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Title: Aroclor 1254 impairs the development of ovarian follicles by inducing apoptosis of granulosa cells

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Abstract

Recent observations have indicated that polychlorinated biphenyls (PCBs) can reduce the number of antral follicles and increase follicular atresia. However, the effect of PCBs on follicles at each developmental stage and its underlying mechanism remain unclear. The aim of the present study was to evaluate the effects of PCB commercial mixtures (Aroclor 1254) on follicles at four developmental stages and apoptosis of mouse granulosa cells *in vivo*. Aroclor 1254 was administered to ICR female mice by intraperitoneal injection at the dose of 4, 20 and 100 mg/kg b.w. once every 3 days for five times. The number of follicles, percentage of apoptosis and ultrastructure of granulosa cells were determined. The results showed that Aroclor 1254 reduced the number of primordial and antral follicles in a dose-dependent manner and increased apoptosis of granulosa cells in the mice treated with 20 mg/kg and 100 mg/kg Aroclor 1254. Transmission electron microscopy analysis showed that mitochondrial dysfunction occurred in the granulosa cells of mice treated with 100 mg/kg Aroclor 1254. Finally, treatment of mice with Aroclor 1254 significantly reduced the expression of *Bcl-2* mRNA and increased the ratio of *Bax* to *Bcl-2* mRNA in granulosa cells. Based on these results, we concluded that exposure to Aroclor 1254 impaired the development of follicle (primordial follicle excluded), possibly via the induction of apoptosis mainly in granulosa cells. These findings suggest that PCB pollutants may cause premature ovarian failure and infertility of humans.

Keywords: Ovarian follicle; Granulosa cell; Aroclor 1254; Apoptosis

Introduction

Polychlorinated biphenyls (PCBs), a family of 209 molecules, are ubiquitous pollutants abundant in the environment despite of the fact that their production was banned since 1970s. Owing to their chemical stability and lipophilicity, PCBs are persistently present in the environment and food chains and can be readily detected in follicular fluid and serum sample from women undergoing assisted reproductive technology (ART).[1] Several reports have shown that PCBs are accumulated in human follicular fluid and human ovarian tissues.[1,2,3] PCBs has also been linked to the adverse health effects on humans and wildlife reproductive function, e.g., human reproductive toxicity and endocrine disruption.[4, 5,6,7].

Aroclor 1254, containing more than 60 PCB congeners, is considered to be environmentally relevant[8] and has been used in several experimental studies. For example, low concentration of Aroclor 1254 disrupts the maturation, fertilization, and developmental competence of bovine oocytes *in vitro*.[9] Pig oocytes exposed to Aroclor 1254 during IVM showed a decrease of developmental competence due to the induction of incorrect relocation of mitochondria.[10] Aroclor 1254 perturbs the gap-junction between pig oocytes and their cumulus cells at GV, MI and MII stages.[10]

Aroclor 1254 significantly increases the expression of the pro-apoptotic gene, *Bax*, and concomitantly reduces the level of the anti-apoptotic gene, *Bcl-2*, leading to the increased apoptosis in the cumulus cells *in vitro*.[11] *In vivo* studies show that exposure to PCB 126 causes widespread ovarian follicular atresia and significant reduction in the total number of antral follicles in rats.[12] Exposure to PCBs during mouse

pregnancy and lactation increases the follicular atresia and reduces the oocyte developmental capacity in the F1 offspring.[13] Similarly, PCB 126 reduces the number of antral follicles and increases the atretic follicles in rats.[14] Rats treated with Aroclor 1016 during the period of utero and lactation have significantly reduced the number of preantral and antral follicles and increased the atresia in the antral follicles.[15] Taken together, these findings provide evidence that PCBs exerts strong inhibitory effects on the development of preantral and antral follicles through inducing follicular atresia. However, the effects of PCBs on primordial and primary follicles following the exposure to Aroclor 1254 in puberty mice are currently unknown. Furthermore, the underlying molecular mechanisms by which PCBs induce follicular atresia are not fully understood.

The aim of the present study was to (1) characterize the effects of Aroclor 1254 on follicles at all developmental stages, and (2) identify the mechanisms of Aroclor 1254 induced follicular atresia in mice. Our results demonstrated that Aroclor 1254 reduced the number of primordial and antral follicles. Apoptosis in granulosa cells is induced by Aroclor 1254 possibly by increasing the expression of pro-apoptotic gene, *Bax*, and decreasing the expression of anti-apoptotic gene, *Bcl-2*. The results from this study provide significant implications on the effect of PCB on human infertility.

Results

Aroclor 1254 treatment did not affect body weight of mice

Body weights were measured on 0, 3, 6, 9 and 12 d after treatment with Aroclor 1254. No significant differences in body weight gains were observed in mice exposed

to Aroclor 1254. These results suggest that Aroclor does not have significant effect on the overall growth of mice.

Table 1 Body weight (g) of female mice exposed to Aroclor 1254

Time following Aroclor1254 treatment (days)	Control	4mg/kg	20mg/kg	100mg/kg
0	22.5±0.3	22.0±0.6	22.8±0.6	22.9±0.7
3	25.4±1.3	24.4±1.5	25.4±1.2	25.2±1.5
6	26.6±1.4	25.4±1.6	26.5±1.4	26.8±1.8
9	28.4±1.7	27.2±1.1	28.0±1.6	28.2±1.9
12	29.0±1.5	28.0±1.1	28.8±1.9	28.8±1.6

Body weights were measured on 0, 3, 6, 9 and 12 d. Values are means±SEM of 8 mice for 0, 4, 20 and 100 mg/kg doses, respectively. No significant differences were found on 0, 3, 6, 9 and 12 d body weight.

Aroclor 1254 treatment reduced the number of primordial, antral and total follicles in mice ovary and had no significant effect on diameter of oocytes

Compared to control group, the number of primordial and antral follicles were significantly decreased in all three experimental groups ($P < 0.01$ or $P < 0.05$, Fig. 1 A), while the number of primary and secondary follicles were significantly reduced only in the group treated with 100 mg/kg Aroclor 1254 ($P < 0.01$, Fig. 1 A). The total number of follicles in all the three experimental groups was significantly lower than that in the control group (Fig. 1 B). These results suggested that Aroclor 1254, one of the most widely used PCB mixtures, reduced the numbers of follicles in the mouse ovary in a dose-dependent manner.

The oocytes' diameter of primordial ($P < 0.05$) and primary follicles ($P < 0.01$) were significantly decreased by Aroclor 1254 at the dose of 20 mg/kg , in comparison to untreated controls (Fig. 1 C). Meanwhile, there were no significant differences in the diameter of oocytes of secondary and antral follicles between Aroclor 1254

treatment groups and the controls (Fig. 1 C).

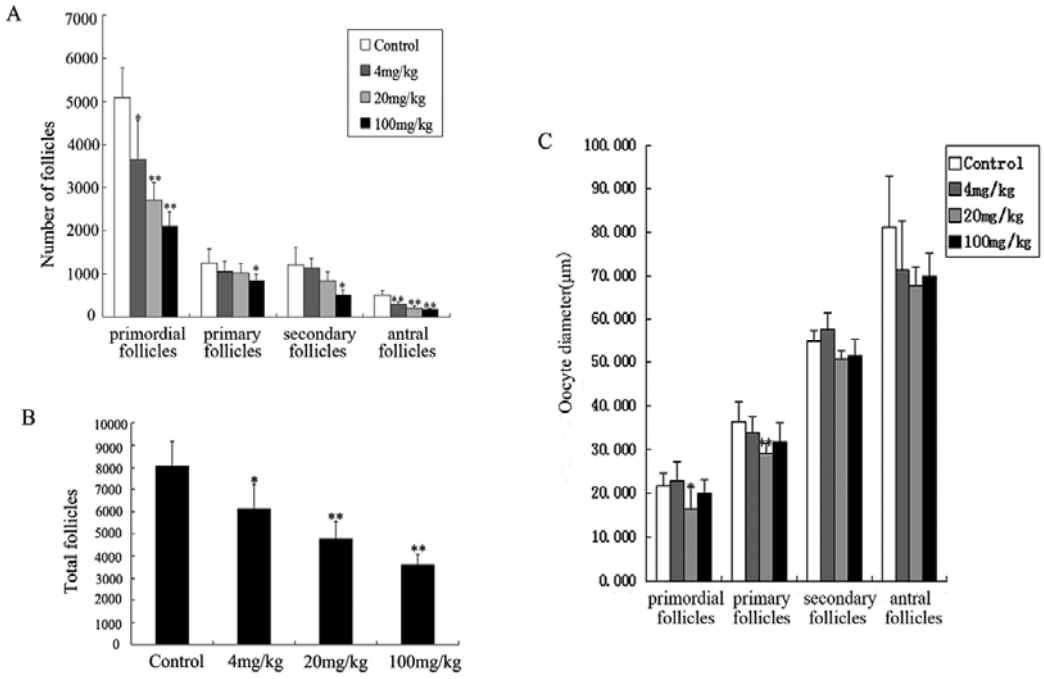

Fig. 1 Effect of Aroclor 1254 on various follicle types and oocyte diameter. Ovaries were collected from mice treated with 4 mg/kg, 20 mg/kg and 100 mg/kg of Aroclor 1254 or vehicle-treated control mice and processed for histological evaluation. Values represent the mean total number of follicles counted in every 5th section of each ovary, n=6. (A) the follicular number of four categories (primordial, primary, secondary and antral follicle) in experimental and control groups. (B) the total number of follicles in experimental and control groups. (C) oocyte diameter of follicles at four development stages in experimental and control groups. Data were analyzed for significant differences using the one-way ANOVA for parametric data (Bars represent means±SEM, *different from control, $P < 0.05$; ** different from control, $P < 0.01$).

Aroclor 1254 treatment induced apoptosis in granulosa cells

Granulosa cell apoptosis of primordial follicles and antral follicles in the 4 mg/kg Aroclor 1254 treatment group was significantly higher than that in the control group ($P < 0.01$) (Fig. 2 A-b, f, 2 B). In contrast, after treatment with 20 mg/kg or 100 mg/kg of Aroclor 1254, granulosa cell apoptosis in all 4 stages of follicles including primordial follicles, primary follicles, secondary follicles and antral follicles was significantly increased compared to those in the control group ($P < 0.01$) (Fig. 2 A-c,

d, g, h, 2 B).

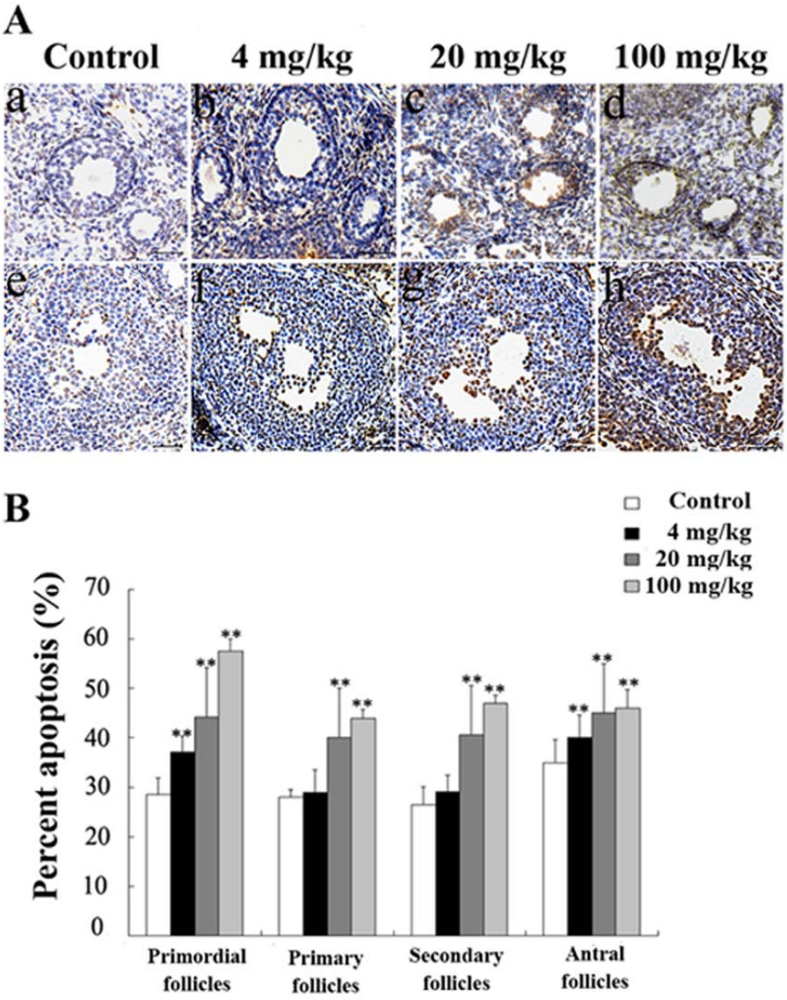

Fig. 2 Aroclor 1254 treatment increases apoptosis in granulosa cells of follicles. TUNEL-positive cells are stained brown and are indicative of apoptosis. (A) Representative field of ovarian sections from control ovary (a, e), 4 mg/kg of Aroclor 1254-treated ovary (b, f), 20 mg/kg of Aroclor 1254-treated ovary (c, g), 100 mg/kg of Aroclor 1254-treated ovary (d, h) was stained by TUNEL assay. (B) The percentage of apoptosis was represented as Mean ± SEM. Percentage of apoptosis in various follicle types from 4, 20 and 100 mg/kg Aroclor 1254-treated mice was increased in comparison to the control mice. Data were analyzed for significant differences using one-way ANOVA for parametric data (**different from control, $P < 0.01$). Bar=50 μm.

Aroclor 1254 altered the ultrastructure of granulose cells

The chromatin, nucleus, rough endoplasmic reticulum, mitochondria and other

cellular organelles were normal in the untreated granulose cells. For example, mitochondria and rough endoplasmic reticulum were abundant and mainly distributed in perinuclear area (Fig. 3 A-a, A-c) and most of the mitochondria had obvious cristae in a traverse arrangement (Fig. 3 A-b). In contrast, in the mice treated with 100 mg/kg Aroclor 1254, the chromatin was condensed in the perinuclear area and rough endoplasmic reticulum membrane swelling occurred in the granulosa cells (Fig. 3 B-a). In addition, treatment with 100 mg/kg Aroclor 1254 reduced the number of mitochondria in granulose cells (Fig. 3 B-a) and induced mitochondrial swelling, leading to the disappearance of the mitochondrial structure (Fig. 3 B-b). The apoptotic process was characterized by the appearance of nuclear fragmentation, fragments of condensed nuclear chromatin, abnormal mitochondria and rough endoplasmic reticulum profiles in the apoptotic body (Fig. 3 B-c). Lipid droplets were randomly accumulated in the peripheral cytoplasmic compartments in the experimental groups (Fig. 3 B-c).

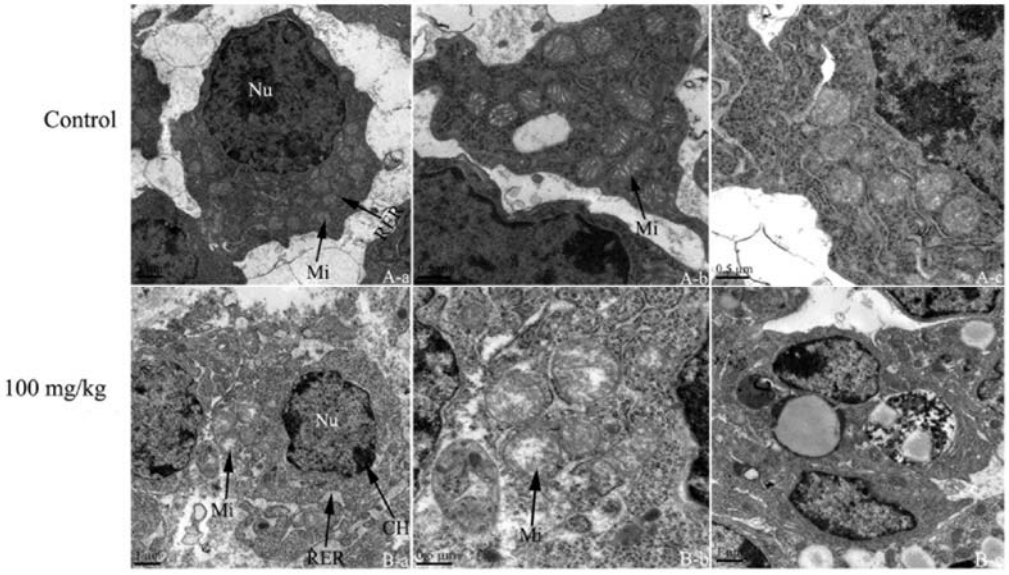

Fig. 3 Electron micrographs of granulosa cells. Nu, nucleus; M, mitochondria; CH, Chromatin; AB, apoptotic body; RER, rough endoplasmic reticulum. (A-a) Granulosa

cells in the control group exhibited abundance of mitochondria in the perinuclear area. Bar = 1 μm. (A-b) Majority of the mitochondria in the control group had obvious cristae and double member in a traverse arrangement. Bar = 0.5 μm. (A-c) Masses of rough endoplasmic reticulum in the control group were surrounded in the perinuclear area. Bar=0.5 μm. (B-a) In the mice treated with Aroclor 1254 at the dose of 100 mg/kg, chromatin had a very marked aggregation pattern in the perinuclear area and local edema of rough endoplasmic reticulum is visible. Bar = 1 μm. (B-b) Aroclor 1254 significantly decreased the number of mitochondria in granulosa cells, and the mitochondrial swelling caused complete disappearance of the mitochondrial structure. Bar = 0.5 μm (B-c) An apoptotic body was enclosed by double membrane and contained chromatin fragmentation, abnormal mitochondria and rough endoplasmic reticulum profiles in degeneration process. Bar = 0.5 μm. Upper panel: control mice; lower panel: mice treated with 100 mg/kg of Aroclor 1254.

Aroclor 1254 enhanced the expression of pro-apoptosis genes in granulosa cells

Immunohistochemistry analysis revealed that BAX and CASPASE-3 proteins were weakly expressed in the control ovaries (Fig. 4 A b_1, c_1, B), while their expression was increased after Aroclor 1254 treatment (Fig. 4 A b_2, b_3, b_4, B). The protein level of CASPASE-3 was increased by 73 % ($P < 0.01$) and 129 % ($P < 0.01$) in mice treated with 20 mg/kg and 100 mg/kg Aroclor 1254, respectively, compared to the control group (Fig. 4 B). The protein level of BCL-2 was decreased by 60 % ($P < 0.01$), 65 % ($P < 0.01$) and 82 % ($P < 0.01$) in the mice treated with 4, 20 and 100 mg/kg Aroclor 1254, respectively, compared to the control group (Fig.4 A a_2, a_3, a_4, B). The ratio of BAX to BCL-2 in all the three experimental groups was significantly higher than that in the control group ($P < 0.01$). These results indicated that Aroclor 1254 treatment affected BAX, BCL-2 and CASPASE-3 expression.

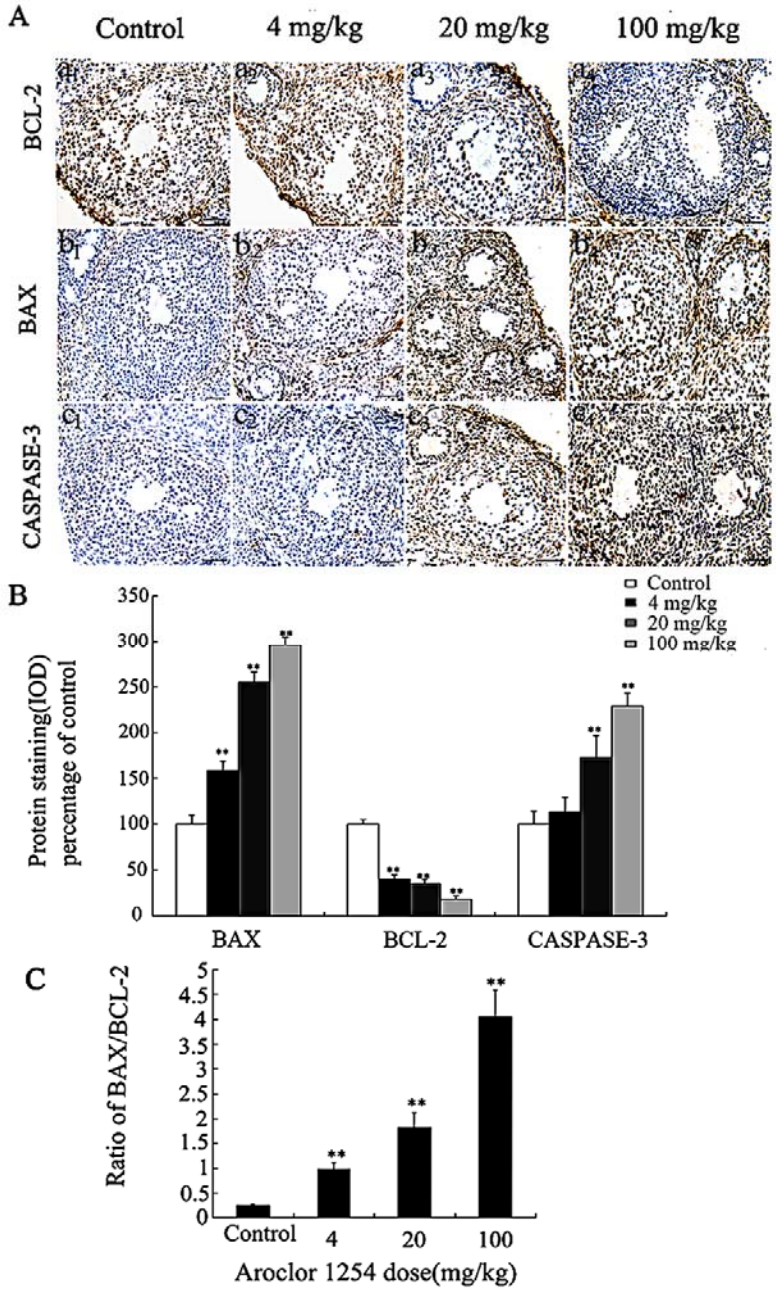

Fig. 4 Immunohistochemistry analyses of Aroclor 1254-induced expression of BAX, BCL-2 and CASPASE-3 proteins in granulosa cells. (A) In situ analysis of BAX, BCL-2 and CASPASE-3 proteins in the ovaries exposed to soybean oil, 4 mg/kg, 20 mg/kg and 100 mg/kg of Aroclor 1254. (B) Quantitative analysis of the relative expression of BAX, BCL-2 and CASPASE-3 in the ovaries exposed to above treatments. The results of the experimental groups were presented as percentage of the control animals (100%). (C) The effect of Aroclor 1254 on the ratio of BAX to BCL-2. Statistical significance was determined using one-way analysis of variance (ANOVA), and significant difference was indicated by $^{**}P < 0.01$, compared to the control. Bar = 50 μm.

Semi-quantitative RT-PCR analysis revealed that the expression of *Bcl-2* mRNA

was decreased by 8.1 % ($P < 0.01$) in 4 mg/kg group, 20.9 % ($P < 0.01$) in 20 mg/kg group and 31.78 % ($P < 0.01$) in 100 mg/kg group (Fig. 5 B), compared to the control group. In contrast, the level of *Bax* mRNA was increased significantly in the mice treated with 20 mg/kg and 100 mg/kg Aroclor ($P < 0.01$) (Fig. 5 A) compared to the control group. Moreover, the levels of *Caspase-3* were increased by 110 % ($P < 0.01$), 373 % ($P < 0.01$) and 695 % ($P < 0.01$) in mice treated with 4 mg/kg, 20 mg/kg and 100 mg/kg Aroclor 1254, respectively, compared to the control group (Fig. 5 C). The ratio of *Bax* to *Bcl-2* was increased by 102 % and 313% in the mice treated with 20 mg/kg and 100 mg/kg Aroclor 1254, respectively, compared to the control group ($P < 0.01$) (Fig. 5 D).

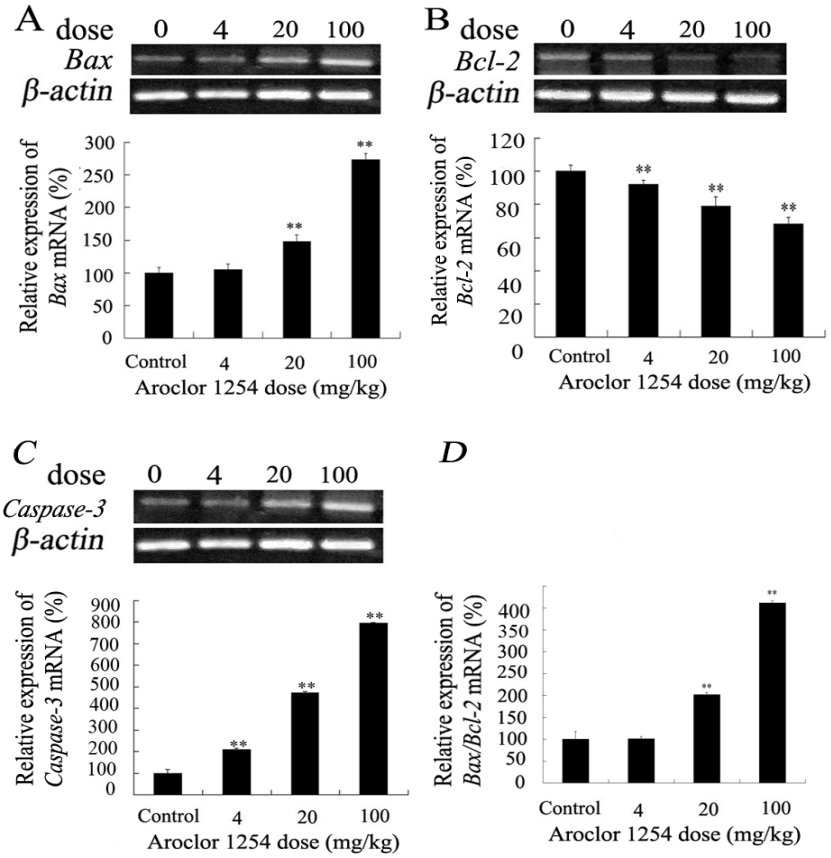

Fig. 5 RT-PCR analyses of Aroclor 1254-induced expressions of *Bax*, *Bcl-2* and

***Caspase-3* mRNA in granulosa cells.**

RT-PCR analysis of *Bax*, *Bcl-2* and *Caspase-3* mRNA expression in ovarian. (A) The upper panel is a representative gel image showing mRNA level of Bax gene in ovaries from control and Aroclor 1254 treated groups. The lower panel was the quantification of mRNA level by the densitometric analysis of three replicates (n=3). **Means significant change compared with control groups ($P < 0.01$). (B) The upper panel is a representative gel image showing mRNA level of *Bax* gene in ovaries from control and Aroclor 1254 treated groups. The lower panel was the quantification of mRNA level the densitometric analysis of three replicates (n=3). **Means significant change compared with control groups ($P < 0.01$). (C) The upper panel is a representative gel image showing mRNA level of *Caspase-3* gene in ovaries from control and Aroclor 1254 treated groups. The lower panel was the quantification of mRNA level the densitometric analysis of three replicates (n=3). **Means significant change compared with control groups ($P < 0.01$). (D) The effect of Aroclor 1254 on the *Bax* to *Bcl-2* ratio. The *Bax* to *Bcl-2* ratio was increased significantly at the dose of 20 mg/kg and 100 mg/kg Aroclor 1254 compared with control group (**$P < 0.01$). Statistical significance was determined using one-way analysis of variance (ANOVA).

Discussion

The effect of PCBs on the follicles throughout the developmental stages, especially the earlier stage (primordial and primary follicles) has not been investigated. The present study demonstrated that at high dose of Aroclor 1254, the development of follicles at all stages was inhibited, while at low dose of Aroclor 1254, only primordial and antral follicles were inhibited. Thus, it is possible that Aroclor 1254 exerts different effects on the follicles at different developing stages and causes follicle loss via acceleration of the natural process of atresia. Muto et al. showed that rat ovaries with prenatal exposure to PCB126 had widespread ovarian follicular atresia, which results in a significant reduction in the total number of antral follicles.[12] Baldridge et al. reported that rats treated with Aroclor 1016 during the period of utero and lactation had significantly reduced number of preantral and antral follicles and increased atresia in the antral follicles.[15] The present results were consistent with previous reports showing that PCBs significantly decreased of the number of follicles

at later stages. More importantly, we also showed that Aroclor 1254 even at the low doses reduced the number of primordial and antral follicles and the total number of follicles.

Studies have suggested that the apoptosis of ovarian granulosa cells plays a major role in follicular atresia.[16,17,18] It has been reported that oocytes are removed by physiological cell death instead of the classical apoptosis during follicular atresia.[19] Oocyte loss is assumed to be a result of oocyte-intrinsic deficiencies or damage.[20] We also found that there was no TUNEL positive oocyte in three Aroclor 1254 treatment groups although TUNEL positive cumulus cells were present in cumulus-oocyte complexes (COCs) obtained from the mice treated with Aroclor 1254 (12.5, 25 and 50 mg/kg) (data unpublished). Meanwhile there was no significant difference in the diameter of oocytes in comparison to the untreated controls (Fig.1 C). During development, the majority of follicles undergo atresia, and the theca cells are often the final follicular cell type to die.[21] In atretic porcine ovary follicles, apoptotic TUNEL staining was seen in scattered granulosa cells located on the inner surface of the follicular wall, but not in internal, external theca cells, or oocytes.[22] In our experiment, less theca cells were observed positive TUNEL staining in three treatment groups. Therefore, Aroclor 1254 impaired the development of follicle possibly via the induction of apoptosis mainly in granulosa cells.

Apoptosis is recognized as a normal process of cell death, and is characterized by distinct morphological features, e.g., plasma membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation, and breakdown of the cell into

apoptotic bodies.[23] In the present study, we showed that Aroclor 1254 treatment had no effects on the mice body weight (Table 2) and ovarian weight (data not shown). However, treatment of mice with 100 mg/kg Aroclor 1254 caused an accelerated depletion of ovarian primordial, primary, secondary and antral follicles. Moreover, increased apoptosis at all stages of follicles were observed in the mice treated with 100 mg/kg Aroclor 1254. Therefore, mice treated with 100mg/kg Aroclor 1254 was selected for electron microscopy analysis.

Ultrastructure analysis of apoptotic granulosa cells indicated that Aroclor 1254 induced abnormal mitochondria, endoplasmic reticulum and condensed chromatins. Apoptosis in granulose cells of follicles were increased in mice treated by 20 mg/kg or 100 mg/kg Aroclor 1254. These results were consistent with previous studies showing that the number of mitochondria is decreased and functional and morphological abnormalities occurred in sheep blastocysts exposed to Aroclor 1254.[24] PCBs were believed to act as uncouplers of the aspiratory chain, which results in the disturbance of the permeability of the inner mitochondrial membrane.[25,26] Brevini et al. demonstrated that exposure to PCBs mixtures altered mitochondria relocation during the maturation of porcine oocyte.[10] Sheep blastocysts exposed to Aroclor 1254 were also characterized by the occurrence of high amount of cell debris (indicative of necrosis), high number of TUNEL-positive cells (indicative of apoptosis) and non-programmed cell death.[24] The results of the present study using TUNEL to identify apoptotic follicles showed that Aroclor 1254 treatment increased apoptosis in various follicle types, which were in agreement with these previous findings. These

results indicated that the mechanisms of Aroclor 1254-induced follicles loss may be related to the activation of apoptotic signaling pathways.

Mitochondria are nowadays regarded as a crucial compartment where numerous proapoptotic pathways converge and execution phase of apoptosis is initiated.[27] *Bax* and *Bcl-2* are mitochondria-dependent pro-apoptotic gene and anti-apoptotic genes, respectively, in rat granulosa cells during follicular development.[28] The ratio of *Bax* to *Bcl-2* determines the susceptibility of a cell to apoptosis.[29] *Caspases*, especially *Caspase-3*, are known to act in the downstream of *Bax/Bcl-2* to control the execution of apoptosis.[30] Hwang et al. has shown that the PCB induces apoptosis in human neuronal cells.[31] Sánchez-Alonso et al. show that the increase of Aroclor-induced apoptosis in neuronal cells correlates with a reduction in the expression of anti-apoptotic *Bcl-2* and an increase in the expression of pro-apoptotic *Bax*.[32] Bcl-2 and Bcl-xL containing four Bcl-2 homology domains (BH1–BH4) have been shown to block the release of mitochondrial apoptogenic factors[33]. Activated Bax undergoes a conformational change, allowing its C-terminal end to be inserted into the mitochondrial outer membrane[34]. Membrane insertion is followed by oligomerization, an event thought to precede cytochrome *c* release[35, 36]. The binding of the released cytochrome *c* with apoptosis-activating factor 1 (Apaf-1) activates downstream caspaseses, e.g., caspasese-3.[37] Caspases act as molecular chainsaw to induce characteristic of apoptosis by cleaving a number of cytoplasmic and nuclear substrates.[38]

Our results showed that exposure to 20mg and 100mg Aroclor 1254 resulted

significant decrease in total number of follicles (Fig. 1 B) and significantly higher percent of apoptosis in granulosa cell of follicles (Fig. 2). Meanwhile, the relative expression of *Bax/Bcl2* mRNA (Fig. 5 D) and the protein ratio of BAX/BCL-2 (Fig. 4 C) were also significantly higher in the two Aroclor 1254 treatment groups compared to control. Similarly, Aroclor 1254 at all the three doses decreased the total number of follicles (Fig. 1 B), relative expression of *Bcl2* mRNA (Fig. 5 B) and the integranted optical density of BCL2 (Fig. 4 B). The role of *Bcl2* in ovarian apoptosis of granulose cells has been suggested by several previous studies. First, the numbers of follicles are decreased in *Bcl2*-deficient mice[39] and excessive expression of *Bcl2* leads to decrease follicular apoptosis and atresia[40]. Second, localization of the BAX protein in the human ovary was abundant in granulosa cells of early atretic follicles, whereas BAX protein was extremely low or non-detectable in healthy or grossly-atretic follicles[41]. In present study, the decreased expression of BCL2 and increased expression of BAX protein (Fig. 4 B) may result in the reduction of total number of mice follicles (Fig. 1 B). These results indicated that follicular loss induced by Aroclor 1254 might be due to the disruption of the balance between pro- and anti-apoptotic molecules in granulose cells, which activates the mitochondria apoptotic signaling pathways.

Materials and methods

Chemicals

Aroclor 1254 was purchased from Santa Cruz Biotechnology, Inc. USA (SC-257096). All other reagents were obtained from Sigma Chemical Co. unless stated otherwise.

Animals and treatment

Animal care and use were conducted in accordance with the Animal Research Institute Committee guidelines of the Ethics Committee of Shandong Normal University, China. The Ethics Committee of Shandong Normal University had approved the experiment. Mice were housed in a temperature-controlled room with proper darkness-light cycles, fed with a regular diet, and maintained under the care of the Laboratory Animal Unit. Euthanization of mice was achieved by cervical dislocation.

ICR mice (purchased from Beijing China Fukang Biological Technology Co. Ltd, China) were acclimatized for one week prior to experiment. After one week, adult female mice (22 ± 2g) without adverse clinical signs were divided randomly into four groups, including three experimental groups and one control group (10 mice in each group). In our previous study, female ICR mice were treated with different doses (12.5, 25 and 50 mg/kg) of Aroclor 1254 for three times (the range of total concentrations was 37.5-150 mg/kg).[42] In the present study, the dosage of 4, 20 and 100 mg/kg Aroclor 1254 was used for the three experimental groups of mice, respectively. The total concentrations (20-500 mg/kg) chosen in present study ranged from 37.5mg/kg to 150 mg/kg. The duration of Aroclor 1254 intraperitoneal administration was once every 3 days for five times as reported previously.[42, 43] Control mice received an equal volume of the soybean oil (0.1 mL/10 g). The mice were sacrificed by cervical dislocation after treatment. Ovaries were removed and placed in 4 % formaldehyde (Fluka 76240) for 24 h, or quickly frozen in liquid

nitrogen and then stored at -70 °C for RNA extraction, TUNEL assay for *in situ* detection of apoptosis or immunohistochemistry.

Follicle counts and oocyte diameter measurement

Ovarian samples were fixed in 4% formaldehyde and then embedded in paraffin block. Sections of paraffin-embedded tissue (5 μm thick) were obtained for routine histology examination. After deparaffinization and rehydration, sections were stained with hematoxylin–eosin (H&E). In every fi8fth ovarian section, the number of primordial, primary, secondary and antral follicle was counted. Only follicles with a nucleolus being present in the nucleus were counted in order to avoid counting the same follicle twice. The received number of follicles was then multiplied by five (to correct for slice thickness) and then by five (to account for the unanalyzed sections) to get an estimate of the total number of follicles per ovary.[44] The numbers of each type of follicles were counted in the whole ovarian cutting surface (200×). Eight ovaries were counted in each treatment for follicle counts.

Follicles were classified into primordial (oocyte surrounded by a single layer of predominantly flat or squamous granulosa cells), primary (oocyte surrounded by a single layer of 20-26 cuboidal-shaped granulosa cells), secondary (two to three layers of granulosa cells surrounded by a thecal tissue, but no evidence of an antrum), and antral (follicles containing a fluid-filled antrum) follicles and each type follicles was counted.[45,46] The final follicle numbers did not represent the absolute follicle number but were relative to the counting procedure.[47]

Oocyte diameter: software Image J (National Institutes of Health, Bethesda,

Maryland, USA) was applied to measure oocyte diameter. In order to avoid measuring the same oocyte twice, only oocyte with a nucleolus being present in the nucleus were counted. The data was collected and the average was calculated as described by Campos-Junior[48]. Six ovaries were counted in each treatment for measurement of oocyte diameter.

TUNEL assay

Apoptosis was determined by TUNEL assay (in situ cell death detection kit, POD, Roche Applied Science, Germany, 12156792910) according to the manufacturer's instructions. Briefly, ovaries were embedded in Tissue Tek O.C.T (Sakura Finetek Inc., Torrance, CA, USA) and sectioned at 8-µm thickness onto charged microscope slides using a cryostat. Subsequently, ovarian sections were fixed with 4 % neutral buffered paraformaldehyde for 20 min at room temperature and washed for 30 min with PBS. Endogenous peroxidase was blocked by incubation in 0.3 % hydrogen peroxide for 10 min. After three washes with PBS, ovarian sections were treated for 2 min on ice with 0.1 % Triton X-100 (T9284). After rinsing twice with PBS and drying areas around the sample, the entire ovarian tissue specimens were incubated with 50 µL of TUNEL reaction mixture at 37 °C for 60 min in a humidified chamber, followed by rinsing three times in PBS for 5 min each. The slides were incubated with 50 µL of Converter-POD at 37 °C for 30 min in a humidified chamber to enable analysis of stained cells under light microscope. After 10 min washing in PBS, the POD retained in the immune complex was visualized as a color reaction by incubation with 3, 3′-diaminobenzidine (DAB, D8001) for 10 min in RT, followed by washing in PBS.

Counterstaining was achieved by incubating the slides with hematoxylin (H9627) for 30 s. The slides were mounted with glycerol-gelatin for long-time storage at room temperature. Positive control was obtained by treating the slides with 1000 U/mL DNase I (promega Applied Science) in 10 X Reaction Buffer (400 mM Tris–HCl pH 8.0, 100 mM $MgSO_4$ and 10 mM $CaCl_2$) for 10 min at room temperature to induce DNA strand breaks prior to labeling procedures. Negative control was obtained by adding TdT in PBS during the reaction. The number of apoptotic cells was determined by counting the labeled cells from follicles in 400× microscopic fields (three sections per ovary; six ovaries) and expressed as the mean apoptotic cell per follicle. Cells were considered TUNEL-positive when they were brown-stained with characteristic apoptotic appearance (marginated chromatin, pyknosis or multiple fragmentations). Apoptotic percentage of granulosa cells is calculated by dividing the number of apoptotic cells by the total number of cells. Images were acquired on an Olympus BX51 microscope.

Electron microscopy

For electron microscopy, multiple thin slices from different regions of ovary of each animal were cut into $1mm^3$ blocks and immediately fixed with 2.5% glutaraldehyde (G5882) in 0.2 M phosphate buffer saline (PH 7.2 to 7.4) at 4°C for 24 h. The samples were then postfixed in a mixture of 2 % OsO4 (O5500) for 2 h at 4 °C. After dehydratation in increasing concentrations of ethanol (50 %, 70 %, 90 %, 95 % and 100 %, each for 15 min) and acetone (15 min), the samples were embedded in Epon 812 (Fluka, 45345). Semithin and ultrathin sections were cut on the

ultramicrotome. Semithin sections were stained with 1 % toluidine blue (198161) and examined by light microscopy. From representative areas, thin sections were double-stained on copper grids with uranyl acetate (P9041) and lead citrate and then examined using a JEM-1OOSX transmission electron microscope (JEM-lOOSX, JEOL) at 80 kV.

Immunohistochemistry

For immunohistochemical staining, cryosections at 5-μm thickness were fixed by acetone (650501) (-20 °C) for 10 min. After three washes with PBS (pH 7.2), sections were treated with 0.3 % Triton X-100 for 30 min in RT, followed by washing in PBS. Endogenous peroxidase activity was blocked by treatment with 3 % hydrogen peroxide in absolute methanol for 10 min. Ovarian sections were incubated with 5 % (w/v) bovine serum albumin (Roche, Applied Science, Germany, 10711454001) for 30 min at 37 °C. Following blocking, sections were incubated with Bcl-2 antibody (Abcam Inc, USA; ab692, 1:200 dilution), Bax antibody (Abcam Inc, USA; ab7977, 1:200 dilution) or Caspase-3 antibody (Abcam Inc, USA; ab44976, 1:200 dilution) overnight at 4 °C and then incubated with peroxidase-conjugated second antibody (Santa Cruz Biotechnology Inc, 1:1000 dilution) for 1 h at 37 °C. Immunodetection was performed by incubating the ovarian tissue specimens with 3, 3′-diaminobenzidine (DAB). Counterstaining was achieved by hematoxylin incubation for 15 min and then destained in HCl-alcohol for 4 s before dehydration. The slides were mounted with glycerol-gelatin for long-term storage. To evaluate the specificity of the antibodies, PBS without primary antibody was used as negative

contro1. Under high-power magnification (200×), photographs of five representative fields were captured by the Olympus Image-Pro Plus v6.2 software. Identical settings were used for each photograph. The intensity of the immunoreactivity was analyzed by integral optical intensity (IOD) with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).[49,50] Five high power fields (200×) were selected from each slide to measure the optical intensity. Photographs of five representative fields can cover about 50% area of each slide of mouse ovary. Six slides were chosen from one ovary. Total ten ovaries were used in each group. The results of the experimental groups are presented as the percentage of the corresponding control animals (100%).

Semi-quantitative RT-PCR analysis

Total RNA of the ovary was extracted using TRIZOL reagent (Life technologies Carlsbad, CA, 12183-555) according to the manufacturer's instructions. RNA was suspended in 25 μL DEPC water and stored at -80 °C. The quantity of RNA was measured by the optical density at 260nm and 280 nm using a spectrophotometer, and integrity was confirmed by running 5 μL RNA on a 1 % agarose gel.

Reverse transcription was performed in a volume of 20 μL reaction mixture containing 3 μg of total RNA, 1 μL oligo $(dT)_{18}$ primers , 1 μL Ribolock™ RNase Inhibitor (20 μ/μL, 2 μL 10 mM dNTP Mix, 4 μL 5 × Reaction Buffer , 1 μL RevertAid™ M-MULV Reverse Transcriptase (200/μL) and nuclease-free water. After reaction at 42 °C for 60 min, reverse transcriptase was inactivated by heating at 70°C for 5 min and the resulting cDNA was stored at -20 °C until use.

PCR was carried out in a volume of 25 μL reaction mixtures containing 1 μL

cDNA, 12.5 μL 2 × Master Mix and 10 μM of each specific PCR primer. The samples were heated to 94 °C for 5 min followed by 28-33 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min, and a final extension of 8 min at 72 °C. PCR of β-actin was used as an internal control. PCR products were run on a 1 % agarose gel containing ethidium bromide and viewed under UV light. Signal intensities for the PCR products were analyzed by Quantiscan software (Bio-soft, Cambridge, MA, USA). The detailed sequences of the primers, the expected size of PCR products and the cycles performed for each gene were shown in Table 2.

Table 2 Sequences of primers, cycles used in RT-PCR analysis and expected size of PCR product for *Bax*, *Bcl-2* and *Caspase-3* genes.

Gene	Gene ID	Sequences (5′-3′)	Size of PCR products (bp)	PCR cycles
Bax	12028	CAGCGGCAGTGATGGACGGG TCTCGGGGGGAGTCCGTGTC	280	33
Bcl-2	12043	GCATTGCGGAGGAAGTAGAC ACTCGTAGCCCCTCTGTGAC	320	33
Caspase-3	12367	AACGCTAAGAAAAGTGACCA ACTTTTTCAGTTCAACAGGC	433	30
β-actin	11461	TGCTGTCCCTGTATGCCTCT GTCACGCACGATTTCCCTCT	217	28

Statistical analysis

All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). All data were expressed as means ± S.E.M. from at least three different experiments. Analysis of variance (one-way ANOVA) was used to determine the significant

differences in the body weights, ovarian follicle numbers, BAX, BCL-2, CASPASE-3 protein levels, *Bax*, *Bcl-2*, *Caspase-3* mRNA levels and percentages of apoptotic granulose cells among the experimental groups treated with the different doses of Aroclor-1254 and the control group. The ratio of *Bax*/*Bcl-2* mRNA was first subjected to square root transformation before statistical analysis. If F-value was significant, the data were subjected to Duncan′s new multiple range test ($P < 0.05$) for multiple sample comparisons.

Conclusion

Exposure of cumulus–oocyte complexes to Aroclor -1254 increased the level of apoptosis in cumulus cells, enhanced the expression of the pro-apoptotic gene *Bax*, and reduced the expression of the anti-apoptotic gene *Bcl*-2. We hypothesized that Aroclor 1254 depletes follicles by inducing granulosa cell apoptosis through mitochondrial pathways. These results provide important implication in the role of PCB in the disruption of reproductive function, infertility and premature ovarian failure.

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References

1 E. M. Petro, J. L. Leroy, A. Covaci, E. Fransen, D. De Neubourg, A. C. Dirtu, I. De Pauw and P. E. Bols, *Hum. Reprod.*, 2012, 27, 1025-1033.

2 H. Schlebusch, U. Wagner, H. Van der Ven, S. Al-Hasani, K. Diedrich and D. Krebs, *Clin. Chem. Lab. Med.*, 1989, 27, 663-668.

3 M. Trapp, V. Baukloh, H. Bohnet and W. Heeschen, *Fert. Steril.*, 1984, 42, 146-148.

4 A. Agudo, F. Goñi, A. Etxeandia, A. Vives, E. Millán, R. López, P. Amiano, E. Ardanaz, A. Barricarte and M. D. Chirlaque, *Environ. Res.*, 2009, 109, 620-628.

5 D. Bachelet, T. Truong, M. A. Verner, P. Arveux, P. Kerbrat, C. Charlier, C. Guihenneuc-Jouyaux and P. Guénel, *Environ. Res.*, 2011, 111, 861-870.

6 K. Tsukimori, S. Tokunaga, S. Shibata, H. Uchi, D. Nakayama, T. Ishimaru, H. Nakano, N. Wake, T. Yoshimura and M. Furue, *Environ. Health Persp.*, 2008, 116, 626-630.

7 M. L. Yu, Y. L. Guo, C. C. Hsu and W. J. Rogan, *Int. J. Epidemiol.*, 2000, 29, 672-677.

8 A. S. Stack, S. Altman-Hamamdzic, P. J. Morris, S. D. London and L. London, *Toxicology*, 1999, 139, 137-154.

9 P. Pocar, F. Perazzoli, A. M. Luciano and F. Gandolfi, *Mol. Reprod. Dev.*, 2001, 58, 411-416.

10 T. A. Brevini, R. Vassena, A. Paffoni, C. Francisci, U. Fascio and F. Gandolfi, Eur. *J. Histochem.*, 2004, 48, 347-356.

11 P. Pocar, D. Nestler, M. Risch and B. Fischer, *Reproduction*, 2005, 130, 857-868.

12 T. Muto, N. Imano, K. Nakaaki, H. Takahashi, H. Hano, S. Wakui and M. Furusato, *Toxicol. Lett.*, 2003, 143, 271-277.

13 P. Pocar, N. Fiandanese, C. Secchi, A. Berrini, B Fischer, J. S. Schmidt, K. Schaedlich, S. M. Rhind, Z. Zhang and V. Borromeo, *Toxicol. Sci.*, 2012, 126, 213-226.

14 M. Shirota, M. Mukai, Y. Sakurada, A. Doyama, K. Inoue, A. Haishima, F. Akahori and K. Shirota, J. *Reprod. Develop.*, 2006, 52, 751-761.

15 M. G. Baldridge, R. L. Stahl, S. L. Gerstenberger, V. Tripoli and R. J. Hutz, *Reprod. Toxicol.*, 2003, 17, 567-573.

16 F. Matsuda-Minehata, N. Inoue, Y. Goto and N. Manabe, *J. Reprod. Dev.*, 2006, 52, 695-705.

17 A. T. Peter and N. Dhanasekaran, *Reprod. Domest Anim*., 2003, 38, 209-213.

18 B. Gumus, S. Kilic and E. Zulfikaroglu, *Int. J. Genet. Mol. Biol.*, 2013, 5, 54-63.

19 P. J. Devine, C. M. Payne, M. K. McCuskey and P. B. Hoyer, *Biol. Reprod.*, 2000, 63,

1245-1252.

20 I. Ben-Aharon, H. Bar-Joseph, G. Tzarfaty, L. Kuchinsky, S. Rizel, S. M. Stemmer and R. Shalgi, *Reprod. Biol. Endocrinol.*, 2010, 8, 20.

21 J. M. Young and A. S. McNeilly, *Reproduction*, 2010, 140, 489–504.

22 N. Manabe, Y. Goto, F. Matsuda-Minehata, N. Inoue, A. Maeda, K. Sakamaki and T. Miyano, *J. Reprod. Dev.*, 2004, 50, 493-514.

23 W. C. Earnshaw, *Curr. Opin. Cell Biol.*, 1995, 7, 337-343.

24 G. Ptak, F. Zacchini, M. Czernik, A. Fidanza, C. Palmieri, L. Della Salda, P. A. Scapolo and P. Loi, *Hum. Reprod.*, 2012, 27, 1034-1042.

25 K. V. Ebner and W. E. Braselton Jr, *J. Toxicol. Env. Health*, 1987, 22, 45-62.

26 Y. Nishihara and K. Utsumi, *Biochem. Pharmacol.*, 1987, 3453-3457.

27 N. Zamzami and G. Kroemer, *Nat. Rev. Mol. Cell Bio.*, 2001, 2, 67-71.

28 D. Choi, S. Hwang, E. Lee, S. Yoon, B. K. Yoon and D. Bae, *J. Soc. Gynecol. Invest.*, 2004, 11, 311-317.

29 Z. Oltvai, C. Milliman and S. J. Korsmeyer, *Cell*, 1993, 74, 609-619.

30 T. Rudel, *Herz*, 1999, 24, 236-241.

31 S. G. Hwang, H. C. Lee, D. W. Lee, Y. S. Kim, W. H. Joo, Y. K. Cho and J. Y. Moon, *Toxicology*, 2001, 165, 179-188.

32 J. A. Sánchez-Alonso, P. López-Aparicio, M. N. Recio and M. A. Pérez-Albarsanz, *Toxicol. Lett.*, 2004, 153, 311-326.

33 N. R. Brady, A. Hamacher-Brady and R. A. Gottlieb, *Biochim. Biophys. Acta.*, 2006, 1757, 667-678.

34 I.S. Goping, A. Gross, J. N. Lavoie, M. Nguyen, R. Jemmerson, K. Roth, S.J. Korsmeyer and G.C. Shore, *J. Cell Biol.*, 1998, 143, 207 – 215.

35 S. Shimizu, M. Narita and Y. Tsujimoto, *Nature*, 1999, 399, 483 – 487.

36 M. Saito, S.J. Korsmeyer and P.H. Schlesinger, *Nat. Cell Biol.*, 2000, 2, 553 – 555.

37 S. A. Susin, N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens and G. Kroemer, *J. Exp. Med.*, 1996, 184, 1331-1341.

38 S. Gupta, H. F. Su, R. F. Bi, S. Agrawal, and S. Gollapudi, *Immun. Ageing*, 2005, 2, 12.

39. V. S. Ratts, J. A. Flaws, R. Kolp, C. M. Sorenson and J.L. Tilly, *Endocrinology*, 1995, 136,

3665-3668.

40. S. Y. Hsu, R. J. Lai, M. Finegold and A. J. Hsueh, *Endocrinology*, 1996, 137, 4837-4843.

41. K. Kugu, V.S. Ratts, G. N. Piquette, K. I. Tilly, X. J. Tao, S. Martimbeau, G. W. Aberdeen, S. Krajewski, J. C. Reed, G. J. Pepe, E. D. Albrecht and J. L. Tilly, *Cell Death Differ.*, 1998, 5, 67-76.

42 S. Z. Liu, L. G. Jiang, X. Q. Meng, X. Y. Han, D. Cheng, T. L. Zhang and Y. L. Miao, *PLoS One*, 2014, 9, e102064. doi: 10.1371/journal.

43 A. Bergman, E. Klasson-Wehler and H. Kuroki, *Environ. Health Perspect*, 1994, 102, 464-469.

44 J. L. Tilly, *Reprod. Biol. Endocrinol.*, 2003, 1, 1-4.

45 J. Flaws, K. Salyers, I. Sipes, P. Hoyer, *Toxicol. Appl. Pharm.*, 1994, 126, 286-294.

46 T. Pedersen and H. Peters, *J. Reprod. Fertil.*, 1968, 17, 555-557.

47 W. B. Murrill, N. Brown, J. X. Zhang, P. Manzolillo, S. Barnes and C. Lamartiniere, *Carcinogenesis*, 1996, 17, 1451-1458.

48 P.H. Campos-Junior, A. C. Marinho, B.C. Carvalho, R. I. Batista, R.M. Garcia and J.H. Viana, *Reprod. Biol.*, 2012, 12, 41-55.

49 H. X. Xu, X. D. Zhu, P. Y. Zhuang, J. B. Zhang, W. Zhang, L. Q. Kong, W. Q. Wang, Y. Liang, W. Z. Wu, L. Wang, J. Fan, Z. Y. Tang and H. C. Sun, *Int. J. Cancer.*, 2011, 128, 1559-69.

50 X. D. Zhu, J. B. Zhang, P. Y. Zhuang, H. G. Zhu, W. Zhang, Y. Q. Xiong, W. Z. Wu, L. Wang, Z. Y. Tang and H. C. Sun, *J. Clin. Oncol.* 2008, 26, 2707-16.

Aroclor 1254 impairs the development of follicle (primordial follicle excluded), possibly via the induction of apoptosis mainly in granulosa cells.