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Polycyclic Aromatic Hydrocarbons Cause Follicle Atresia and Apoptosis in Mouse Ovarian Follicles Cultured in Vitro that can be Reduced with the Activator of PI3K/Akt Pathway, 740Y-P

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Abstract

Objective: To explore the effect of the environmental toxicants polycyclic aromatic hydrocarbons (PAH) on the ovary.

Methods: Female mice of child-bearing age were randomized to a control group and three PAH exposure groups at a dose of 0.1 μg/mL, 1 μg/mL or 10 μg/mL. The ovary was removed using aseptic technique, cultured in vitro for 10 days, and HE stained to observe structural change by light microscopy. Ovarian granulosa cell apoptosis was detected by immunohistochemistry, and the number of ovarian follicles undergoing atresia was counted. Western blot was carried out to detect the expression of apoptosis-related proteins (Caspase3,Caspase9, bax and bcl-2). Then we detected if these damages can be prevented by 740Y-P, an activator of PI3K/Akt pathway.

Results: The number of ovarian follicles in the three PAH groups was significantly lower than that in the control group ($P<0.05$). Compared with the control group, the expression of apoptotic protein Caspase-3, Caspases9 and Bax was increased, and the expression of Bcl-2 was decreased in PAH groups. PI3K/Akt pathway prevented the apoptosis of the ovary.

Conclusion: Exposure of female mice of reproductive age to PAH induced ovarian granulosa cell apoptosis that can be prevented with the activator of PI3K/Akt pathway 740Y-P.

Keywords: Polycyclic aromatic hydrocarbons; PI3k/Akt; Ovarian granulosa cell; Follicle apoptosis

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a well-known group of persistent organic pollutants widely existing in soil, water, or air. Volcanic eruption, forest fires and biosynthesis are the main natural sources of PAH, and organochlorine manufacturing, paper bleaching processes, waste incineration, metal manufacturing, fossil fuels and wood burning are the main artificial sources, including human activities such as indoor heating, smoking, career in PAH-exposure industries, cooking and food contact to polycyclic aromatic hydrocarbons [1]. The most common kind of polycyclic aromatic hydrocarbons including benzo(a)pyrene (BaP), dimethyl-benzene -pyrene-anthracene (DMBA), benzo [e] pyrene (BeP), benzo(a)anthracene (BaA), dibenzo(a,h)anthracene (DBA), benzo(k)fluoranthene (BkF), chrysene (CRH), acenaphthene (ANA), acenaphthylene (ANY), and anthracene (ANT). PAH are carcinogenic and toxic to bone marrow and female reproduction. Related animal experiments [2-5] demonstrated that exposure to PAH during pregnancy of female mice damaged germinal cell proliferation of their offspring, and reduced the volume of the offspring ovary and the number of primordial follicles. Neonatal female mice exposed to PAH diminished primary follicles in ovary. Zebrafish experiments showed that PAH in polluted water affected the fish reproductive function including the ability of spawning. The ovary is an important female reproductive organ that is easily affected by environmental factors, including environmental toxicology of ovarian premature senility, which is regarded as one of the important causes of female infertility. High lipophilicity and stability of PAH make it easy to accumulate in the biological body and food chain, thus forming a potential threat to human health. To better understand the toxic effects of PAH on the ovary, we cultured the ovary of childbearing age mice in vitro and exposed them to different concentrations of PAH to observe the impact of PAH on ovarian cell apoptosis, in an attempt to clarify the toxic effect of PAH on female reproduction.

Methods and Materials

Animals

Clean grade healthy female C57BL/6 mice were brought from the Second Military Medical University Animal Center (Shanghai, China) and raised in plastic cages with the environmental temperature controlled at 22 ± 1 in 12-hour day and night cycles with free access to food and water. Mice at the age of 10 weeks were sacrificed by cervical dislocation under aseptic conditions to remove the bilateral ovaries.

Ovaries cultured *in vitro*

In vitro culture of the ovary was in accordance with the Devine literature [6] with minor modifications. The animals were assigned to eight groups, each group was treated with one of the following: DMSO(Sigma–Aldrich, CAS NO.67-68-5, USA; vehicle control), PAHs (0.1 μg/ml, 1 μg/ml, 10 μg/ml, 7,12-Dimethylbenz[a]anthracene (Sigma–Aldrich,CAS:57-95-6) and Benzo[a]pyrene (Sigma–Aldrich,CAS: 50-32-8) in 1:1 mix), and a PI3K activator (740Y-P,10 μg/ml) or 740Y-P + PAHs(0.1 μg/ml, 1 μg/ml, 10 μg/ml). F-12/DEME medium with 1 mg/mL BSA, 1 mg/mL Albumax 50 μg/mL ascorbic acid, 27.5 μg/mL transferrin, 5 μ/mL antibiotic and 500 μl medium were added to each well of a 24-well plate, in addition to different concentrations of PAH. The plate was balanced for at least 1 h in a 37 and 5% CO_2 incubator with a Millicell-CM filter membrane insert before use. The 10-week-old mice were sacrificed by cervical dislocation using the aseptic technique to remove the bilateral ovaries freshly. After cutting off the surrounding fat and connective tissue, they were quickly and randomly placed on the 24-well plate. To prevent drying, 2-3 drop medium was dripped to the surface of the ovary, with two ovarian tissues in each well and every two wells for a group. Culture the 24 well plate in the 37 and 5% CO_2 incubator for 10 days, with the medium changed every two days.

Ovarian follicle counting

After culture, the ovary was fixed in 4% paraformaldehyde, paraffin embedding for 4 h, sliced to 5-micron-thick sections, H&E stained, and counted for the number of follicles according to follicle counting reference Gougeon published in 1986 follicle classification method: primordium follicle: oocytes around particles by simple squamous cells; primary follicles: oocytes by monolayer around at least three of the cube of granular cells; secondary follicle: oocytes by two or more than two layers of granular cell, but excluding sinus cavity structure; sinus follicle and mature follicle: oocytes around particles containing at least two or more layers of cells, and sinus cavity structure [7]. The number of follicles per whole ovary was determined in every tenth section, as previously described. The original follicles, primary follicles, secondary follicles, sinus follicles and atresia follicles were observed respectively.

Immunohistochemical analysis and TUNEL assays

Five ovaries were chosen from the control and the three experimental groups respectively, paraffin embedded, sliced into 5-micron-thick sections, repaired with EDTA (PH9.0) antigen repair buffer, incubated in 3% hydrogen peroxide solution for 25 min at room temperature away from light to block endogenous peroxidase and then in 5% BSA at room temperature for 30 min. after addition of caspase3 antibody (1:500), caspase 9 (1:1000), Bax (1:300) and Bcl-2 (1:500) antibody, they were incubated at 4 overnight, washed, and added with the second antibody (1:200), and incubate at room temperature for 50 min for immunohistochemical analysis. Cell apoptosis was assessed by Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays. The Cell Apoptosis Detection Kit (Beyotime, Jiangsu, China) was used to detected TUNEL assays according to the manufacturer's protocol. The results were quantified with Image-Pro plus 6.0 software.

Western blot

Five ovaries were chosen from the control and the three experimental groups respectively. Protein was extracted and the concentration was determined using the BAC quantitative determination kit. After SDS-PAGE gel electrophoresis to separate proteins, they were transferred to nitrocellulose membranes, blocked, added with corresponding antibodies at 4, incubated overnight, added with the second antibody (1:5000), incubated at room temperature for 1 h, washed with TBS-T, and finally imaging scanned and analyzed.

Statistical analysis

All the data were expressed as mean ± SD, and all the statistical analyses were performed using the SPSS statistical software package (SPSS Inc, Chicago, IL, USA) by using Student's t-test. P-Values<0.05 were considered statistically significant.

Results

PAHs exposure affect the ovarian morphology

To observe the influence of PAHs on the ovarian follicles, ovaries were cultured in media containing vehicle control or PAHs (0.1 μg/mL, 1 μg/mL or 10 μg/mL) for 10 days. Light microscopy of the HE stained sections showed that the ovarian morphology structure and the number of atresia follicles varied with different groups. As shown in **Figure 1**, the ovarian tissue structure and the number of follicles at different levels were normal in the control group (**Figure 1A**), while varying degrees of follicular cavity collapse and interstitial fibrosis were seen in PAH groups.

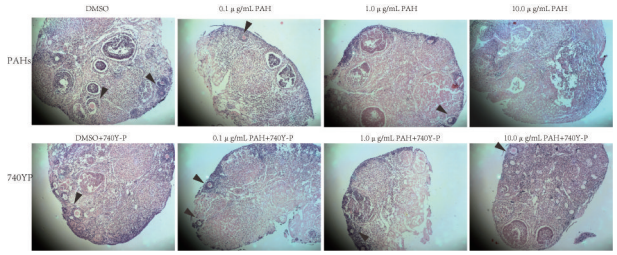

Figure 1 Effect of PAHs exposure on the ovarian morphology. HE staining of the ovarian tissue: A, the control group; B, the low-concentration (0.1 μg/ml) PAH group; C, the medium-concentration (0.1 μg/ml) PAH group; D, the high-concentration (0.1 μg/ml) PAH group. Compared with the control group, the ovary morphologic structure of the PAH groups was changed, the number of follicles was decreased, and the number of atretic follicles was increased. compare the PAHs treated groups with the 740Y-P +PAHs groups, the figures show that the PI3K-pathway activator prevent the follicular atresia. (The arrow "▼" refers the follicle.)

PAHs decrease the number of ovarian follicles

In the three PAH groups, the PAH concentrations was increased, the number of total follicles was decreased gradually, and the proportion of atretic follicles was increased, as compared with the control group (P<0.05). To investigate the progressive reduction of follicles, we quantified the follicles numbers after 10 days cultures. All of the PAHs treated groups shows a significant descrease of follicles, especially in the high concentration group compare to the control group. Correspondingly, the number of the folliclular atresia increased, compared with the control group the PAHs treated groups have a notably increase of the follicluar atresia (**Figure 2**).

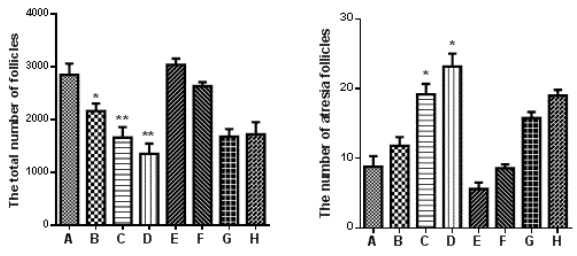

Figure 2 PAHs decrease the number of ovarian follicles. A, the control group; B, the low-concentration (0.1 μg/ml) PAH group; C, the medium-concentration (0.1 μg/ml) PAH group;D, the high-concentration (0.1 u g/ml) PAH group. E: 740Y-P, F: 0.1 μg/ml PAH+740Y-P, G: 1.0 μg/ml PAH+740Y-P, H: 10.0 μg/ml PAH+740Y-P. Single-factor analysis of variance showed that the number of follicles decreased with the concentration of PAH increasing. All data were presented as mean ± SD values and were compared with the control group. "*" indicates P <0.05, and the difference is significant.

PAH promote granulosa cell apoptosis and follicular atresia.

The result of immunohistochemistry showed that compared with the control group, the expression level of apoptosis-related caspase3, caspase9 and Bax in the granulosa cells of follicles was increased, and the expression of apoptosis-inhibiting bcl-2 was decreased in the three PAH groups. To a certain extent, the apoptosis of granulosa cells reflected the follicle atresia. This trend was also shown by Western blot analysis, further confirming that PAH had a toxic effect on female reproduction (**Figure 3**). In addition, comparison of immunohistochemical positive cells in all groups suggested that the percentage of apoptotic granulosa cells increased with the PAH concentration increasing, especially in M-PAH and H-PAH groups (P<0.05).

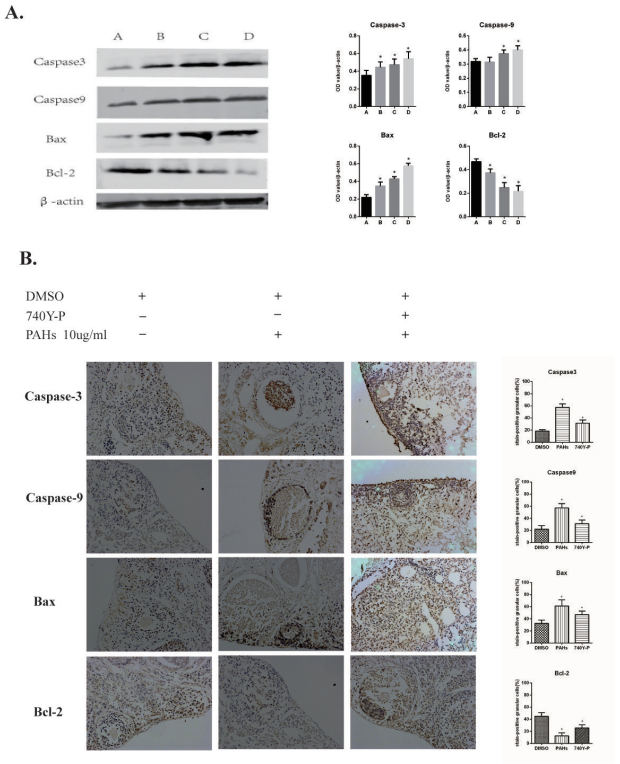

Figure 3 PAH promote granulosa cell apoptosis and follicular atresia. (A, the control group; B, the low-concentration (0.1 μg/ml) PAH group; C, the medium-concentration (0.1 μg/ml) PAH group; D, the high-concentration (0.1 μg/ml) PAH group) (A). The western blot shows that with the increasing concentration of PAHs, these apoptosis-related protein expression was up-regulated. And the expression of the anti-apoptosis protein Bcl-2 become decreased (B). Immunohistochemical detection of the expression of caspase3, caspase9, Bax and Bcl-2. Comparison of the immunohistochemical positive rate between the control and three PAH groups. All data were compared with the control group. "*" indicates P <0.05, and the difference is significant.

Effect of the PI3K activator 740Y-P on granulosa cell apoptosis and follicles atresia

The 740Y-P is an activator of PI3K pathway. With the treatment of 740Y-P, the follicle number increased compared with the DMSO control group. And the results of the HE stains show that the total follicles number were up-regulated in the 740Y-P treated groups compared with the PAHs group, though the P values have no significance (**Figure 2**). To further confirm the apoptosis of the follicle cells, TUNEL assays shows that apoptosis cells were increased in PAHs group compared with the control group, while the addition of 740Y-P decreased the apoptotic trend (**Figure 4**).

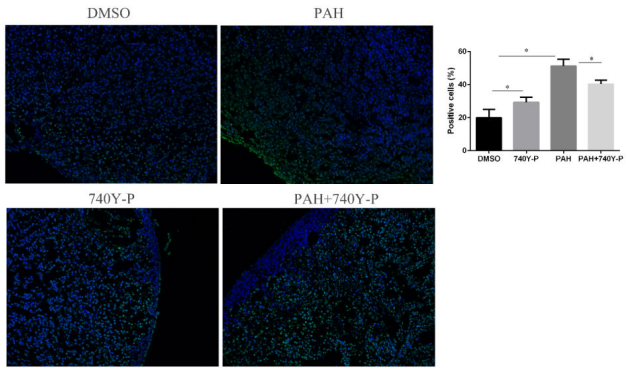

Figure 4 Effect of the PI3K activator 740Y-P on granulosa cell apoptosis and follicles atresia. The follicular cell apoptosis assessed by TUNEL shows that compared with the control group the percentage of positive cells (green) were increased in PAHs group. While add the 740Y-P, the positive cells percentage were decreased. Representative images are shown. Objective vision, 200X.

Discussion

Polycyclic aromatic hydrocarbons are a group of lipophilic environmental toxic substances. Incomplete fuel burning, flue gas, automobile exhaust and cigarette smoking are the main sources of PAH in the atmospheric pollution. In addition, smoked foods also contain a considerable amount of PAH. According to the US Environmental Protection Agency, the concentration of 16 PAH in water should be less than 0.2 μg/L, and according to the World Health Organization (WHO) the concentration of PAH in water should be less than 0.02 μg/L. However, the concentration of PAH in industrial wastewater in China is as high as 1 mg/mL [1]. The lipophilic characteristic of PAH makes it easy to deposit in lipid-rich tissues and organs, affecting human health.

Related studies [8] demonstrated that exposure of the mouse ovary to BaP, a component of the PAHs, delayed the follicular development and shortened service life. Fish experiments [9] showed that high concentrations of PAH could disrupt endocrinology, inhibit the body formation and steroid hormone secretion, and affect fish development and reproduction. Cigarette smoke contains multiple kinds of PAH. Related studies [10] showed that exposure of female mice to cigarette smoke decreased the number of primordial follicles due to various chemical poisons in the smoke, among which PAH may play an important role. Another [11] reported that the risk of fetal exposure to PAH is increased in women smokers, thus affecting the fetal ovarian development and hormone secretion. In a cigarette smoke inhalation trachea model to simulate human smoking, the observation [12] about that the egg quality of the mice was decreased in 12 weeks in the mice exposed to cigarette smoke, as compared with the normal and healthy mice, confirming that PAH have a toxic effect on female reproduction. Studies on the mechanism underlying the ovarian toxicity of PAH showed that this toxic effect occurred in the earliest stage of follicle development, thus decreasing in the number of primordial and primary follicles, eventually leading to ovarian premature failure and reduced fertility. An experiment [13] on rats shows that prenatal exposed to BaP leads offsprings premature ovarian failure and ovarian tumorigenesis. A Study concerning the effect of PAH on follicular growth [14] showed that mice exposed to cigarette smoke could reduce antral follicles, therefore leading to appropriate preovulatory follicles decreased before ovulation. Relevant epidemiological studies [15,16] also showed that smoking women of childbearing age were usually accompanied with a low rate of pregnancy or a high rate of abortion and/or infertility. A study [17] showed that the BaP and other PAH levels in the follicular fluid of smoking women were significantly higher than those of nonsmoking women. As a coal-based city, the high PAH contamination in the air of Shanxi Province, a coal-based province in China, puts women living there at a high risk of contacts with PAH, which is reported to be the main cause of the high rate of birth defects in that province. It was reported [18] that the PAH concentration in the venous blood of women whose offspring were affected with neural tube defects was significantly higher than that in women who gave birth to healthy babies (258 ng/ml vs. 120 ng/ml, $p<0.05$).

But the air we breathe is polluted with a mixture of PAHs. Also, there are few studies about the effect of PAH mixtures on human or animal's reproduction capacity. In this experiment we attempt the common two components of PAHs to treat the ovary in different concentrations. The results show that apoptosis could be induced in preovulatory follicles by PAH. The number of ovarian follicles may reflect the ovarian function. The childbearing age ovary has large numbers of growing follicles. Follicular growth and atresia play an important role in maintaining the pool of primordial follicles, and the abnormal atresia or activation may result in premature ovarian failure and other ovarian diseases. It was found in our experiment that after 10-day exposure to PAH, the number of atretic follicles was increased, the number of normal follicles was decreased, and the percentage of apoptotic granulosa cells was increased significantly, as compared with the control group, suggesting that the toxicity of PAH may alter the ovary morphologically and affect the follicles in vitro.

Apoptosis is a programmed cell death mode regulated by relevant genes, in which the Caspase family and Bcl-2 family play an important role. The Caspase family cascade is an important component in regulating apoptosis. Caspase9 is a top cascade, whose activation can activate downstream apoptotic molecules

such as caspase3. As a key executive molecule in this reaction, caspase3 can lead to apoptosis by lysing the corresponding substrate in the cytoplasm or nucleus [19]. The Bcl-2 family can be divided into the anti-apoptotic protein and the pro-apoptotic protein, like Bcl-2 and Bax. The balance between the anti-apoptotic molecule Bcl-2 and the pro-apoptotic Bax can regulate cell apoptosis. Dominance of Bax expression promotes apoptosis, while dominance of Bcl-2 expression inhibits apoptosis [20]. Immunohistochemistry of our experiment showed that the expression of apoptotic molecules caspase3, caspase9 and Bax in the three PAH groups was significantly higher than that in the control group, while Bcl-2 was down-regulated, which further suggests that PAHs can up-regulate caspase3, caspase9 and Bcl-2, and down-regulate Bcl-2, thus inducing apoptosis of granulosa cells. And the apoptosis of granulosa cells may result in the ovarian reserve damaged and infertility or other reproductive-related diseases. The 740Y-P is an activator of the PI3K-Akt pathway [21], and the PI3K-Akt pathway plays an important role to follicle development, activate or survival [22]. We tested weather the follicular atresia and apoptosis caused by PAHs could be suppressed by activation of the PI3K-Akt pathway. Our results show that after treatment of the PI3K activator, the follicular atresia and apoptosis were significantly reduced, thus indicating the participation of the PI3K-Akt pathway in PAH-induced ovary damage.

In conclusion, current study suggests that the exposure to the PAH mixtures result the follicles deletion and activate the apoptosis of the granular cells. Though the carcinogenic effect of PAH and its effects on birth and reproduction have already been confirmed, but the mechanism of PAH toxicity on the reproductive system remains to be further studies. General studies have suggested that PAH may have an anti-estrogen-like effect, and can activate the Ahr receptor, or any other mechanism to act reproductive toxicity on the ovary. It was found in our study that PAHs could interfere with the expression of casepase3, casepase9 and Bcl-2/Bax to promote follicular apoptosis and affect the ovary function. But this trend can be reduced through activating the PI3K/Akt pathway wjth the activator 740Y-P. Perhaps in the future it can be a means of protect the ovarian function.

Acknowledgments

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Declaration of Interest

We declare that there is no conflict of interest.

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