ESTROUS CYCLE-DEPENDENT CHANGES IN STEROID SECRETION BY PIG OVARIAN CELLS EXPOSED IN VITRO TO POLYCHLORINATED BIPHENYL (PCB 153)

Anna K. Wojtowicz, [1]Erik Ropstad, Ewa L. Gregoraszczuk[2]

Laboratory of Physiology and Toxicology of Reproduction, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Kraków, Poland.
[1]Department of Reproduction and Forensic Medicine, Norwegian School of Veterinary Science, Oslo, Norway.
[2]E-mail: greg@zuk.iz.uj.edu.pl

Objective. To characterize PCB 153 action on the ovary, the direct effect of PCB153 was investigated in vitro using a co-culture of pig granulosa and theca cells collected during different stages of follicular development.

Methods. The cells were cultured in the absence or presence of 5, 10, 50 or 100 ng/ml of PCB 153. Media were changed after 48, 96 and 144 h and frozen until further estradiol (E2), progesterone (P4) and testosterone (T) analysis.

Results. 48 hrs exposure of follicular cells collected from small-size follicles to all the investigated doses of PCB 153 caused statistically significant decrease in progesterone (P4) secretion and at doses of 50 ng and 100 ng/ml in testosterone (T) secretion. No effect on estradiol (E2) secretion was observed. After 96h and 144h exposure to PCB an increase in P4 secretion with concomitant drastic decrease in T secretion and a tendency to decrease in E2 secretion was observed. Similarly as in the case of small follicles, the action of PCB on steroid secretion by cells collected from medium follicles depended on time of exposure. The increase in T secretion and no influence on P4 and E2 secretion was observed after 2 days of exposure to PCB. Antiestrogenic action of PCB was noted after 4 and 6 days of exposure to PCB. In large, preovulatory follicles 2 days exposure to PCB had no effect on steroids secretion while longer exposition to this congener caused statistically significant antiestrogenic action.

Conclusion. The presented paper suggests various actions of PCB 153 as an endocrine disrupter on estradiol, progesterone and testosterone secretion from ovarian cells *in vitro* which were dependent on both exposure length and stage of the follicular development.

Key words: PCB 153 – Ovarian follicles – Estradiol – Progesterone – Testosterone - Steroid secretion – Estrous cycle

Among the environmental chemicals that may be able to disrupt the endocrine systems of animals and humans, the polychlorinated biphenyls (PCBs) are a chemical class of considerable concern. Polychlorinated biphenyls (PCBs) are lipophilic industrial chemicals that are regularly detected in human breast milk, serum, and tissues. One of the most prominent environmental contaminant is non-coplanar 2,2', 4,4', 5,5'-hexachlorobiphenyl (PCB 153). This congener is the most prevalent in biological tissues (Safe 1994; Kimbrough 1995; Krogenaes et al. 1998). Barsotti et al. (1976) showed that one of the most serious results of PCB exposure in the female monkey was the effect on reproduction. They observed menstrual cycle's irregularities and prolonged menstrual bleeding. The precise mechanism of PCBs' endocrine disrupting effect is unknown, but the effects may be caused via the interference with the synthesis, secre-

tion, transport, binding, action or elimination of endogenous hormones. McKinney and Waller (1994) and Pauwels et al. (1999) showed strong correlation between levels of PCB 153 in serum and follicular fluid obtained from women undergoing in vitro fertilization and embryo replacement. There are relatively few reports on PCB 153- induced endocrine reproductive changes. Recently, our data from studies involving isolated ovarian follicular cells, collected from preovulatory follicles and cultured as a monolayer or in co-culture, showed the dose-, time-, and tissue-dependent action of PCB 153 on steroid secretion (Wojtowicz et al.2000a, 2000b).

Taking into consideration that the disorders in folliculogenesis involve not only preovulatory follicles but mostly the early preantral and antral follicles, we attempted to characterize PCB 153 action on follicular steroidogenesis using a system of porcine theca and granulosa cells in co-culture collected from follicles of different size.

Material and Methods

Reagents. Parker medium M199, trypsin, and calf serum were purchased from the Laboratory of Sera and Vaccines, Lublin Poland. Antibiotic antimycotic solution (100x) and testosterone were obtained from Sigma Chemical Co. St. Louis, MO, USA. Stock solution of PCB 153 (2,2', 4,4', 5,5'-CB; 25 mg/ml) was prepared by dissolution of the pure powder in ethanol (Prochem GmbH, Wesel, Germany; purity 0.997).

Tissue preparation and cultures. Pig ovaries in the early, mid and late follicular phase of the estrous cycle were obtained from a slaughterhouse. Follicles were classified as small (1-3 mm in diameter), medium (4-6 mm in diameter) or large (7-10 mm in diameter) according to Gregoraszczuk and Skalka (1996). The separation of Gc from the theca layer was performed according to the technique described by Stoklosowa et al. (1982). Granulosa cells and theca cells were inoculated at a concentration of $6.8x10^6$ and $2.1x10^6$ cells/ml respectively, thus at the concentration comparable to that observed in vivo (Gc: Tc = 3:1). Cells were cultured in Parker Medium (M199) supplemented with 10 % calf serum with or without PCB153. The cultures were maintained at 37 °C in humidified atmosphere of 5 % CO_2.

The cells were cultured with 5.0, 10.0, 50 or 100 ng of PCB 153 for 48, 96, and 144 hrs and the media were frozen (-20 °C) prior to steroid analysis. Every treatment was conducted in 5 wells and each experiment was repeated 3 times.

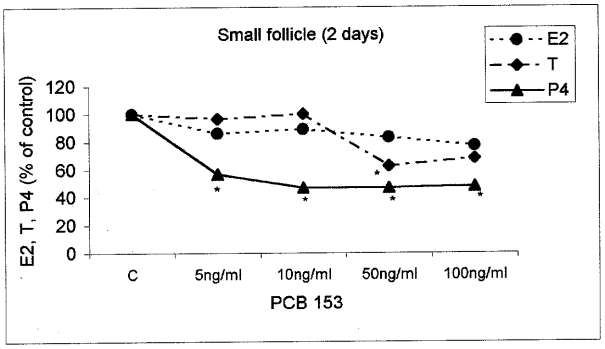
Steroid analysis. Progesterone, estradiol and testosterone concentrations were determined radioimmunologically using Spectria RIA kits (Orion, Diagnostica, Finland), supplied by Polatom (Świerk, Poland).

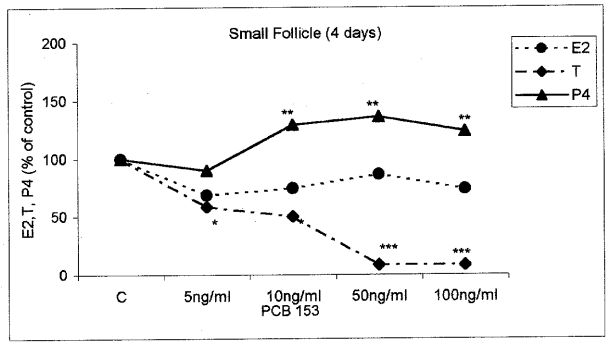
Statistical evaluation. All data points are expressed as means ± SEM from at least three different experiments (n=3), each in triplicates. Significance of differences between the concentrations of progesterone, estradiol and testosterone in the control and experimental cultures were compared by analysis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

Results

Small follicles. The exposure of follicular cells to all investigated doses of PCB 153 for 48 h caused significant decrease in progesterone secretion (56 %, 46.9 %, 46.8 % and 47.9 % of the control culture after 5, 10, 50 and 100 ng/ml of PCB 153, respectively, $P< 0.05$). After the doses of 50 and 100 ng/ml also a decrease of testosterone (T) secretion was found ($P<0.05$), while no effect on estradiol (E2) secretion was observed. (Fig. 1a). In addition, the increase in progesterone secretion was found after 96 h (129 %, 135 %, 123 % of the control cultures after 10, 50 and 100 ng/ml of PCB, respectively; $P< 0.01$) and after 144 h (260 %, 260 % of control level after 50 and 100 ng/ml of PCB, resp, $P< 0.001$) Exposure to PCB had no effect on estradiol secretion (Figs. 1b-c).

Medium follicles. Similarly as in the case of small follicles, the action of PCB 153 on steroid secretion depended on exposure length. PCB added for 48 h to the culture medium of cells isolated from medium-size follicles remarkably increased testosterone secretion (606 %, 585 % and 570 % of that in the control culture after 10, 50 and 100 ng/ml of PCB, respectively; $P<0.001$) and had no influence on progesterone and estradiol secretion (Fig. 2a). However, after the exposure has been prolonged to 96 h, a decrease in estradiol secretion after all doses pf PCB was found (64 %, 36.3 % and 34.1 % of the





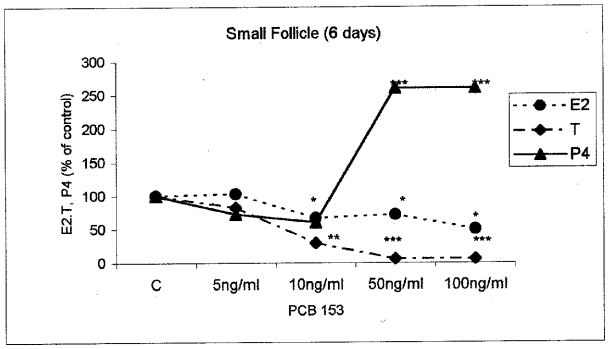
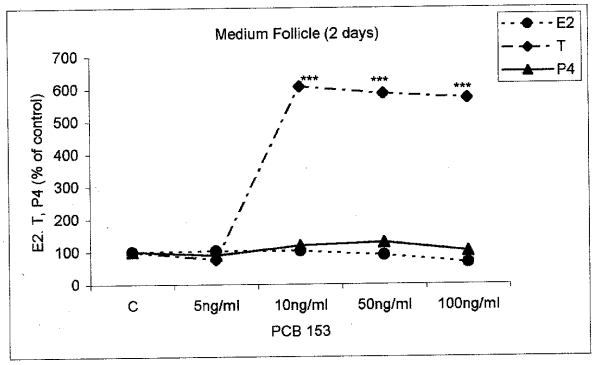
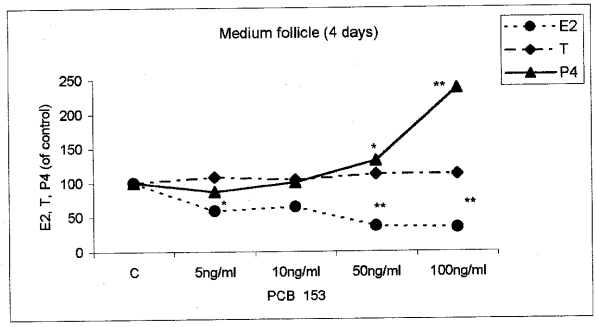


Fig 1 Dose-dependent effect of in vitro treatment with PCB 153 on progesterone (P4), testosterone (T) and estradiol secretion (E2) by small follicles. Data are means of 4 replicates. * P<0.05; ** P<0.01; *** P<0.001.





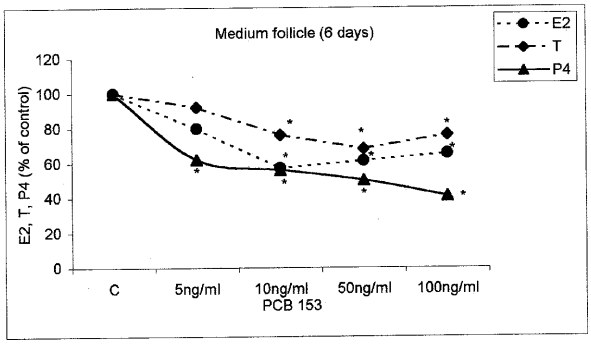


Fig. 2 Dose-dependent effect of in vitro treatment with PCB 153 on progesterone (P4), testosterone (T) and estradiol secretion (E2) by medium size follicles. Data are means of 4 replicates. * P<0.05; ** P<0.01; *** P<0.001.

control culture after 10, 50 and 100 ng/ml of PCB 153, respectively; P< 0.01) with simultaneous increase (P<0.01) in progesterone secretion under the influence of the highest dose of PCB (Fig. 2b). Decreased secretion of all the investigated steroids after 6 days of exposition to PCB was noted (Fig. 2c).

Large follicles. PCB153 added for 2 days to the culture media of cells collected from the preovulatory follicles did not influence their steroid secretion (Fig. 3a). However, after 4 days of exposure to this congener the tendency to decrease progesterone, testosterone and estradiol secretion was noted (Fig. 2b). Moreover, high antiestrogenic action of PCB 153 was observed after 6 days of exposure to PCB 153 resulting in a striking decrease of estradiol secretion(67.6 %, 17.1 %, 14.1 %, 12.7 % of controls after 5, 10, 50 and 100 ng/ml of PCB, respectively, P< 0.05 and <0.001) (Fig, 3c).

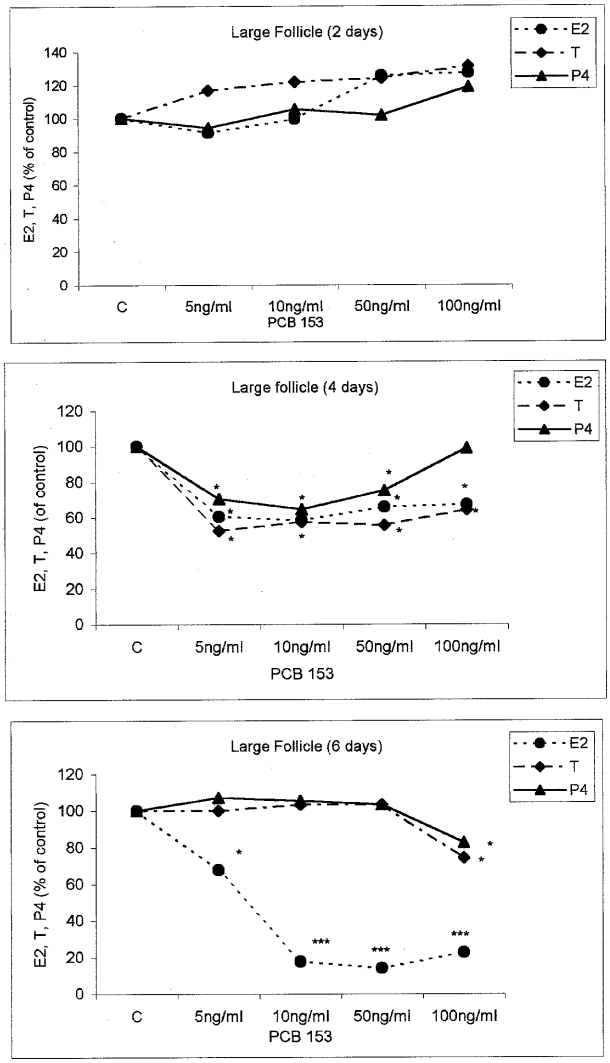


Fig. 3 Dose-dependent effect of in vitro treatment with PCB 153 on progesterone (P4), testosterone (T) and estradiol secretion (E2) by large preovulatory follicles. Data are means of 4 replicates. * P<0.05; ** P<0.01; *** P<0.001.

Discussion

The present study *in vitro* clearly demonstrates the alterations in follicular steroidogenesis induced by the polychlorinated biphenyl PCB 153. Ovarian follicles are remarkably vulnerable to agents that cause DNA damage such as ionizing radiation and chemotherapeutic drugs (Himelstein-Braw et al. 1977).

The observed decrease in progesterone (P4) and testosterone (T) secretion and no statistically significant effect on estradiol (E2) secretion by cells from small follicles exposed for 48 h to PCB suggests that it acts at the stages before the progesterone formation. This mechanism of action has been suggested for dioxin as an endocrine disrupter in follicular cells. Richards (1980) showed atretogenic action of testosterone on preantral follicles. However, longer exposure caused an increase in progesterone secretion with concomitant drastic decrease in testosterone secretion and a tendency to decrease estradiol secretion thus suggesting inhibition of P450 17a-hydroxysteroid dehydrogenase, an enzyme converting progesterone to testosterone.

The observed PCB153 induced increase in progesterone secretion by cells collected from small antral follicles with concomitant decrease in estradiol secretion accounts for the induction of luteinization and in this case inhibition of aromatization process in the follicles.

Previously it has been shown that high level of progesterone might have an inhibitory effect on aromatase activity (Schreiber et al. 1980; Gregorszczuk 1994). Taking into consideration that during the early stage of follicular differentiation, androgen acts as an enhancer of FSH-stimulated follicular differentiation, a failure in this process, as observed under the influence of PCB 153, might result in inappropriate androgenic stimulation, and consequently follicular atresia.

As follicular differentiation progresses, under increasing stimulation of FSH and LH (Tetsuka and Hillier 1997) androgen is mainly utilized as a substrate for estrogen synthesis.

In these follicles, the expression of androgen receptor (AR) is decreased and androgen acts mainly as a substrate for estrogen synthesis. For healthy follicular development a smooth transition of androgen utilization from action via AR to metabolism via $P450_{arom}$ is necessary. The relative expression of AR and $P40_{arom}$ may be an important factor determining the mode of androgen utilization.

In these experiments we observed that, similarly as in the case of small follicles, the action of PCB on steroid secretion by medium size follicles depended on the duration of exposure. The increase in testosterone and no influence on progesterone and estradiol secretion was observed after 2 days. Remarkable increase in testosterone levels in cultures of cells collected from medium antral follicles and then af-

ter longer exposure to PCB153 a decrease in estradiol secretion (96 h) or all investigated hormones after even longer exposure (144 h) points to possible action on follicular atresia. Antiestrogenic action of PCB noted during long-term exposure confirms this suggestion.

Heinrichs et al. (1971) suggested that exposure of women to high levels of DDT might account for the increased incidence of polycystic ovarian disease. In patients with polycistic ovary syndrome (PCOS), folliculogenesis does not proceed normally (Erickson and Yen 1984). The initial steps, recruitment and growth to the small graafian stages are functional in PCOS, but the terminal step, the selection of dominant follicles that can ovulate, does not occur regularly. In some unexplained way, this condition leads to the accumulation of large numbers of small graafian follicles (commonly referred to as cysts) in which the theca interstitial cells produce abnormally large amounts of androgen, but the granulosa cells fail to aromatize it to estradiol. A likely explanation for these changes is related to progesterone-testosterone-estradiol imbalance in the medium follicles, these hormones being responsible for follicular development and selection for ovulation. Fauser and Hsueh (1995) showed that final stages of follicular maturation are suppressed during chronic hyperandrogenism, as observed in the polycystic ovary syndrome.

In large preovulatory follicles the alteration of steroidogenesis was noted only after long term exposition to PCB 153. Drastic decrease in estradiol secretion after all doses of PCB used in the presented experiment was noted after 144 h of culture. The secretion of estradiol-17β in the maturing preovulatory follicles is obligatory for granulosa differentiation and induction of estrous at the proper time in relation to the preovulatory maturation (Ainsworth et al. 1990). This is in accordance with Li et al. (1994) who found that PCB 153 has an estrogenic effect in rats. Reduced circulating levels of estradiol without difference in serum concentration of progesterone, were observed also by Alvarez et al. (2000) in HCB (hexachlorobenzene - polychlorinated aromatic hydrocarbon) treated cycling rat. Low estrogen level in PCB treated follicles seem to be involved in blocked ovulation.

In conclusion, the current study demonstrated that the exposure of porcine follicular cells to PCB 153 influenced the steroidogenesis and the action of that congener was dependent on the follicular stage and duration of exposure. Taking into consideration that sites and effects of individual steroids vary with the developmental state of the follicles, it may be suggested that possible mechanism of PCB action consists of its ability to mimic natural hormones. It could be also concluded that PCB showed greater and faster effect on steroidogenesis when administered during early follicular development than during later stages of development.

Acknowledgements

The authors wish to thank Dr M. Mika for RIA determination of steroid hormones. This work was supported by a grant from State Committee for Scientific Research 0888/P06/2000/18

References

Ainsworth L, Tsang BK, Downey BR, Marcus GJ: The synthesis and actions of steroids and prostaglandins during follicular maturation in the pig. J Reprod. Fert. (Suppl) **40**, 137-150, 1990

Alvarez L, Randi A, Alvarez P, Piroli G, Chamson-Reig A, Lux-Lantos V, Kleiman de Pisarev D: Reproductive effects of hexachlorobenzene in female rats. J Appl Toxicol **20**, 81-87, 2000

Barsotti DA, Marlar A, Allen JR: Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248) Fd Cosmet Toxicol 14, 99-103, 1976

Erickson GF , Yen SSC: New date on follicle cells in polycystic ovaries: a proposed mechanism for the genesis of cystic follicles. Sem Reprod Endocrinol **2**, 231-243, 1984

Fauser BCM, Hsueh AJW: Genetic basis of human reproductive endocrine disorders. Hum Reprod **10**, 826-46, 1995

Gregoraszczuk EL, Skalka M: Thyroid hormone as a regulator of basal and human chorionic gonadotrophin-stimulated steroidogenesis by cultured porcine theca and granulosa cells isolated at different stages of the follicular phase. Reprod Fertil Dev **8,** 961-961, 1996

Gregoraszczuk EL: Is the progesterone a modulator of luteal steroidogenesis in pig? A tissue culture approach. Folia Hist. Cytobiol 32, 31-33, 1994

Heinrichs WL, Gellert RJ, Bakke JL, Lawrence NL: DDT administered to neonatal rats induced persistent

estrus syndrome. Science **13**;173 (997), 542-543, 1971

HIMELSTEIN-BRAW R, PETERS H, FABER M: Influence of irradiation and chemotherapy on the ovaries of children with abdominal tumours. Br J Cancer **36**, 269-275, 1977

KIMBROUGH R: Polychlorinated biphenyls (PSBs) and human health: an update. Crit Rev Toxicol **25**, 133-63, 1995

KROGAENAS AK, NAFSTAD I, SKARE JU, FARSTAD W, HAFNE AL: In vitro reproductive toxicity of polychlorinated biphenyls congeners 153 and 126. Reproductive Toxicology **12**, 575-580, 1998

LI MH, ZHAO YD, HANSEN LG: Multiple-ose toxicokinetic influence on the estrogenicity of 2,2',4,4'5,5'-hexachlorobiphenyl. Bull Environ Contam Toxicol **53**, 583-590, 1994

MCKINNEY JD, WALLER CL: Polychlorinated biphenyls as hormonally active structural analogues. Environmental Health Perspectives **102**, 290-297, 1994

PAUWELS A, COVACI A, DELBEKE L, PUNJABI U, SCHEPENS PJ: The relation between levels of selected PCB congeners in human serum and follicular fluid. Chemosphere **39**, 2433-2441, 1999

RICHARDS JS: Maturation of ovarian follicles: actions and interactions of pituary and ovarian hormones on follicular cell differentiation. Physiology Review **60**, 51-89, 1999

SAFE S: Polychlorinated biphenyls (PCBs) : environmental impact, biochemical and toxic responses and implications for risk assesment. Crit Rev Toxicol **24**, 87-147, 1994

SCHREIBER JR, NAKAMURA K, ERICSON F: Progestins inhibit FSH-stimulated steroidogenesis in cultured rat granulosa cells. Mol Cell Endocrinol **19**, 165-173, 1980

STOKLOSOWA S, GREGORASZCZUKL EL, CHANNING CP: Estrogens and progesterone secretion by isolated cultured porcine thecal and granulosa cells. Biol Reprod **26,** 943-952, 1982

TETSUKA M, HILLIER S.G: Differential regulation of aromatase and androgen receptor in granulosa cells. J Steroid Biochem. **61**, 233-239, 1997

WOJTOWICZ AK, GREGORASZCZUK EL, MIKA M: PCB 153 action on steroid secretion by cultured in vitro porcine theca and granulosa cells. Organohalogen Compounds **49**, 363-366, 2000b

WOJTOWICZ AK, GREGORASZCZUK EL, LYCHE JL, ROPSTAD E: Time-dependent and cell-specific action of polychlorinated biphenyls (PCB 153 and PCB 126) on steroid secretion by porcine theca and granulosa cells in mono- and co-culture. Journal Physiol and Pharmacol **51**, 555-568, 2000a

Corresponding author:

Anna K. Wojtowicz, M. D.
Dept. of Animal Physiology
Jagiellonian University
Ingardena 6
30-060 Krakow
Poland