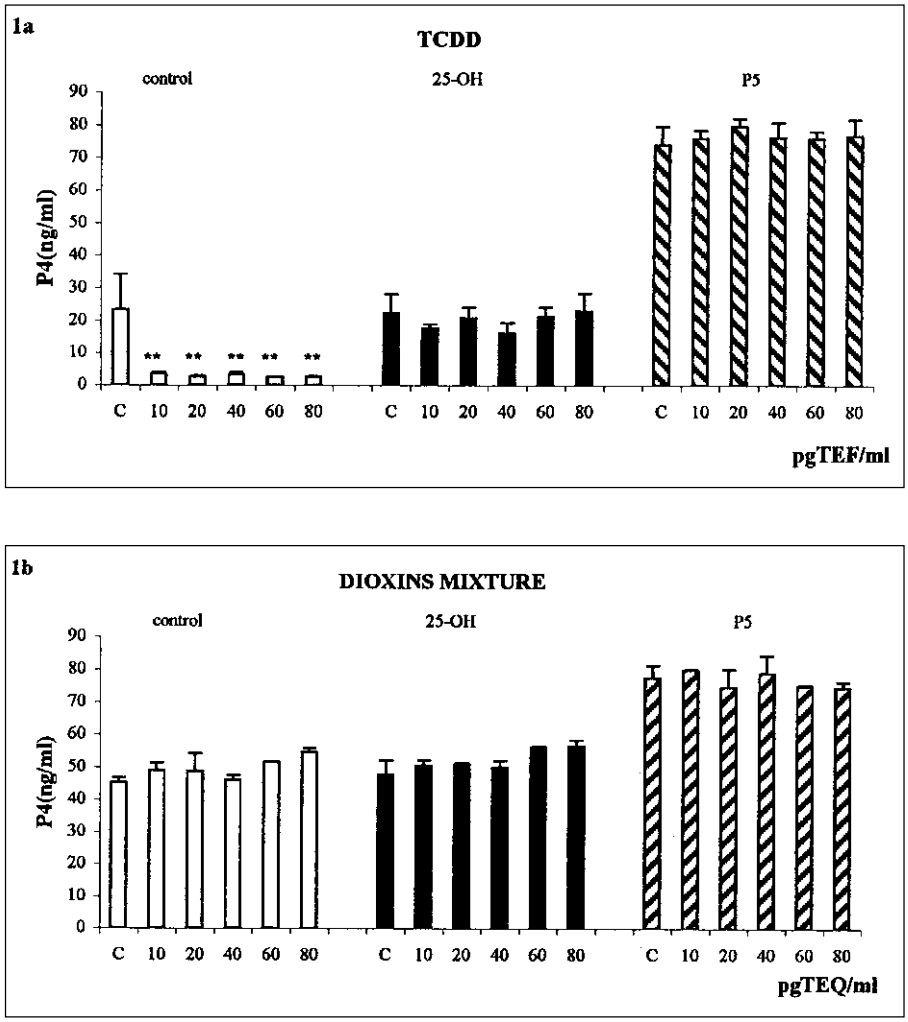

Fig 1 The influence of a) 2,3,7,8-tetrachlorodibenzo-p-dioxin and b) PCDDs and PCDFs natural congener mixture on basal (C), 25-hydroxycholestrol (25-0H) and pregnenolone (P5) stimulated progesterone secretion by placental cells cultured in vitro. * P<0.05; ** P<0.01; *** P<0.001.

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

Results

Alteration of progesterone secretion. To examine TCDD and dioxin mixture action on P450 scc and *3β-HSD* activity placental cells were cultured either in basal condition or with addition of 25-hydroxycholesterol or pregnenolone. In control culture, progesterone secretion by placental cells was 33.3 ng/ml. The secretion of progesterone did not changed after 25-hydroxycholesterol was added to the medium, however 2-fold increase of progesterone secretion was noted in pregnenolone supplemented cultures. TCDD in all doses used decreased basal progesterone secretion (P<0.001) while had no effect on 25-OH and P5 supplemented cultures. (Fig. 1a). In all variants of culture the mixtures of dioxins and furans were without effect on basal and substrate supplemented cultures. (Fig. 1b).

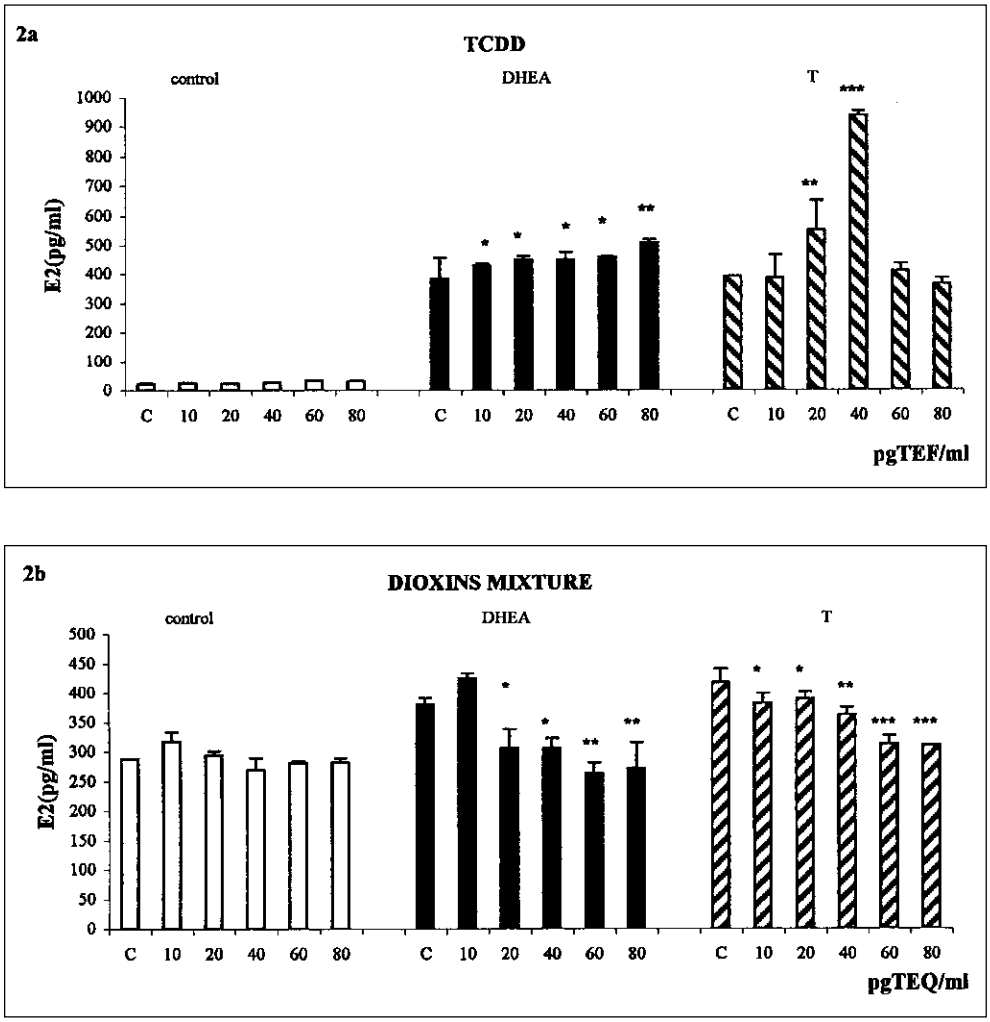

Fig 2 The influence of a) 2,3,7,8-tetrachlorodibenzo-p-dioxin and b) PCDDs and PCDFs natural congener mixture on basal (C), dehydroepiandrosterone (DHEA) and testosterone (T) stimulated estradiol secretion by placental cells cultured in vitro. * P<0.05; ** P<0.01; *** P<0.001.

Alteration of estradiol secretion To examine TCDD and dioxin mixture action on P450arom activity placental cells were cultured either in basal condition or with addition of DHEA or T. In control culture estradiol secretion by placental cells was 251.21 pg/ml, while. 1.5-fold increase of estradiol secretion was noted in DHEA and T supplemented cultures. Small, but significant increase of estradiol secretion under the influence of TCDD was noted in DHEA supplemented cultures (450, 456, 458, 460, 505 pg/ml vs. 380 pg/ml in DHEA treated cells). In T supplemented cultures the increase of estradiol secretion was observed in doses 20 and 40 pgTEF/ml (548 and 935 pg/ml vs. 390 pg/ml in T treated cells (Fig. 2a). However, dioxin mixture in doses 20, 40, 60 and 80 pg TEQ/ml decreased the conversion of DHEA to estradiol (351, 277, 262, and 271. pg/ml vs. 380 pg/ml in DHEA alone treated cells) and in all doses conversion of testosterone to estradiol (381, 389, 361, 312 pg/ml and 299.82 vs. 416.86 pg/ml in T alone treated cells) (Fig. 2b).

Discussion

In this study the effects of TCDD and dioxin mixture on particular step of steroidogenesis in human

placenta were investigated. It is well known that during pregnancy increasing quantities of progesterone are synthesized in human placenta (KLOPPER and FUCHS 1977). The human syncytial trophoblast is known to play several roles in pregnancy. Thus, it mediates the transport of nutrients and immunoglobulins from the maternal to fetal circulation and also functions as an endocrine organ secreting steroid and protein hormones (LOKE 1983). Placental transport of dioxins and furans from the mother to fetus is possibly related to the fatty acid transport. Between 10 and 20 % of fatty acids in a full-term baby is of maternal origin. In adipose tissue of children that died in the early neonatal period up to 25 percent were found of three dioxin and furan congeners, e.g. 12378 P5CDD, 123678 H6CDD and 23478 P5CDF (KOPPE et al. 1992).

In this investigation it was found that TCDD remarkably decreased basal progesterone production by placental cells. This action could be due to increasing concentration of non-active cholesterol metabolites. HASSOUN et al. (1995) showed that TCDD administration to pregnant CF1 mice resulted in an increase in the amniotic fluid levels of lipid metabolites such as formaldehyde, acetaldehyde and acetone thus suggesting that reactive oxygen species may participate in the teratogenic effects of TCDD. However, also other mechanism can be proposed taking into consideration the fact that TCDD did not inhibit progesterone secretion by placental tissue when the culture was fortified by exogenous 25-OH cholesterol – a substrate to pregnenolone formation and by pregnenolone – a substrate for progesterone production. These results suggest that such inhibition of progesterone may be due to the reduction in the activity of the mitochondrial enzyme converting cholesterol into pregnenolone (cytochrome $P450_{scc}$) or pregnenolone to progesterone. Inappropriate progesterone secretion could be the cause of adverse pregnancy outcomes. The decreased progesterone level increases the susceptibility of uterine muscle to contractile stimuli which may result in a preterm labor. It is known that human exposure to halogen aromatic hydrocarbons is associated with intrauterine growth retardation and developmental deficits (CHEN et al. 1992; ROGAN et al. 1988; MILLER 1985). On the other hand humans are daily exposed to mixtures of chemicals, rather than to individual chemicals. From a public health point of view, it is most relevant to answer the question of whether or not the components in a mixture interact in such a way which results in an increase in their overall effect compared with the sum of the effects of the individual components. In the presented paper we observed no effect of short-term treatment of dioxin mixture on progesterone secretion.

Conversely, progesterone has effects on 17 β-HSD oxidoreductase and thus the metabolism of estradiol. The placenta during late pregnancy is the major source of estrogens, deriving its substrate from the fetal adrenal (SIMPSON and MCDONALD 1981) due to the inability of the placenta to synthesize androgen. Fetal adrenal DHAS can be converted to estrone and estradiol in the placenta. Since estriol may affect uterine CAP (cystyl aminopeptidase) gene expression (DARNE et al. 1987), it could contribute to the progressive increase in uterine responsiveness in primate pregnancy during the third trimester of gestation, and its measurements may be of predictive value in delineating patients at risk of premature delivery (DARNE et al. 1987; FERRE et al. 1975).

The presented data clearly showed that TCDD increased conversion of DHEA to E2 and T to E2. As a matter of fact, estrogen regulates LDH uptake and P450scc, and thus apparently is involved in generating substrate for progesterone production within the placenta there are reports of an increase in the estrogen-progesterone (E: P) ratio in amniotic fluid of women during the labour (ROMERO et al. 1988). It have been previously showed that placental microsomes and mitochondria incubated with single dose of E2 showed a decrease in the progesterone formation by inhibition of 3-beta HSD (FERRE et al. 1975; YOSHIDA et al. 1988; DEPP et al. 1973). GENTI-RAIMONDI et al. (1983) showed that physiological doses of E2 had stimulatory effect on the conversion of pregnenolone to progesterone, however supraphysiological doses showed an inhibitory effect. The observed under the influence of TCDD increase of estradiol secretion by placental cells could be also due to the other mechanism by which TCDD can disrupt progesterone secretion observed in control culture.

The decrease of progesterone secretion and estrogen-like action of TCDD in placental tissue as observed in this study can be taken into consideration in view of the data by CHEN et al. (2001) examined

the transfer of polychlorinated dibenzo-p-dioxins, dibenzofurans (PCDFs) and non-orto biphenyls to offspring and placenta. They showed dose-dependent increase in hepatic sequestration with TCDD, PeCDD, 4-PeCDF, OCDF. TCDD and three PCBs reached equlibration between the foetus and placenta. In the presented paper we observed antiestrogenic action of the PCDDs/PCDFs mixture. Aromatase activity is one of the proposed enzymes disrupting under the influence of xenobiotics. The human placenta expresses high levels of aromatase activity and thus regulates the balance of estrogens in uterus. An alteration in aromatase function in uterus has been shown to permanently affect human embryo (Simpson et al. 1994). Human JEG-3 and IAR choriocarcinoma cell cultures of cytotrophoblast from malignant placental tissue have been used as in vitro models for investigating the effects of xenobiotics in placental toxicity (Albrecht and Peppe 1990).

The presented results suggested that both estrogenic action of TCDD and antiestrogenic action of mixture is due to action on the activity of P450 arom. This observation is in accordance with data of McMurry and Dickerson (2001) who showed that mixture of six different endocrine disrupters indicate effects very different from either or both mixture components, indicating the lack of predictability of chemicals when combined in mixture. Also, last data of Chu et al. (2001) indicated that the mixture of PCBs and TCDD may be additive or antagonistic depending on the dose level and endpoints measured. The effects of mixture dioxin may be additive or antagonistic depending on dose level. For this purpose of predicting mixture effects, knowledge of mechanism of action and toxicokinetics is required.

In conclusion, short-term exposition to dioxin mixture present in fly ash did not significantly alter the placental steroidogenesis at the dose level tested, indicating that in case of exposure to complex dioxin mixtures at environmental levels, only marginal effects can be expected on the placental hormonal status. The same results was noted by van der Plas et al. (2001), who indicating that exposure to complex PCB mixture at the environmental level no effect or had marginal effects on thyroid hormone status. The placenta is able to metabolise many foreign chemical compounds (Hakkola et al. 1996; *Juchau* et al. 1980). Xenobiotic-metabolising cytochrome P450 (CYPs) are very important in metabolising a number of endogenous substrates and xenobiotics (Nelson et al. 1993; Guengerich 1994). Relatively little is known about the individual CYPs presence in human placenta. To understand the differences in action of TCDD and dioxin mixture on placental steroidogenesis further studies on the induction of CYP izosymes mRNA in human placenta by this compound are needed.

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