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APOPTOSIS IN CHICKEN OVARIAN FOLLICLES FOLLOWING *IN VITRO* EXPOSURE TO TCDD, PCB 126 AND PCB 153*

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Abstract

The study was conducted in order to compare the *in vitro* effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) on the number of apoptotic cells and the activity of caspase-3 in chicken ovarian follicles. The ovarian stroma, white (WF) and yellowish (YF) prehierarchical follicles and fragments of the theca and granulosa layers of the 3 largest preovulatory follicles (F3–F1) were *in vitro* exposed to TCDD (10 nM), PCB 126 (10 nM) and PCB 153 (10 µM) for 24 h. After incubation the number of apoptotic cells and caspase-3 activity were determined by TUNEL method and fluorometric assay, respectively. PCB 126 and PCB 153 increased the number of apoptotic cells in the ovarian stroma while TCDD and PCB 126 elevated it in the WF follicles. Under the control conditions, caspase-3 activity steadily increased along with maturation of the follicles, reaching the highest level in the theca layer of the F1 follicle. The activity of this enzyme in the granulosa layer of F3–F1 follicles was on average 60% lower in comparison to the stroma. Exposure to TCDD elevated caspase-3 activity in prehierarchical follicles and in the granulosa layer of F2 and F1 preovulatory follicles. On the contrary, PCB 126 exerted a suppressive effect on caspase-3 activity in the WF follicles and the granulosa layer of the F2 follicle, and PCB 153 in the theca layer of F2 and F1 and the granulosa layer of the F3 follicle. In conclusion, the results indicate that TCDD and PCBs affect apoptosis in chicken ovarian follicles and in consequence may disrupt follicle development.

Key words: apoptosis, caspase-3 activity, TCDD, PCBs, chicken ovarian follicles

Dioxins and polychlorinated biphenyls (PCBs) are persistent environmental contaminants which exert various endocrine toxic effects (Schecter et al., 2006). Dioxins are mainly formed as unwanted by-products in thermal and industrial processes.

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PCBs, however, were intentionally manufactured for industrial purposes and commercial applications from 1929 to 1979 (Kulkarni et al., 2008). The endocrine toxicity of dioxins and PCBs is primarily mediated through the aryl hydrocarbon receptor (AHR) (Antkiewicz et al., 2006; Marshall and Kerkvliet, 2010). Several lines of evidence indicate that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PCBs exert a negative effect on reproduction in birds (Giesy et al., 2003; Ottinger et al., 2009; Hrabia et al., 2013; Sechman et al., 2014). Exposure to TCDD causes inhibition of egg laying (Ikeda et al., 2004), increases embryo mortality, decreases hatchability and the body weight of hatched chicks (Blankenship et al., 2003; Bruggeman et al., 2003) and decreases the number of prehierarchical white follicles in adult chickens (Bruggeman et al., 2005). Since the chicken ovary expresses the AHR (Antos et al., 2015; Wójcik et al., 2015), it provides an exceptional model for studying the disturbing action of dioxins and other chemicals acting via this receptor. Our previous studies revealed that TCDD decreases secretion of sex steroid hormones and expression of genes encoding major steroidogenic enzymes (Sechman et al., 2014) and alters the transcription and activity of detoxification enzymes in chicken ovarian follicles (Antos et al., 2015).

The chicken ovary contains the stroma with primordial follicles, white (1–4 mm; WF) and yellowish (4–8 mm; YF) prehierarchical follicles, and five to eight preovulatory follicles that are arranged in a distinct hierarchy according to size (9–35 mm), with the largest (F1) ovulating first. White follicles are highly susceptible to atresia; only 5% reach a diameter of 6–8 mm and enter the follicular hierarchy. In contrast, hierarchical follicles are committed to ovulation and under normal physiological conditions do not become atretic (Gilbert et al., 1983; Johnson, 1996; Proszkowiec-Węglarz et al., 2005). Tissue homeostasis is maintained mainly by apoptosis, which eliminates cells without the initiation of an inflammatory response (Johnson and Bridgham, 2002). One of the most important ways leading to apoptosis is activation of caspases (cysteine proteases). This activation leads to the cleavage of numerous cellular proteins. It is widely considered that the most important of the executioner caspases is caspase-3 (McIlwain et al., 2013). In the chicken, TCDD and PCBs increase the number of apoptotic thymocytes and cause thymic atrophy (Goff et al., 2005). Moreover, TCDD has an influence on cardiac apoptosis (Ivnitski et al., 2001) and PCBs may initiate apoptosis in the chicken shell gland (Hrabia et al., 2013). In avian pre-B cells, TCDD stimulates the apoptosis mediated via AHR (Puebla-Osorio et al., 2004).

Despite the above-mentioned data regarding the effects of TCDD and PCBs on chicken gonadal function, there are, surprisingly, no data in the available literature concerning the effects of dioxins and PCBs on the apoptosis in the chicken ovary. Therefore, the present study was undertaken to examine the *in vitro* effect of TCDD, 3,3',4,4',5-pentachlorobiphenyl (PCB 126; co-planar ongener) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153; non-coplanar congener) on the ovarian cell apoptosis and activity of caspase-3 in chicken ovarian follicles.

Material and methods

Chemicals

Antibiotic-antimycotic solution, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Trizma® buffer substance (Sigma, St. Louis, USA); TCDD, PCB 126, PCB 153 (Dr. Ehrenstorfer GmbH, Germany), Eagle's medium (Laboratory of Sera and Vaccines, Lublin, Poland); *In situ* cell-death detection kit POD-horseradish peroxidase (Roche Diagnostics, Mannheim, Germany), Caspase-3 fluorometric assay kit (BioVision, Milpitas, USA). All other reagents were purchased from ICN Biomedicals (USA) or POCH (Poland).

Animals

All procedures were approved by the First Local Animal Ethics Committee in Kraków, Poland. The experiments were performed on 28-week-old Hy-Line Brown laying hens. The birds were caged individually at a neutral temperature (18–20°C), under a 14L:10D photoperiod with free access to commercial feed and water. Oviposition was checked every 15 min between 7:00 and 15:00. Ovulation was considered to occur within 15 min after oviposition of the previous egg in the series. Hens (n = 6 in each experiment) were sacrificed by decapitation approximately 2 h after a midsequence ovulation (i.e. about 22.5 h before the next ovulation).

Effects of TCDD, PCB 126 and PCB 153 on the number of apoptotic cells in the chicken ovarian stroma and white follicles (Experiment I)

The ovarian stroma and white prehierarchical follicles (1–4 mm; WF) were isolated from the ovary. The fragments of the ovarian stroma and groups of three intact WF were placed in separate wells and incubated in 1 mL of Eagle's medium supplemented with 0.05% bovine serum albumin, 2 µL/mL antibiotic-antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 µg amphoteracin B/mL) in the following four groups: control, medium with the addition of TCDD (10 nM), PCB 126 (10 nM), and PCB 153 (10 µM). Stock solutions of TCDD and PCBs were dissolved in DMSO and subsequently diluted in Eagle's medium (the final concentration of DMSO in medium was below 0.1%). The TCDD concentration in the medium was chosen on the basis of the dose-response curve from our previous studies (Sechman et al., 2014) while PCB 126 and PCB 153 concentrations were established on the basis of the toxicity equivalent factor (TEF) in birds shown by van den Berg et al. (1998), and the dose-response curve carried out in the previous *in vitro* experiment (Sechman et al., 2016) where the fragments of the stroma and the WF were incubated in the medium supplemented with increasing concentrations of PCB 126 (0.01–1000 nM) or PCB 153 (0.01–1000 µM); data not shown. The incubation was performed in 6 replications (n = 6) at 39°C for 24 h in an atmosphere of 95% air and 5% CO_2. After incubation, the tissues were fixed in freshly prepared 4% buffered (0.1 M phosphate buffer, pH = 7.6) formalin and embedded in paraffin wax for subsequent localization of apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method.

Effects of TCDD, PCB 126 and PCB 153 on the caspase-3 activity in the chicken ovarian follicles (Experiment II)

The white (1–4 mm; WF) and yellowish (4–8 mm; YF) prehierarchical follicles and the 3 largest preovulatory follicles (20–36 mm; F3–F1; F3<F2<F1) were isolated from the ovary. With respect to the preovulatory follicles, the granulosa layer was separated from the theca one. The theca and granulosa layers of F3–F1 follicles were divided into 4 equal pieces. The prehierarchical follicles were incubated as intact ones. The YF were incubated individually while the WF were incubated in groups of three. The incubation was processed in accordance with the schedule presented in experiment I, and with application of the same xenobiotic doses. After incubation, the tissues were collected and stored at –80°C until determination of caspase-3 activity.

TUNEL assay for apoptosis

Analysis was performed as described previously by Hrabia et al. (2011). Briefly, deparaffinized and rehydrated slides (6 μm thick) of the stroma and the WF follicles were incubated with proteinase K (20 μg/ml) in 10 mM TRIS-HCl, pH 7.4, at 37°C for 20 min and apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) method by using an *in situ* cell death detection kit POD according to the protocol provided by the manufacturer. Negative controls were prepared by incubating slides without TdT. To visualize the immunoreaction products, sections were incubated with a mixture of DAB and H_2O_2. Slides were examined and apoptotic (TUNEL-positive) cells were counted with a computerized image-analysis system (MultiScanBase v. 14.02, Computer Scanning System, Warsaw, Poland) in 10 random areas (50 μm × 50 μm) of each examined ovarian stroma and white follicles and averaged for each bird. The mean value was calculated from six chickens.

Caspase-3 activity determination

The enzymatic activity of caspase-3 was measured according to Monroe et al. (2002) and Hrabia et al. (2014) with a ready-to-use fluorometric assay kit (BioVision, USA). Briefly, tissue samples were homogenized in lysis buffer (100 mg tissue per 300 μl buffer) and centrifuged. Supernatants were collected and total protein concentrations were determined by the Bradford method. A 100 μg aliquot of protein from each sample was transferred in duplicate to 96-well microplates. Immediately after a 2 h incubation at 37°C in reaction buffer with substrate, fluorescence at 400 nm excitation and 505 nm emission was read by a Fluorescence Microplate Reader FLx800 (BioTek Instruments, Winooski, USA). The activity of caspase-3 in the samples was normalized and expressed as the relative activity.

Statistical analysis

Data of the experiments were statistically analyzed by one-way ANOVA followed by Duncan's multiple range test using Sigma Stat 2.03 (Systat Software GmbH, Germany). Log transformations were performed as needed to maintain homogeneity of variance. Figures were prepared using Grapher 10.3 (Golden Software Inc., USA). Values are expressed as the mean ± SEM and are considered significantly different at $P<0.05$.

Results

Number of apoptotic cells

The apoptotic cells were present in ovarian stroma with primordial follicles and WF (Figure 1). In the ovarian stroma, TUNEL-positive cells were mainly detected in the connective tissue and the granulosa layer of primordial follicles. In the WF, apoptotic cells were localized in the epithelium as well as in the theca and granulosa layers. The TUNEL-positive cells were more numerous in the WF than in the stroma (Figure 2 a, b). In comparison with the control group, exposure to TCDD increased the number of apoptotic cells per unit area (50 μm × 50 μm) in the WF by 20.4% ($P<0.05$; Figure 2 b). PCB 126 increased the number of TUNEL-positive cells in the ovarian stroma and the WF by 53.4% and 29.6%, respectively ($P<0.05$; Figure 2 a, b) and PCB 153 elevated their number in the ovarian stroma by 47.4% ($P<0.05$; Figure 2 a). It should be noted that after 24 h of incubation the tissue structure was partly degenerated, which caused difficulties in histochemical analysis.

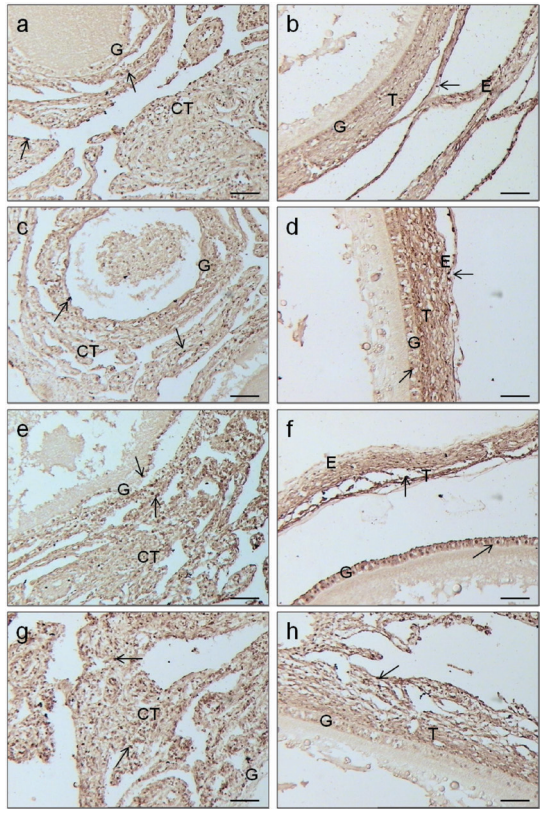

Figure 1. Localization of apoptotic cells (TUNEL-positive; arrows) in the ovarian stroma with primordial follicles (a, c, e, g) and white follicles (b, d, f, h) incubated for 24 h in control medium (a, b) and with addition of TCDD (c, d), PCB 126 (e, f) and PCB 153 (g, h). Apoptotic cells are present in the granulosa (G) layer and connective tissue (CT) of ovarian stroma and in epithelium (E), the theca (T) and the granulosa (G) layer of white follicles

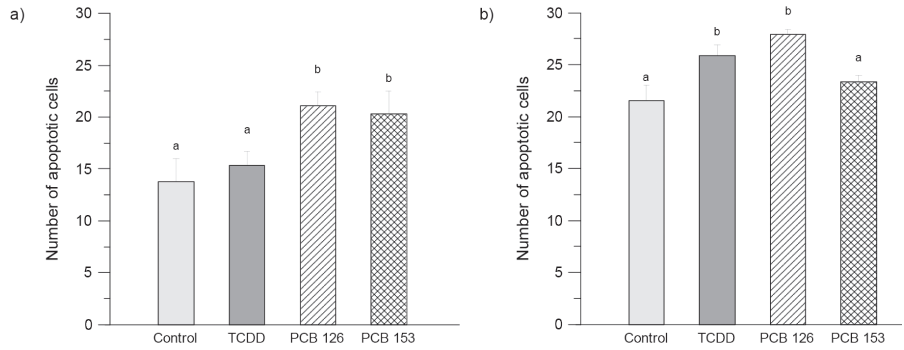

Figure 2. Effect of TCDD, PCB 126 and PCB 153 added to incubation medium on number of apoptotic cells in the ovarian stroma (a) and the white follicles (b) of the laying hen. Data represent the mean ± SEM from 6 replicate experiments. TUNEL-positive cells counted on 10 randomly chosen fields (50 μm × 50 μm) of each stroma and WF were average for each individual and for that microscopic field size and subsequently the mean value for six chickens was calculated. Values marked with different letters differ significantly (P<0.05)

Caspase-3 activity in chicken ovarian follicles

In the control conditions the activity of caspase-3 was detected in prehierarchical follicles (WF and YF) as well as in the theca and granulosa layers of F3–F1 preovulatory follicles. The level of caspase-3 activity in the WF was set as 1. The lowest caspase-3 activity (0.37) was observed in the granulosa layer of the F2 follicle and the highest activity (2.08) in the theca layer of the F1 follicle (Figure 3).

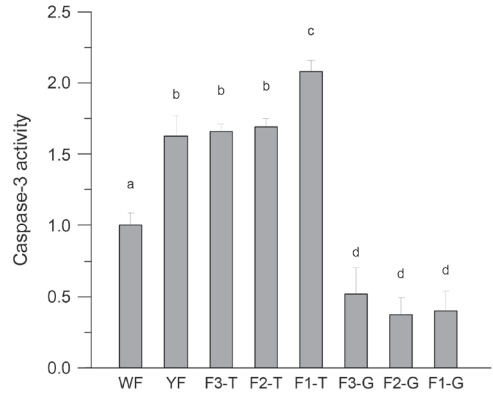

Figure 3. Caspase-3 activity in the white (WF) and yellowish (YF) prehierarchical follicles, the theca (T) and granulosa (G) layers of preovulatory follicles (F3–F1) of the laying hen incubated in a control medium for 24 h. Each value represents the mean ± SEM from 6 determinations. Values marked with different letters differ significantly (P<0.05)

In comparison with the control group, TCDD increased (P<0.05) caspase-3 activity in the WF and YF by 39% and 53%, respectively (Figure 4 a). Exposure to TCDD also stimulated caspase-3 activity in the granulosa layer of F2 and F1 follicles by

82% and 141%, respectively (P<0.01; Figure 4 c). PCB 126 decreased caspase-3 activity in the WF and granulosa layer of F2 follicles by 47% and 33%, respectively (P<0.05; Figure 4 a, c). Exposure to PCB 153 decreased caspase-3 activity in the theca layer of F2 and F1 follicles by 23% and 21%, respectively (P<0.05; Figure 4 b) and in the granulosa layer of F3 follicle by 40% (P<0.05; Figure 4 c).

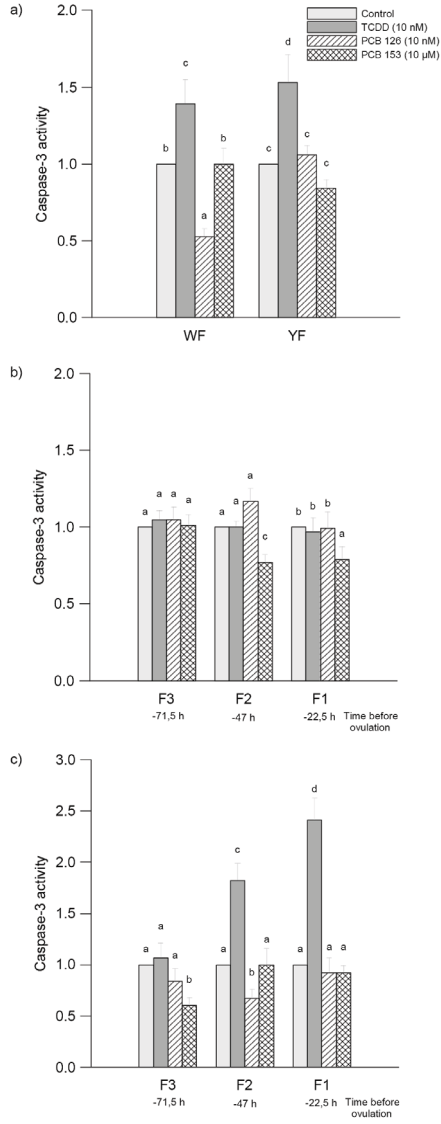

Figure 4. Effect of TCDD, PCB 126 and PCB 153 added to incubation medium on caspase-3 activity in the white (WF) and yellowish (YF) prehierarchical follicles (a), and the theca (T) (b) and granulosa (G) layers (c) of preovulatory follicles (F3–F1) of the laying hen. Data represent the mean ± SEM from 6 replicate experiments standardized to control activity in each follicle, which was set as 1. Values marked with different letters within each follicle differ significantly (P<0.05)

Discussion

The present study demonstrated, for the first time, the *in vitro* effects of TCDD, PCB 126 and PCB 153 on incidence of apoptosis in the ovarian follicles in chickens. Follicular atresia mediated via apoptosis is one of the most important processes involved in the function of the ovary. The intensity of atresia is high in smaller chicken ovarian follicles, while it is rare in large follicles (Gilbert et al., 1983). Results of this study showed that following PCB 126 and PCB 153 exposure the number of apoptotic cells (TUNEL-positive) increased in the ovarian stroma by about 50%. In respect to the white follicles, the number of apoptotic cells also increased, however after exposure to both coplanar compounds, TCDD and PCB 126. The non-coplanar congener PCB 153 did not affect the number of apoptotic cells in these follicles. These observations may indicate different levels of susceptibility of the follicles to TCDD and PCB toxicity.

One of the most important ways leading to apoptosis is the activation of a caspase cascade, therefore next in the study we assessed the *in vitro* effect of TCDD and two PCB congeners on caspase-3 activity. Under the control conditions, in comparison with the white follicles the caspase-3 activity was higher in yellowish follicles and the theca layer of F3 and F2 follicles, and highest in the theca layer of the F1 follicle. These results indicate that the activity of caspase-3 increases gradually during growth and maturation of the follicle. Unsurprisingly, the caspase-3 activity in WF and YF was higher than in the granulosa layer of F3–F1 follicles, since granulosa cells of prehierarchical follicles are much more susceptible to apoptosis compared to granulosa cells of preovulatory follicles. In F3–F1 preovulatory follicles the activity of this enzyme was 3.2-, 4.6- and 5.2-fold higher in the theca layer than in the granulosa one, respectively. This observation is consistent with findings reported by Johnson and Bridgham (2000), who for the first time showed the higher expression of procaspase-3 protein in the theca layer than in the granulosa layer of preovulatory follicles of the hen. However, they showed that the level of procaspase-3 protein does not change significantly in the theca layer during follicle development, which was inconsistent with our results concerning caspase-3 activity. Moreover, the low activity of caspase-3 in the granulosa layer of preovulatory follicles may be associated with the functional differentiation of the granulosa cells, as well as resistance of these cells to apoptosis, which occurs after selection into the preovulatory hierarchy (Johnson, 1996; Johnson and Woods, 2009).

Exposure to TCDD steadily elevated caspase-3 activity in prehierarchical follicles similarly to the number of TUNEL-positive cells. The gradual increase of caspase-3 activity following TCDD exposure was also found in the granulosa layer of the preovulatory follicles. On the other hand, TCDD had no effect on caspase-3 activity in the theca layer of these follicles. Our results clearly indicate a stimulatory effect of TCDD on caspase-3 activity in the granulosa layer which elevates with the maturational state of the follicle. Previous studies on mammals consistently showed that the effect of TCDD on apoptosis differs by species. For instance, Piasecka-Strader et al. (2016) did not find any effect of TCDD on the apoptosis in granulosa cells from antral follicles. The exposure to TCDD had also no influence on atresia in

mouse antral follicles (Karman et al., 2012). On the other hand, the induction of apoptosis was observed in Chinese Hamster Ovary cell line following PCB77, a planar dioxin-like PCB congener, treatment (Murati et al., 2015).

Contrary to TCDD, polychlorinated biphenyls exerted only a suppressive effect on caspase-3 activity in most of the examined follicles. Similar effects were observed by Gregoraszczuk et al. (2003), who found a reduction in caspase-3 activity in cultured granulosa and theca cells of large (8–12 mm) porcine ovarian follicles following PCB 126 or PCB 153 exposure. The inhibitory effect of PCBs on activity of this enzyme in preovulatory follicles seems to be very interesting since the reduction of caspase-3 activity was found in the granulosa layer of the F3 follicle, both layers of the F2 follicle and the theca layer of the F1 follicle. Surprisingly, PCB 126 elevated the number of apoptotic cells but decreased caspase-3 activity. It is worth noting that the PCB-induced number of apoptotic cells may not be related with caspase-3 activity. It is known that mitochondria can be triggered to release their proapoptotic factors, e.g. apoptosis-inducing factor (AIF) by a mechanism that does not require caspase activation (Yu et al., 2002). The apoptotic inductors may activate caspases or release the AIF without activation of caspase cascade. AIF protein migrates to the nucleus and activates the endonuclease G (Cregan et al., 2004). It cannot be excluded that PCB 126 stimulates the apoptosis of cell acting via a pathway, which omits caspase-3 activation. Although the TUNEL assay is widely applied as a specific indicator of apoptosis, the positive reaction in TUNEL assay is also given by necrotic and autolysed cells (Grasl-Kraupp et al., 1995). Thus, the elevation in the incidence of TUNEL-positive cells observed in our investigation may be caused at least in part by the cytotoxic effect of PCBs. Moreover, in cell culture, unlike *in vivo*, apoptotic cells are not phagocytosed and eventually undergo secondary necrosis, so the metabolic changes seen in cultures may be due to the transition from apoptosis to necrosis, rather than to the induction of apoptosis itself (Cejna et al., 1994). This suggestion may be supported by the partly degenerated structure of tissues seen in this study after 24 h of incubation. Further investigations are necessary to explain the molecular mechanism of PCB 126 action in the chicken ovary.

In conclusion, we presented the basal caspase-3 activity in chicken ovarian follicles. This activity depended on the maturational state and the layer of the follicle. Furthermore, the results support the suggestion that chicken ovarian follicles are a target tissue for the action of dioxins and structurally related compounds. Taking under consideration the results of the present study, we suggest that TCDD and PCBs affect ovarian cell apoptosis and in consequence may disrupt follicle development.

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