Apoptosis in bovine cumulus–oocyte complexes after exposure to polychlorinated biphenyl mixtures during *in vitro* maturation

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Abstract

Aroclor-1254 (A-1254) is a commercial mixture of coplanar (dioxin-like) and non-coplanar (non dioxin-like) polychlorinated biphenyls (PCBs) affecting bovine oocyte *in vitro* maturation (IVM) and developmental competence. In the present study, the role of cumulus cell apoptosis in mediating the toxic effects of PCBs during *in vitro* maturation has been investigated. Results indicate that exposure of cumulus–oocyte complexes (COCs) to A-1254 significantly induced apoptosis of cumulus cells. Furthermore, A-1254 significantly increased the expression of the pro-apoptotic gene, Bax, concomitantly reducing the level of the anti-apoptotic gene, Bcl-2, in the cumulus cell compartment. The effects of pure mixtures of coplanar (PCB 77, 126 and 169) or non-coplanar (PCB 52, 101 and 153) PCBs were examined. Exposure of COCs to coplanar PCBs affected maturation at doses as low as 100.6 pg/ml. Furthermore, a significant increase in apoptosis and in Bax mRNA expression was observed. No variations in maturation or apoptosis were observed in the non-coplanar PCB group. To further analyze the role of cumulus cells, COCs and denuded oocytes (DOs) have been exposed to A-1254 or coplanar PCBs during IVM. Exposure of COCs significantly reduced the percentage of matured oocytes after 24 h of culture in both treatments. In contrast, exposure of DOs significantly decreased the maturation rate only at the highest dose investigated (100-fold greater than that affecting COCs). Taken together, the results indicate a direct role of cumulus cell apoptosis in mediating PCB toxicity on bovine oocytes, and a direct relationship between congener planarity and toxicity in bovine oocytes is suggested.

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Introduction

Polychlorinated biphenyls (PCBs) are a structurally related group of halogenated aromatic hydrocarbons that consist of 209 isomers and congeners with different numbers and positions of chlorine atoms substituted on the biphenyl moiety. PCBs have gained widespread industrial applications due to their physical and chemical properties. They were synthesized for approximately 60 years, from the early 1920s until they were banned in many countries during the late 1970s. Because of their chemical stability and extensive industrial use, it became evident in the mid 1960s that PCBs had become ubiquitous and persistent environmental contaminants (Cantlon 1983). To date, it is estimated that 10^8 kg PCBs still reside in the biosphere (Boyle *et al.* 1992) and about 113 different congeners are most frequently detectable in the environment (Crisp *et al.* 1998). PCBs are small molecules with a low solubility in water but a high solubility in organic solvents, oils and fat

(Ballschmiter *et al.* 1989). As a result of this lipophilic nature, and their stability and resistance to degradation, PCBs can be found at all levels of the food chain and can accumulate in the animal and human body (Jones 1988, McFarland & Clarke 1989).

PCBs occur in mixtures of multiple congeners that differ in the numbers and positions of chlorine around the biphenyl ring. It has been reported that different congeners can exert distinct effects (Geisy & Kannan 1998). PCB congeners without ortho-chlorine substitution have a coplanar structure similar to 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) and bind to the arylhydrocarbon receptor (AhR), causing TCDD-like toxicity. However, congeners with ortho-chlorines favor a nonplanar conformation and do not bind to the AhR, eliciting different toxicity from TCDD (Kodavanti & Tilson 1997).

PCBs exert a variety of toxic effects such as carcinogenicity, immunotoxicity, teratogenicity and reproductive toxicity (Battershill 1994, Safe 1994). Reproductive disorders

caused by PCBs were first described in the Baltic seal (Reijnders 1986). PCB exposure during pregnancy is reported to cause altered motor function (Pantaleoni *et al.* 1988) as well as decreased levels of neurotransmitters in the brain (Seegal *et al.* 1986, 1991). Maternal PCB exposure can lead to pre- and post-implantation embryo loss and reduced survival of the offspring (Ahlborg *et al.* 1992, Battershill 1994, Seiler *et al.* 1994). Particularly, PCBs have been reported to decrease implantation rate and litter size in mice and rats (Lindner *et al.* 1974) and to cause reproductive failure in mink (Platonow & Karstad 1973). Several reports described the presence of various environmental pollutants, including PCBs, in human follicular fluid (Trapp *et al.* 1984, Pflieger-Bruss & Schill 2000) and human ovarian tissue (Mes 1990).

We have recently demonstrated that PCBs have a severe negative effect on reproductive functions in cattle (Pocar *et al.* 2001*b*). An environmentally relevant mixture of over 60 PCB congeners (Aroclor-1254, A-1254) affected oocyte maturation, fertilization and embryo development at doses that ranged between 0.001 and 1 µg/ml, the minimum effective dose (0.001 µg/ml) being approximately 10-fold lower than the mean level found in human follicular fluid in non-exposed women (Trapp *et al.* 1984). In addition, we observed that, besides a decreased ability of the oocyte to complete the nuclear maturation, a significant impairment of cytoplasmic maturation was observed upon exposure to A-1254 (Pocar *et al.* 2001*a*).

During the process of oocyte development, a close association exists between the oocyte and the surrounding cumulus cells. The cumulus cells form a multilayered mass of cells that surround the oocyte during the maturation process within the follicle. Their function is to protect the developing oocyte and to provide nutrients to it through gap junctions within the cumulus mass and between the latter and the oocyte (Mori *et al.* 2000, Tatemoto *et al.* 2000, Fatehi *et al.* 2002). Cumulus cells are not required for meiosis resumption, but are necessary for both final nuclear and cytoplasmic maturation and can influence the development of the oocyte (Perez & Tilly 1997, Sun *et al.* 2001). Due to this close association between oocyte and somatic cells, it is reasonable to hypothesize that cumulus cells may be a target for toxicological injury during oocyte maturation.

One of the ovarian regulative events that may be susceptible to modulation by PCBs is apoptosis. The development of the follicle and its cumulus–oocyte complex (COC) is influenced by various apoptotic mechanisms (Tilly 1996). The spatiotemporal pattern of apoptosis during follicle growth and oocyte maturation is tightly regulated. Disruption of either timing or the magnitude of apoptosis can alter the cell connectivity in the cumulus mass and between cumulus and oocyte, causing deficits in oocyte quality. In fact, the degree of apoptosis has been correlated with the developmental competence of the enclosed oocytes in the bovine (Ikeda *et al.* 2003).

It has been reported that apoptosis may contribute to PCB-induced toxicity. For example, it was demonstrated that apop-

totic cell death induced by 2,2',4,6,6'-pentachlorobiphenyl (PCB 104) or Aroclor-1254 may be one possible mechanism of PCB-mediated immunosuppression of human monocytic cells and murine splenocytes (Yoo *et al.* 1997, Shin *et al.* 2000). In addition, results published by Hwang *et al.* (2001) indicated that 2,2',5,5'-tetrachlorobiphenyl (PCB 52) can induce apoptosis of neuronal SK-N-MC cells in a process that involves down-regulation of Bcl-2 expression. Finally, it has been reported that exposure to 3,3',4,4'-tetrachlorobiphenyl (PCB 77) and 2,2',4,6,6'-pentachlorobiphenyl (PCB 104) can stimulate apoptotic cell death of endothelial cells (Slim *et al.* 2000, Lee *et al.* 2003).

The goals of the study reported herein were threefold: (1) to determine whether the effects of PCBs on bovine oocytes are linked to increased apoptosis in the cumulus cell mass during *in vitro* maturation, (2) to explore whether congener planarity is relevant for PCB toxicity in bovine oocytes and (3) to investigate the role of cumulus cells on the PCB-induced toxicity previously observed in bovine COCs.

Materials and Methods

Polychlorinated biphenyls

Aroclor-1254 was purchased from Supelco (Bellefonte, PA, USA), dissolved in ethanol (Merck, Darmstadt, Germany) and stored at a final concentration of 100 mg/ml. The stock solution was serially diluted in maturation medium to obtain the desired working concentrations as described in the experimental design, with a maximum content of ethanol in the medium of 0.1% (v/v). The same amount of ethanol was present in the control medium.

The PCB congeners employed in the present study were highly purified (99.2 to 99.8 purity according to the supplier's certification after HPLC/DAD and HRGC/MS analysis; Ökometric GmbH, Bayreuth, Germany). Two mixtures of non-*ortho*-substituted PCB congeners and of *ortho*-substituted PCB congeners respectively (designated as co-planar and non-coplanar) were selected. The calculated amount of powder was dissolved in DMSO to a final concentration of 3 µg total PCBs/ml, in the proportions listed in Table 1. Serial dilutions were freshly made to obtain the desired final concentration as described below.

Table 1 Composition of the PCB mixtures used in the present study.

	IUAPC no.	Weight (%)
Coplanar PCB mixture		
3,3',4,4'-tetrachlorobiphenyl	77	33.3
3,3',4,4'-tetrachlorobiphenyl	126	33.3
3,3',4,4',5,5'-hexachlorobiphenyl	169	33.3
Non-coplanar PCB mixture		
2,2',5,5'-tetrachlorobiphenyl	52	60.0
2,2',4,5,5'-pentachlorobiphenyl	101	20.0
2,2',4,4',5,5'-hexachlorobiphenyl	153	20.0

Samples with DMSO without PCBs were used as controls (final concentration of DMSO in medium 0.3%).

Cumulus–oocyte complexes (COCs) collection

Ovaries were collected from a local slaughterhouse and transported, within 2 h, to the laboratory in Dulbecco's phosphate balanced saline (PBS), supplemented with 100 000 IU penicillin, 100 mg streptomycin and 250 µg amphotericin B per liter, maintained at 32–34 °C. All subsequent procedures were conducted at a constant temperature of 36 °C.

COCs were collected from ovarian follicles by slicing with a razor blade in modified Dulbecco's PBS (cat # D6650) supplemented with 2 IU heparin and 0.1% BSA Fraction V. Intact COCs were collected in tissue culture medium (TCM) 199 (cat # M 5017) supplemented with 0.4% BSA (cat # A 3156), 25 mM Hepes and 10 µg/ml heparin. COCs were then washed three times in the same medium. Only COCs with at least 3 complete layers of cumulus cells and finely granulated homogeneous ooplasm were selected as suitable for *in vitro* maturation (IVM) and were used for the following experiments, as previously described (Gandolfi *et al.* 1997). Cumulus-free oocytes (denuded oocytes, DOs) were obtained by mechanically removing cumulus cells from selected COCs with a narrow-bore pipette.

In vitro maturation

Basic maturation medium (bMM) was TCM 199, supplemented with 0.68 mM L-glutamine, 25 mM NaHCO₃, 10% (v/v) fetal calf serum, 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 5 IU/ml human chorionic gonadotropin (hCG) (Suigonan, Intervet, Wiesbaden, Germany) and 1 µg/ml 17β-estradiol. Groups of 25–35 COCs or DOs were matured in 500 µl bMM in four-well dishes (Nunc, Roskilde, Denmark). COCs were incubated for 24 h at 39 °C in a humid atmosphere of 5% CO₂ in air.

Evaluation of nuclear maturation

To assess the rate of meiosis at the end of IVM, a total of 373 oocytes, separated in groups according to the treatment, were analyzed. Oocytes were completely denuded of cumulus cells by repeated pipetting, recovered under a stereomicroscope and transferred onto glass slides in a small drop of fluid. Silicone was used to maintain a coverslip in contact with the oocytes without exerting excessive pressure. The slides were immersed in a 3:1 fixative solution of ethanol/acetic acid for a minimum of 24 h. Nuclear morphology was assessed by staining with 1% lacmoid and specimens were examined under a phase contrast microscope. Oocytes were classified as: immature (germinal vesicle (GV) and GV breakdown stage), intermediate (metaphase I and anaphase I), and matured (telophase I and metaphase II). Oocytes showing either multipolar meiotic spindle, irregular chromatin clumps or

no chromatin were considered as degenerated (Luciano *et al.* 1999).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

To assess the rate of apoptotic cells, a total of 162 COCs, separated in groups according to the treatment, were analyzed. Briefly, COCs were fixed in 4% PBS-buffered paraformaldehyde for 20 min at room temperature. An *in situ* cell death detection kit using fluorescein-conjugated dUTP and TUNEL (Roche Molecular Biochemicals, Mannheim, Germany) was used for labeling apoptotic cells. COCs were washed three times in PBS-0.1% PVA (polyvinyl alcohol) and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. COCs were washed twice with PBS-0.1% PVA before labeling. Positive control COCs were treated with 50 U/ml RNase-free DNase in cacodylate buffer for 1 h at 37 °C and then washed twice with PBS-0.1% PVA prior to labeling. The TUNEL reagent was prepared immediately before use and kept on ice. COCs were placed in 30 µl drops of TUNEL reagent and incubated in the dark for 1 h at 37 °C in a humidified chamber. The COCs were washed three times with 0.5% BSA in PBS. Negative controls were performed by omitting the TUNEL reagent. Total cell nuclei were labeled with 10 µg/ml Hoechst in 2.3% Na-citrate for 5 min in the dark. After washing twice in PBS-0.1% PVA, COCs were mounted in Fluoroguard antifade mounting reagent (BioRad, München, Germany). Samples were examined under a Nikon Diaphot microscope equipped with epifluorescence. Apoptosis was determined as the percentage of labeled cells to the total cell number. For each COC, a minimum of 400 cells, divided into four randomly chosen fields of the cumulus mass, was examined.

mRNA isolation and complementary DNA synthesis

Poly(A)⁺RNA from pooled COCs was extracted using Dynabeads mRNA DIRECT kit (Deutsche Dynal, Hamburg, Germany). Briefly, pools of 3–4 COCs were lysed for 10 min at room temperature in 200 µl lysis buffer (100 mmol Tris–HCl, pH 8.0, 500 mmol LiCl, 10 mmol EDTA, 1% (w/v) SDS, 5 mmol dithiothreitol). After lysis, 10 µl pre-washed Dynabeads oligo(dT)₂₅ were pipetted into the tube and binding of poly(A)⁺RNAs to oligo(dT) was allowed for 5 min at room temperature. The beads were then separated with a Dynal MPC-E magnetic separator, washed twice with 30 µl washing buffer A (10 mmol Tris–HCl, pH 8.0, 0.15 mmol LiCl, 1 mmol EDTA, 0.1% (w/v) SDS) and three times with 30 µl washing buffer B (10 mmol Tris–HCl, pH 8.0, 0.15 mmol LiCl, 1 mmol EDTA). Poly(A)⁺RNAs were then eluted from the beads by incubation in 11 µl diethyl pyrocarbonate-treated (DEPC) sterile water at 65 °C for 2 min. Aliquots were immediately used for reverse transcription using the Perkin Elmer (Foster City, CA, USA) PCR Core kit, using 2.5 µmol random hexamers to get the widest array of cDNAs.

RT reaction was carried out in a final volume of 20 µl at 25 °C for 10 min, 42 °C for 1 h, followed by a denaturation step at 99 °C for 5 min and immediate cooling on ice.

Oligonucleotide primers for PCR

Based on the mRNA sequences available at the EMBL databank, the following specific primer pairs were designed: β -actin (accession number U39357) sense primer: CCAAGGCCAACCGTGAGAAG, antisense primer: CCATCTCCTGCTTCGAAGTCC; Bcl-2 (accession number U92434) sense primer: GAGATGTCCAGTCAGCTGCACC, antisense primer: ATAGGCACCCAGGTGATGC; Bax (accession number NM173894) sense primer: TGCTTCAGGGTTTCATCCAG, antisense primer: GTGTCCCAAAGTAGGAGAGG. PCR products were sequenced to verify their identity and homology to corresponding mRNA sequences in the EMBL databank.

Semiquantitative PCR

To normalize signals from different RNA samples, β -actin transcripts were co-amplified as an internal standard. The amplification reaction was stopped before leaving the exponential phase (29 cycles for β -actin and 40 cycles for other fragments investigated). Amplifications were performed on 2 µl first strand cDNA in a 30 µl final volume containing 0.2 µM of the primer combinations listed above, 1 U Taq polymerase (Life Technologies, Karlsruhe, Germany), 0.2 mM dNTPs, 1.5 mM MgCl₂, 1 × PCR buffer. Amplification cycles comprised a 30 s step at 94 °C for denaturation, a 30 s step at 57 °C for annealing, and a 45 s step at 72 °C for elongation. A water control was included to identify possible contamination. In addition, all samples were amplified with an intron-exon spanning primer pair to detect possible genomic DNA contamination.

A volume of 20 µl per reaction was subjected to electrophoresis on a 1.5% agarose gel in Tris–acetate-EDTA (TAE) buffer, containing 0.2 µg/ml ethidium bromide. After separation, the fragments were visualized on a 312 nm UV-transilluminator. The image of each gel was digitalized using a CCD camera and the intensity of each band was quantified by densitometric analysis using a computer-assisted image analysis system (BioProfil, LTF software, LTF Labortechnik, Wasserburg/B, Germany). The relative amount of the mRNA of interest was calculated as a percentage of the intensity of the β -actin band for the corresponding sample. For each mRNA, experiments were replicated at least three times.

Statistical analysis

Data for *in vitro* maturation were analyzed using a binary logistic regression. Controls were assumed as reference group between replicates. Experiments were replicated at least 3 times and each replicate was fitted as a factor. The log likelihood ratio statistic was used to detect between treatment differences using the SPSS statistical package

(SPSS, Chicago, IL, USA). Data for cell number and gene expression were assessed using analysis of variance followed by Duncan's test. Data are presented as mean percentages ($\pm$ S.E.M.) of a minimum of three independent experiments. In all cases the criterion for significance was set at $P < 0.05$.

Experimental design

Experiment 1. Effects of PCBs on the incidence of apoptosis in cumulus cells

Between 25 and 35 COCs per treatment group were cultured for 24 h in wells containing 500 µl bMM or bMM + 1 µg/ml A-1254. The concentration was chosen based on previous results showing the concentration able to affect oocyte maturation without inducing unspecific cytotoxicity (Pocar *et al.* 2001a). After maturation, two groups per treatment (i.e. 3–4 COCs/group) of the matured COCs were snap-frozen in liquid nitrogen and stored for RNA analysis (see above). The remaining COCs (a total of 82) were fixed in 4% paraformaldehyde for the assessment of apoptosis (see above). Experiments were replicated at least 3 times.

Experiment 2. Congener-related effects of PCBs during oocyte maturation

The effects of either coplanar or non-coplanar PCB mixtures on the nuclear maturation of bovine oocytes were evaluated. Two concentrations of PCBs (100.6 and 201.2 pg/ml coplanar PCBs and 84 and 168 ng/ml non-coplanar PCBs respectively) were compared with vehicle-treated controls. The chosen concentrations were based on previous results reported by Krogenaes *et al.* (1998), and are considered to be representative of actual *in vivo* levels of individual PCB congeners (Pauwels *et al.* 1999). The nuclear status of the oocytes was examined at 24 h. In addition, the incidence of apoptotic cell death was investigated in a total of 80 COCs.

Experiment 3. Role of cumulus cells on PCB-induced toxicity in bovine COCs

To determine the role of cumulus cells in mediating PCB toxicity, cumulus-enclosed oocytes (COCs) and cumulus-free oocytes (DOs) were exposed during the maturation period to increasing concentrations of A-1254 (between 1 and 100 µg/ml) or coplanar PCBs (between 0.1 and 10 ng/ml). The nuclear status of the oocytes was examined at 24 h.

Results

Effects of Aroclor-1254 on the incidence of apoptosis in cumulus cells

As the quality of cumulus cells has been indicated as a crucial factor influencing the outcome of oocyte maturation

Figure 1 Representative images of bovine cumulus-oocyte complexes after *in vitro* maturation subjected to TUNEL analysis to determine apoptosis. COCs representing samples from (A) the control group, (B) the DMSO-treated group, (C) the Aroclor-1254-treated group, (D) the coplanar PCB-treated group, (E) the non-coplanar PCB-treated group, and (F) a positive control for TUNEL analysis are shown. Green staining indicates fragmented DNA in cells undergoing apoptosis, whereas intact cell nuclei are stained blue. Scale bar represents 50 μm .

and subsequent developmental competence, the incidence of apoptosis in cumulus cells after exposure to PCBs has been investigated first. As shown in Figs 1 and 2a, the incidence of apoptosis, demonstrated by TUNEL assay in COCs treated with 1 $\mu\text{g/ml}$ A-1254 was significantly higher compared with controls ($P < 0.001$). The expression of Bcl-2 and Bax mRNA, as anti- and pro-apoptotic genes respectively, was examined in the different groups. Results are depicted in Fig. 2b. In accordance with the results obtained by TUNEL, a significant increase in Bax expression was observed in A-1254-treated COCs compared with controls ($P < 0.05$). In A-1254-treated COCs, a significant down-regulation of Bcl-2 gene was observed ($P < 0.05$).

Toxic effects of PCB on bovine COCs are congener specific

Aroclor-1254 is a highly complex mixture of coplanar and non-coplanar PCB congeners. Therefore, the effects of two

pure mixtures of coplanar (PCB 77, 126 and 169) or non-coplanar (PCB 52, 101, and 153) PCBs on bovine oocyte maturation were examined.

Results are summarized in Table 2. Compared with controls without DMSO, the presence of 0.3% DMSO in the maturation medium did not affect the rate of degenerated oocytes or the rate of incompletely matured oocytes (any stage prior to metaphase II) (data not shown). Similarly, the percentage of completely matured, incompletely matured and degenerated oocytes did not differ significantly between the control group and the group treated with two different concentrations of non-coplanar PCB mixture. Furthermore, there was no difference in the percentage of immature oocytes between COCs treated with or without coplanar PCB mixtures. However, the maturation rate of COCs at the metaphase II stage was significantly decreased by treatment with coplanar PCBs, at doses as low as 100.6 $\mu\text{g/ml}$ compared with that of untreated COCs. In parallel, an increased percentage of intermediate and degenerated oocytes was observed.

Figure 2 Effects of Aroclor-1254 exposure during *in vitro* maturation on (a) apoptosis incidence and (b) Bax and Bcl-2 mRNA expression in bovine cumulus-oocyte complexes. The relative amount of the mRNA of interest was calculated as a percentage of the intensity of the β -actin band for the corresponding sample. The values are expressed as means $\pm$ S.E.M. Values with different superscripts are significantly different ($P < 0.05$). (c) Representative gels of independent experiments.

Table 2 Effects of coplanar or non-coplanar PCB mixtures on *in vitro* maturation of bovine oocytes.

Treatment	No. of COCs*	Immature** (%)	Intermediate** (%)	Mature** (%)	Degenerate** (%)
Control	82	2.27	17.04 ^a	80.68 ^a	0.00 ^a
Copl PCB 100.6 pg/ml	93	2.11	32.70 ^b	59.41 ^b	5.77 ^{a,b}
Copl PCB 201.2 pg/ml	84	8.93	23.21 ^{a,b}	57.14 ^b	10.71 ^b
Non-copl PCB 84 ng/ml	88	0.00	13.64 ^a	81.82 ^a	1.79 ^{a,b}
Non-copl PCB 168 ng/ml	78	0.00	15.48 ^a	80.95 ^a	4.54 ^{a,b}

* Total number of oocytes allocated for each treatment, $n = 3$ replicates per treatment.

** Categorical culture data are expressed as mean percentages of oocytes at the germinal vesicle and germinal vesicle breakdown (immature), metaphase I (intermediate), metaphase II (mature) and degenerate stages of the total number of oocytes evaluated.

^{a,b} Different superscripts within the same column denote significant differences ($P < 0.05$). Control has been assumed as reference.

Copl, coplanar; non-copl, non-coplanar.

Finally, no significant differences were observed in the presence of non-coplanar PCBs at doses as high as 168 ng/ml compared with vehicle-treated COCs for any of the parameters observed.

The incidence of apoptosis in cumulus mass in coplanar or non-coplanar PCBs was investigated. As shown in Figs 1 and 3a, a significant increase in apoptotic cells was observed after exposure to 100.6 pg/ml coplanar PCBs ($P < 0.001$), whereas no difference in the apoptotic rate was observed upon exposure to 84 ng/ml of a non-coplanar PCB mixture compared with controls. In addition, the expression of Bcl-2 and Bax mRNA was examined in the different groups. Results are depicted in Fig. 3b. A significant increase in Bax expression was observed in coplanar PCB-treated COCs compared with controls ($P < 0.05$), whereas no differences were observed in Bcl-2 expression level. No variations in mRNA expression of apoptotic-related genes were observed in non-coplanar PCB-treated COCs.

Role of cumulus cells on PCB-induced toxicity in bovine COCs

To analyze the role of cumulus cells on the PCB-induced toxicity in bovine oocytes, COCs and DOs were exposed to increasing concentrations of A-1254 (dose range between 1 and 100 µg/ml) or coplanar PCBs (dose range between 0.1 and 10 ng/ml) during IVM. Results are shown in Tables 3 and 4. As expected, the maturation rate of DOs to metaphase II was significantly lower than that of COCs, regardless of the concentration of PCBs in the maturation medium ($P < 0.05$). In agreement with previous results, a decrease in the percentage of cumulus-enclosed oocytes able to reach the metaphase II stage was observed with A-1254 at doses as low as 1 µg/ml compared with controls ($P < 0.001$). In parallel, an increased percentage of degenerated and not fully matured oocytes was observed ($P < 0.001$). In contrast, in the absence of cumulus cells, a decrease in matured oocytes was observed only upon exposure to 100 µg/ml A-1254 ($P < 0.001$). The lower doses were not effective. Similarly, upon exposure of COCs and DOs to increasing concentrations of coplanar PCBs the rate of incompletely matured oocytes increased in a linear manner with PCB concen-

trations only in the COCs group ($P < 0.001$) at doses as low as 0.1 ng/ml. An increased percentage of degenerated and not fully matured oocytes was observed ($P < 0.001$). There were no effects of coplanar PCBs on DOs at doses as high as 1 ng/ml, and a decrease in matured oocytes was observed only in groups exposed to 10 ng/ml ($P < 0.001$).

Discussion

We previously demonstrated that exposure of bovine cumulus-oocyte complexes during *in vitro* maturation to a commercial PCB mixture, Aroclor-1254, affects oocyte maturation, fertilization and developmental competence (Pocar *et al.* 2001b). Exposure to A-1254 during *in vitro* maturation is able to decrease the percentage of oocytes that can reach the metaphase II stage at concentrations as low as 0.01 µg/ml, whereas fertilization and developmental competence is affected at doses as low as 0.001 µg/ml. Furthermore, the negative effect of PCB exposure during maturation is not only limited to the ability of the oocytes to reach the metaphase II stage, but also includes embryonic development, as there is a significant decrease in the proportion of cleaved embryos reaching the blastocyst stage. Further, we have shown that PCB toxicity in bovine oocytes is related to a reduced competence of the oocytes to undergo normal cytoplasmic maturation, as indicated by an abnormal migration of cortical granules during maturation and subsequent fertilization and by changes in the polyadenylation pattern of several maternal mRNAs (Pocar *et al.* 2001a). Because cumulus cells play an important role in the nuclear and cytoplasmic changes associated with oocyte maturation (Larsen & Wert 1988, Mattioli *et al.* 1988, Fukui 1990, Chian & Sirard 1995, Tanghe *et al.* 2003), in the present study we decided to investigate whether cumulus cells can modulate the adverse reproductive effects of environmental toxicants.

Apoptosis is defined as programmed cell death for homeostasis and is closely involved with most of the reproductive processes, including follicular atresia (Tilly *et al.* 1991). The COC comprises an intimate relation between a cumulus cell syncytium in conjunction with the oocyte involving large gap junctions. When stimulated with follicle-stimulating hormone, and prior to the lutei-

(a)

(b)

(c)

Figure 3 Effects of exposure to PCB mixtures on (a) apoptosis incidence and (b) Bax and Bcl-2 mRNA expression in bovine cumulus-oocyte complexes. The relative amount of the mRNA of interest was calculated as a percentage of the intensity of the β -actin band for the corresponding sample. The values are expressed as means $\pm$ S.E.M. Values with different superscripts are significantly different ($P < 0.05$). (c) Representative gels of independent experiments. Ctrl, control; CP, coplanar; NCP, non-coplanar.

Table 3 Effects of cumulus cells on the meiotic progression of bovine oocytes treated with Aroclor (A)-1254.

Treatment	No.*	Immature** (%)	Intermediate** (%)	Mature** (%)	Degenerate** (%)
Cumulus–oocyte complexes					
Control	81	1.98 ^a	13.39 ^a	82.74 ^a	1.88 ^a
A-1254 1 µg/ml	69	10.53 ^{a,b}	25.57 ^b	53.99 ^b	9.91 ^b
A-1254 10 µg/ml	106	11.51 ^{a,b}	26.69 ^b	41.37 ^c	20.43 ^c
A-1254 100 µg/ml	81	20.85 ^b	23.70 ^b	40.28 ^c	15.16 ^d
Denuded oocytes					
Control	75	24.86	15.96	48.77 ^a	10.41 ^a
A-1254 1 µg/ml	75	36.66	13.23	45.12 ^a	4.99 ^a
A-1254 10 µg/ml	84	28.33	10.56	55.00 ^a	6.11 ^a
A-1254 100 µg/ml	78	21.83	4.31	19.94 ^b	53.90 ^b

* Total number of oocytes allocated for each treatment. $n = 3$ replicates per treatment.

** Categorical culture data are expressed as mean percentages of oocytes at the germinal vesicle and germinal vesicle breakdown (immature), metaphase I (intermediate), metaphase II (mature) and degenerate stages of the total number of oocytes evaluated.

^{a,b,c} Different superscripts within the same column denote significant differences ($P < 0.05$). Control has been assumed as reference.

nizing hormone peak, these gap junctions are open and allow even large molecules to move freely between the cumulus cells and the oocyte. Signaling molecules for the regulation of the apoptotic process can easily be distributed among cells in the COC. It has been shown that Aroclor-1254 acts as an apoptotic agent in various cell types (Yoo *et al.* 1997, Perez-Reyes *et al.* 2001).

The present results clearly demonstrate that exposure to A-1254 during IVM induced apoptotic cell death in cumulus cells, shown by DNA cleavage by TUNEL, the up-regulation of the expression level of the pro-apoptotic Bax gene, and the concomitant down-regulation of the level of the anti-apoptotic Bcl-2 gene in treated COCs. These data are in agreement with the results obtained by Campagna and co-workers (2001), demonstrating that exposure to an organochlorine mixture during IVM significantly impaired the quality of cumulus expansion, concomitantly increasing the rate of apoptotic cells of porcine COCs.

We further observed that bovine oocytes cultured without cumulus cells were approximately 100-fold less susceptible to the effects of A-1254 compared with cumulus-enclosed oocytes, suggesting a direct role of cumulus cells in mediating the toxic effects of A-1254 in bovine

oocytes. These data are in agreement with results obtained in the mouse, showing that fertilization of cumulus-free oocytes was not affected by exposure to A-1254 at doses as high as 10 µg/ml (Greenfeld *et al.* 1998), but fertilization and early embryogenesis of cumulus-enclosed oocytes were significantly reduced (Kholkute *et al.* 1994).

The results shown in the present study have important practical significance since mixtures of PCB congeners have been extensively used industrially and now contaminate the environment. The female germ cells are, for as-yet poorly understood reasons, extremely vulnerable to environmental biohazards, ultimately causing premature ovarian failure and infertility due to accelerated oocyte depletion (Tilly 2001). However, the mechanism of the toxic effect on the cells and the relevance of apoptotic cell death have not been characterized so far. Because Aroclors are complex mixtures of PCB congeners it is possible that different components are responsible for diverse effects. This possibility was examined in this study using two pure mixtures of PCB congeners.

Only coplanar PCBs affected oocyte maturation and this toxic effect was directly correlated with the presence of cumulus cells, cumulus-free oocytes being approxi-

Table 4 Effects of cumulus cells on the meiotic progression of bovine oocytes treated with a coplanar PCB mixture.

Treatment	No.*	Immature**	Intermediate** (%)	Mature** (%)	Degenerate** (%)
Cumulus–oocyte complexes					
Control	81	1.98	13.39 ^a	82.74 ^a	1.88 ^a
Copl PCBs 0.1 ng/ml	98	3.51	33.15 ^{a,b}	57.43 ^b	5.91 ^{a,b}
Copl PCBs 1 ng/ml	90	3.03	23.53 ^b	54.04 ^b	19.39 ^b
Copl PCBs 10 ng/ml	78	0.00	50.00 ^c	38.75 ^c	11.25 ^{a,b}
Denuded oocytes					
Control	75	24.86	15.96	48.77 ^a	10.41 ^a
Copl PCBs 0.1 ng/ml	60	10.41	10.41	54.17 ^a	25.00 ^a
Copl PCBs 1 ng/ml	72	4.17	23.21	58.93 ^a	13.69 ^a
Copl PCBs 10 ng/ml	84	3.57	24.35	27.92 ^b	44.16 ^b

* Total number of oocytes allocated for each treatment. $n = 3$ replicates per treatment.

** Categorical culture data are expressed as mean percentages of oocytes at the germinal vesicle and germinal vesicle breakdown (immature), metaphase I (intermediate), metaphase II (mature) and degenerate stages of the total number of oocytes evaluated.

^{a,b,c} Different superscripts within the same column denote significant differences ($P < 0.05$). Control has been assumed as reference.

Copl, coplanar.

mately 100-fold less sensitive than cumulus-enclosed oocytes. These results are consistent with the experiments of Krogenaes *et al.* (1998), demonstrating that the coplanar PCB 126 increased the rate of incompletely matured oocytes whereas the non-coplanar congener PCB 153 had no effect, at concentrations similar to those employed in the present study. It is important to notice that the concentration range affecting COCs in the present study is comparable to that observed in the serum of non-exposed women (0.001 and 0.4 ng/g for PCB 126 and 153 respectively; Johansen *et al.* 1994). Furthermore, Pauwels *et al.* (1999) showed that a strong correlation exists between serum and follicular fluid PCB content. Our data showed an increase in apoptosis in cumulus cells upon exposure to coplanar compounds while non-coplanar PCBs did not lead to a significant increase in apoptosis compared with controls. A significant increase in the expression level of the proapoptotic gene Bax was observed only upon exposure to coplanar PCBs. These data strongly suggest that the apoptotic cell death of cumulus cells may be a critical factor involved in coplanar PCB toxicity in the bovine oocyte. Krogenaes *et al.* (1998) previously demonstrated that exposure of bovine cumulus-oocyte complexes during IVM to the non-coplanar PCB 153, although not affecting oocyte maturation significantly, reduced the proportion of oocytes able to undergo the first mitotic divisions. Therefore, the hypothesis that exposure to non-coplanar PCBs during *in vitro* maturation affects subsequent embryo development through mechanism(s) of action other than apoptosis cannot be ruled out. In addition, in the present study, no differences in the expression level of Bcl-2 mRNA in the presence of coplanar PCBs was observed, in contrast to the results obtained upon exposure to A-1254, indicating that a possible interference of non-coplanar PCBs can occur when administered to bovine COCs in complex mixtures with coplanar PCBs. It is therefore possible that subtle changes, not detectable with the presently employed analysis, can occur. Finally, both coplanar and non-coplanar PCBs are able to accumulate in the follicular wall and to alter steroid secretion in follicular cells (Wojtowicz *et al.* 2000, 2001). Therefore, it is crucial to determine the significance of the present data for PCB toxicity on oocyte maturation in *in vivo* models, and should be the focus of future research before final conclusions can be drawn.

Coplanar PCBs are structurally related to polychlorinated dibenzofurans and polychlorinated dibenzo-*p*-dioxins. Many of the biochemical changes and toxic responses elicited by these compounds arise from alterations in gene expression due to interactions with the arylhydrocarbon receptor (AhR), a ligand-transcription factor (Whitlock 1990). Several studies implicate the AhR as having a role in modulating or mediating apoptotic processes. For example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces apoptosis in normal mice but AhR-deficient mice are not affected (Fernandez-Salguero *et al.* 1996, Kamath *et al.* 1997, Zaher *et al.* 1998). The AhR also modulates aspects

of ceramide signaling associated with the induction of apoptosis (Reiners & Clift 1999). In addition, recently the presence of consensus AhR response elements (XRE) in the promoter region of the pro-apoptotic Bax gene has been demonstrated. That Bax is a transcriptional target for the AhR was confirmed in reporter assays in microinjected mouse oocytes (Matikainen *et al.* 2002). Furthermore, using the human ovarian xenograft model it was reported that AhR ligands induce Bax expression and apoptosis in human ovarian follicles *in vivo* (Matikainen *et al.* 2002). Finally, we recently demonstrated that the AhR is abundantly expressed in bovine oocytes and that its activation is tightly regulated during oocyte maturation (Pocar *et al.* 2004). The ability of the coplanar PCB mixture used in the present study to activate the AhR signaling pathway was tested in a variety of cell types (Hombach-Klonisch S, personal communication). Based on these data, it is reasonable to hypothesize that the coplanar PCB congeners can induce apoptosis in bovine cumulus-oocyte complexes by a mechanism dependent on AhR receptor activation. Further analyses are in progress to test this hypothesis.

In conclusion, the present study provides evidence that PCB mixtures, at environmentally relevant levels, adversely affect the quality of cumulus cells by inducing apoptosis in a congener-specific manner. Our data point to a specific role of cumulus cells in mediating PCB toxicity during IVM that may account for the reduced maturation and developmental competence of the PCB-treated oocytes.

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