Perfluorooctanoic Acid Exposure in Early Pregnancy Induces Oxidative Stress in The Uterus and Liver in Mice

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Abstract

To investigate the mechanism perfluorooctanoic acid (PFOA)'s toxicity on the uterus and liver of the mice during early pregnancy, pregnant mice were given 0, 1, 5, 10, 20, 40 mg/kg PFOA daily by gavage from gestational day (GD) 1-7, and sacrificed on GD 9. Uterus and liver weight were recorded, liver and uterine indexes were calculated, histopathological changes of the liver and uterus were examined, and levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) in liver were detected by spectrophotometric method. Expression of FAS, FASL, Bax, Bcl-2, and Caspase-3 in decidual cells were detected by immunohistochemistry and the TUNEL method was used to detect apoptotic uterine cells. Results showed that liver weight increased, and the uterus index was significantly reduced at 40 mg/kg compared with the control group. With increasing doses of PFOA, levels of SOD and GSH-PX were significantly decreased, and MDA significantly increased in liver tissue. 20 mg/kg and 40 mg/kg of PFOA caused greater harm to the uterus and congestion and resorption may occur. Expression of FAS, FASL, Bax, and Caspase-3 in decidual cells of the uterus in PFOA treatment groups significantly increased in a dose-dependent manner. The expression of Bcl-2 was down-regulated, which decreased the ratio of Bcl-2/Bax. It is therefore proposed that oxidative damage may be one of the mechanisms by which PFOA induces liver toxicity, and a subsequent increase in uterine cell apoptosis may induce embryo loss or damage.

Introduction

Perfluorinated compounds (PFCs) have been widely used in many areas, including industrial production, household supplies and even biomedical applications, because of their good chemical stability, and hydrophobic and oleophobic properties (A et al. 2020; Kah et al. 2020; Li et al. 2019b; Sivaram et al. 2020). It has been shown that PFCs can be found in the blood, liver, kidney, heart, and muscle, and the toxicity has been demonstrated in the liver(Filgo et al. 2015; Wu et al. 2017; Zhao et al. 2016; Zheng et al. 2017), as well as in neuro(Goulding et al. 2017), cardiovascular(Lv et al. 2018), and reproductive tissues(Lee et al. 2017). And it has also been shown to be genotoxic(Butenhoff et al. 2014), carcinogenic(Tsuda 2016), and immunotoxic (Di Nisio et al. 2020; Liu and Gin 2018). Perfluorooctanoic acid (PFOA), one of the most widely used PFCs, is most frequently found in the environment(Fagbayigbo et al. 2018; Rankin et al. 2016). It contains eight perfluoroalkyl carbons and is widely used in manufacturing consumer products, including surfactants, oil and in water proof coatings of carpets, leather, paper, food packaging and non-stick cookware (Poothong et al. 2012; Post et al. 2012). However, there is increasing evidence suggesting that PFOA is a persistent, bioaccumulative, and toxic substance. Because it is bioaccumulated along the food chain, PFOA is potentially toxic to humans and animals (Vierke et al. 2012). The most important exposure pathways are food (Heo et al. 2014), water 2010(Murray et al. 2010; Schwanz et al. 2016; Xie et al. 2020), and indoor air or household dust(Haug et al. 2011). Perfluorooctanoic acid (PFOA) has been detected in water, air, sediment, and sludge, and as well as in animal and human blood and other tissue samples(Dong et al. 2018; Knight et al. 2020). Results from Lau et al (2006) indicated that PFOA led to an obvious embryo development toxicity in

pregnant mice. It has been reported that potential reproductive toxicity of ammonium perfluorooctanoate on the offspring of rats(Butenhoff et al. 2014), but the mechanism of developmental toxicity remains unclear.

Pregnant animals are very sensitive to toxins. Therefore, the reproductive and developmental toxicity of pollutants such as PFOA can be manifested shortly after exposure. In this study, pregnant mice were gavaged with different doses of PFOA (0, 1, 5, 10, 20, 40 mg/kg) from gestational day 1 to 7. To help understand the mechanism of PFOA's hepato- and developmental toxicity in early pregnancy stage in mice, the relative parameters were measured including uterine and liver indexes, expression of SOD, MDA, and GSH-Px in the liver, pathological changes in the uterus and liver, and the expression of FAS, FASL, Bax, Bcl-2 and Caspase-3 in decidual cells.

Materials And Methods

Chemicals and reagents.

Perfluorooctanoic acid (PFOA, 99.2% pure), purchased from Fluka Sigma-Aldrich, USA, was dissolved in deionized water. SOD, MDA, GSH-PX biochemical parameter kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Antibodies against FAS, FASL (mouse monoclonal), Bcl-2, Bax, and Caspase-3 were purchased from Boster Biological Technology (Pleasanton, CA, USA), and a ready-to-use SABC immunohistochemical kit was sourced from Boster Biological Technology Co Ltd (Wuhan, China).

Treatment of animals.

8-week-old female and male Kunming mice were purchased from Sibeifu Animals Biotech Co. Ltd. (Beijing, China), license number SCXK(Beijing) 2016-0002. They were given free access to mouse chow and water, with a 12 h light cycle from 7:00–19:00. After a week adaptation to their new environment, females in estrous were mated with males 1:1 overnight. And the next morning, if a vaginal plug was detected, that day was designated as gestational day 0 (GD 0). Sixty pregnant mice at GD 0 were randomly divided into six groups (n = 10). Group A as the control (PFOA 0 mg/kg bw), and the rest groups were given PFOA at various concentrations by oral gavage on GD 1–7. Group B (1 mg/kg bw), group C (5 mg/kg bw), group D (10 mg/kg bw), group E (20 mg/kg bw) and group F (40 mg/kg bw).

All mice were weighed on GD 9. Pregnant mice were sacrificed by cervical dislocation after collecting blood on GD9 (48 h after last treatment). Liver and uterus were removed and the absolute and relative weights measured. The left horn of the uterus and part of the liver were promptly fixed in Bouin's Fluid. 0.2 g liver tissue was ground into a 10% homogenate in 0.9% saline at 4℃. The homogenate was centrifuged at 3000 rpm for 15 min, and the supernatant was stored at -80℃. The experiment was conducted in the animal house of Hebei Agricultural University, and all experimental protocols were approved by the Animal Protection Committee of Hebei Agricultural University before the study began.

Organ index.

The liver and uterus were collected discarding adherent fat and fascia, rinsed in saline, then dried with filter paper. Absolute and relative liver and uterus weights were measured and the organ index calculated as follows; Organ index (%) = organ weight (g) / body weight (g) × 100%

Liver and uterine morphology.

Uterus and liver tissues were fixed in Bouin's solution for 48 h, dehydrated in graded ethanol, transparented in xylene, and embedded in paraphine. Then 5 μm thick serial sections were prepared. The sections were stained with hematoxylin-eosin (HE) and the morphology and pathological changes in the uterine and liver sections were observed under a microscope.

Detection of liver oxidation index.

MDA, SOD, and GSH-PX were detected via the thiobarbituric acid method (TBA), xanthine oxidase assay, and the dithiobis nitrobenzoic acid assay, respectively, using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Measurements were conducted in accordance with the manufacturer's instructions. Protein content of the liver homogenate was measured by spectrophotometer using the Coomassie brilliant blue G-250 method.

Immunohistochemical detection of FAS、FASL、Bax、Bcl-2、and Caspase-3 protein expression in the uterus.

The 5 μm uterus sections were mounted on polylysine treated glass slides, placed in an oven, and baked at 60℃ for 60 min to promote adherence. Sections were dewaxed in xylene for 10 min twice, then hydrated. 3% H2O2 was added for 8 min at room temperature to inactivate endogenous enzymes. the sections were rinsed three times in double distilled water before washing in 0.01M citrate buffer (pH6.0) for antigen retrieval. 5% BSA blocking buffer was added dropwise and the slides were incubated at room temperature for 20 min, then incubated with 1:100 dilution of rabbit anti rat antibody at 4℃ overnight. A negative control for each group, 0.01 mol/L PBS solution (pH7.4) without any antibody was used and processed in the same manner. The following day, all slides were removed the refrigerator, left to come up to room temperature, then washed in PBS for 3 x 2 min. Biotinylated goat anti-rabbit IgG was added to the slide and it was incubated at 37℃ for 20 min, washed in PBS for 3 x 2 min. SABC solution was then added for 20 min at 37℃ followed by washing in PBS for 4 x 5 min. Sections were visualized using a DAB kit, dehydrated by the conventional method, mounted by neutral balsam, and observed under a microscope.

Brown particles in the cell membrane or cytoplasm were regarded as positive in each slice, 10 fields (magnification 40x for the objective lens) were selected randomly, the number of positive cells in each field of view was analyzed in Image J software.

TUNEL assay.

Polylysine-treated slides were hydrated in gradient alcohol and incubated with 20 μg/ml Proteinase K at 37 °C for 20 min, then washed twice with PBS. The TUNEL assay was conducted in accordance with the manufacturer's instructions. The images were collected using a microscope, the positive cells were pooled from 5 slices in each group and 8 fields in each section. Each experiment consisted of 10 slices, two negative control sections, and one positive control section.

Statistical analysis.

Statistical analyses were performed using one-way analysis of variance (ANOVA) using SPSS 19.0. Data were presented as means and standard errors (Mean ± SD). $P < 0.05$ shows the significant difference.

Results

Impact of PFOA on organ index

Results showed a gradual increase in body weight in each group of pregnant mice. The control group gained weight faster than the experimental group, and with more PFOA administrated, the lower the increase in body weight, as shown in Fig. 1A.

Results indicated that there was a dose-dependent effect of PFOA on the liver index. The liver index of the mice administered different doses of PFOA was significantly greater than those of the control group ($P < 0.01$), as shown in Fig. 1B. According to the uterus index results, group F showed a significant difference when compared with the control group ($P < 0.01$). No significant differences were observed when groups A, B, C, D, and E compared with the control group.

Histopathological changes in the liver and uterus of mice

Histopathologic changes were observed in the livers of PFOA treated mice. There were visible hemorrhagic spots and necrosis, the liver size increased, and the edge of the liver was congested, as shown in Fig. 2A to C.

Stained sections were observed under a microscope at 400x magnification. In group A, histopathologic examination showed normal liver tissue with homogeneous texture, clear texture of the hepatic duct and sinus, and intact cytoblast of the hepatocyte. However, morphological changes such as a narrowing of the hepatic duct and sinus, uneven cytoplasm, and overflowed or enlarged nucleus of cells were observed in both group E (20 mg/kg) and group F (40 mg/kg). The lesions increased in a dose dependent manner (Fig. 2D to E).

In group A, embryos in the uterus were in the best conditions, even, full, and good transmittance, without bleeding or bruising. Mice treated with PFOA at 1 and 5 mg/kg had minimal histopathologic changes in the uterus. Embryos of group D (10 mg/kg) and group E (20 mg/kg) were congestive and showed different bigger sizes. In 20 and 40 mg/kg group mice, abortion and embryo resorption were observed.

Resorbed embryos were small, dark or purple, some showed hemorrhage and necrosis, and some have been melted into blood clumps, as shown in Fig. 3A to C.

Histopathologic examination of uteruses from group A showed normal tissue with epithelial integrity of the mucous membrane, developed glands, plentiful cytoplasm, hyperchromatic nucleus in the center, loosened intercellular substance, clumps of erythrocyte in the vascular endothelial cells, and vascular cavity. There was no hemorrhage, necrosis, or exfoliation. Compared to the controls, groups E and F changed significantly, characterized by partial hemorrhage, exfoliation, and interstitial cell gap becoming narrower. Some cells showed different degrees of nuclear condensation and crushing, uneven cytoplasm, and vacuolar degeneration. However, there still were normal structures of cells visible, as shown in Fig. 3D to F.

changes of liver oxidation index

As shown in Fig. 4A, PFOA increased the content of MDA in the liver. Compared with the controls, 10 mg/kg groups and 20 mg/kg groups showed significant difference ($P < 0.05$), and 40 mg/kg groups showed a greater significant difference ($P < 0.01$).

As shown in Fig. 4B, PFOA reduced the liver content of SOD. Compared to the control group, SOD in groups 10 mg/kg, 20 mg/kg, and 40 mg/kg was decreased significantly ($P < 0.01$), and in groups 1 mg/kg and 5 mg/kg decreased significantly ($P < 0.05$).

As shown in Fig. 4C, PFOA decreased content of GSH-PX in the liver. Groups 10 mg/kg and 20 mg/kg showed a significant difference ($P < 0.05$), and group 40 mg/kg a greater significant difference ($P < 0.01$) when compared to control group.

Immunohistochemical detection of FAS、FASL、Caspase-3 expression in the uterus

FAS, FASL, and Caspase-3 were expressed in the uterus of the early pregnancy with brown color or brown granules. FAS and FASL were mainly expressed in decidual cells surrounding the uterine blastocyst, with some expression in the myometrium and uterine glandular epithelial cells as shown in Figs. 5. Caspase-3 was shown as a hollow ring, mainly expressed in the decidual cell layer of embryonic contact sites and with less expression in muscle tissues, as shown in Fig. 5.

As shown in Fig. 5(B), the expression of FAS in the 40 mg/kg groups was increased significantly when compared to the control group ($P < 0.01$). The positive expression of FASL and Caspase-3 in the uterus was significantly different between the control group and 40 mg/kg groups ($P < 0.01$).

Immunohistochemical detection of Bax and Bcl-2 expression in the uterus

Bax and Bcl-2 expression was observed in the uterus during early pregnancy. As shown in Figs. 6, expression was seen in decidual tissue and myometriuma.

As shown in Fig. 6, there were significant differences in Bax and Bcl-2 expression between the control and experimental groups ($P < 0.01$). Expression of Bcl-2 in each PFOA group was significantly lower than that in the control group ($P < 0.01$). However, in the 40 mg/kg groups expression was significantly increased when compared to the control group ($P < 0.01$). PFOA groups significantly downregulated the ratio of Bcl-2/Bax expression ($P < 0.01$).

TUNEL detection of uterine cell apoptosis

Apoptotic uterine cells were detected using the TUNEL method. Nuclei that were brown or dark brown were judged to be positive. The results showed that apoptotic cells were found in the uterus of each group. There was a small number of apoptotic cells in the uterus of the normal mice at day 9 of gestation, and the staining was shallow (Fig. 7). In the 40 mg/kg PFOA groups, the number of apoptotic cells in the uterus was significantly higher than the control group ($P < 0.01$).

Discussions

Previous studies have shown that PFOA can induce oxidative damage in liver and uterine cell apoptosis in pregnant mice(Dixon et al. 2012; Li et al. 2019a; Zheng et al. 2017) Our study confirmed that after 7 days of continuous exposure to PFOA the liver weight was increased. Significant differences ($P < 0.01$) were observed in the liver indexes of the PFOA 1 to 40 mg/kg groups Compared with the control group. HE staining showed hepatocyte swelling, and at 20 and 40 mg/kg, PFOA led to a narrowing of the hepatic duct and sinus. Body and organ weights reflect health and nutritional status and are used as a simple and direct indicator of animal health. The results of the present study suggest that PFOA is hepatotoxic in a dose-dependent manner and short-term exposure can cause swelling of the liver cells, which explains the increase in liver index. This result is in consistent with findings of other scholars(Wang et al. 2011; Yahia et al. 2010). Lower doses (1 to 20 mg/kg) of PFOA did not affect the relative weight of uterus, but the high-dose group (40 mg/kg) significantly lowered relative weight of uterus, which might contribute to the abortion or fetal absorption after a high dose of PFOA during early pregnancy. Results of HE staining demonstrated that PFOA treated uteruses showed different degrees of pathological changes, suggesting that PFOA exposure during early pregnancy caused some toxicity to embryos.

High doses of PFOA can increase MDA, and decrease SOD and GSH-PX levels in the liver. SOD and GSH-PX are important antioxidant enzymes, and protect cells from damage by oxidative stress. Therefore, the concentrations of SOD and GSH-PX reflect the activity of free radical scavengers. MDA is generated when free radicals act on unsaturated fatty acids on the cytomembrane. The content of MDA reflects the rate and intensity of lipid peroxidation, and thus, reflects the degree of cell damage (Tsikas 2017). Taken together, MDA, SOD, and GSH-PX levels can reasonably reflect the amount of free radicals and the degree of lipid peroxidation damage. As this study shows, compared with the control group, SOD and GSH-PX levels in the liver homogenate of PFOA groups were decreased at different degrees, and MDA was increased in a dose-dependent manner. Oxidant/antioxidant balance is the basic premise of internal environment stabilization, which directly affects cell proliferation, differentiation, apoptosis, necrosis, and other physiological and pathological processes (Eryılmaz et al. 2018; Ha et al. 2015). It has been shown

in this study that PFOA induces oxidant/antioxidant imbalance in hepatocytes. With increasing doses of PFOA, SOD and GSH-PX decreased along with the antioxidant ability of the animals. If reactive oxygen species cannot be cleared in time in vivo, accumulated free radicals can act on the polyunsaturated fatty acids in the phospholipid bilayer, causing lipid peroxidation and the accumulation of MDA (Trojanowicz et al. 2018). The higher the PFOA levels administered, the lower the SOD and GSH-PX, and the greater the MDA accumulation. The results suggest that oxidative damage is likely to be the mechanism of PFOA's hepatotoxicity. By reducing the activity of antioxidant enzymes SOD and GSH-PX in the liver, the whole antioxidative system of the body is destroyed, and the ability to scavenge free radicals is greatly weakened. This may lead to excess accumulation of free radicals in the body, resulting in excessive oxidative damage to cells, which will interfere with the normal function of the hepatocytes and deteriorate liver damage.

The FAS-FAS ligand (FASL) system contributes to immune tolerance at the feto-maternal site and has been ascribed a role in implantation and placental development by regulating trophoblast invasion and spiral artery remodeling (Eide et al. 2007). Bcl-2 and Bax are two important members of the Bcl2 family. Bax is an apoptotic molecule, and BCL-2 is an anti-apoptotic molecule (Cobellis et al. 2007). In immunohistochemical detection of FAS, FASL, Bcl-2, Bax, and Caspase-3 expression in this study, we found that the apoptosis gene FAS was expressed in yellow or brown, and mainly located in the cytoplasm of decidual cells, partly in the nucleus. Positive expression of FAS in the PFOA treated groups were significantly higher when compared to the controls, in a dose-dependent manner. Mice administered 40 mg/kg of PFOA showed uneven embryo sizes, congestion, or embryo absorption, which may presumably be related to the increased expression of FAS in decidual cells. Apoptosis of decidual cells is coordinated by multiple genes and the expression of these genes play a role in synergism or antagonism thereby controlling the normal apoptotic rate of decidual cells (Liao et al. 2015). If the apoptosis rate of is too high or low, it may induce miscarriage, premature birth, placental abruption, and dysontogenesis (Lv et al. 2016). These results suggest that the mechanism of PFOA's toxicity in embryos may involve upregulating FAS expression in decidual cells, disrupting the balance of FAS/FASL and inducing apoptosis through the FAS/FASL pathway, resulting in embryo loss or damage. Bcl-2 and Bax were expressed in the decidual tissue and also in the myometrium. The expression level of Bcl-2 was higher than Bax. The expression level of Bcl-2 was significantly decreased in each PFOA group and the Bcl-2/Bax ratio was also decreased, positively correlated with PFOA dosage. Around the implantation site in decidual cells, the in situ hybridization signal of Bax gradually increased from day 6 to day 8 after fertilization. More Caspase-3 is expressed in contact with embryos, and it is hardly expressed in muscle tissue. Caspase-3 expressed in the uterus is increased in a dose dependent manner. PFOA administration groups showed different embryo sizes, congestion, or absorption, which may be related to the increased apoptosis of decidual cells. The results showed that Caspase-3 was located at the implantation site of the embryo and expressed adjacent to the embryo. This result is in consistent with findings of Lee et al 's results(Lee et al. 2017).

The TUNEL results showed that there were fewer uterine apoptotic cells in the normal mice which were light colored and distributed in the decidua and uterine smooth muscle. The number and density of

positive cells in the PFOA group was increased and the color darkened, showing a dark brown color, in a dose-dependent manner. This was consistent with the results of the immunohistochemical detection of FAS, FASL, Bcl-2, Bax, and Caspase-3 expression, indicating that PFOA induced a higher level of apoptosis in uterine tissue. Our study result is in consistent with previous findings 's results(Chen et al. 2017).

Conclusion

PFOA has damaging effects on the uterus and liver of mice during early pregnancy. This study provides useful information regarding reproductive and hepatotoxicity in animals induced by the environmental pollutant PFOA.

Declarations

Consent to Participate and Consent to Publish: All the authors participated in the study and agreed to publish their findings in the journal.

Ethical Approval: The experiment was conducted in the animal house of Hebei Agricultural University, and all experimental protocols were approved by the Animal Protection Committee of Hebei Agricultural University before the study began.

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Authors contributions: XW conceived and designed the study, YZ performed most of the experiments and analyzed the results, YZ and XW drafted the manuscript. LL assisted in experimental design, data interpretation and manuscript preparation. YZ and LZ contributed to samples collection, experiment and helped with the data analysis. YZ and JB participated in HE, TUNEL experiments. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

Availability of data and materials: The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere.

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Figures

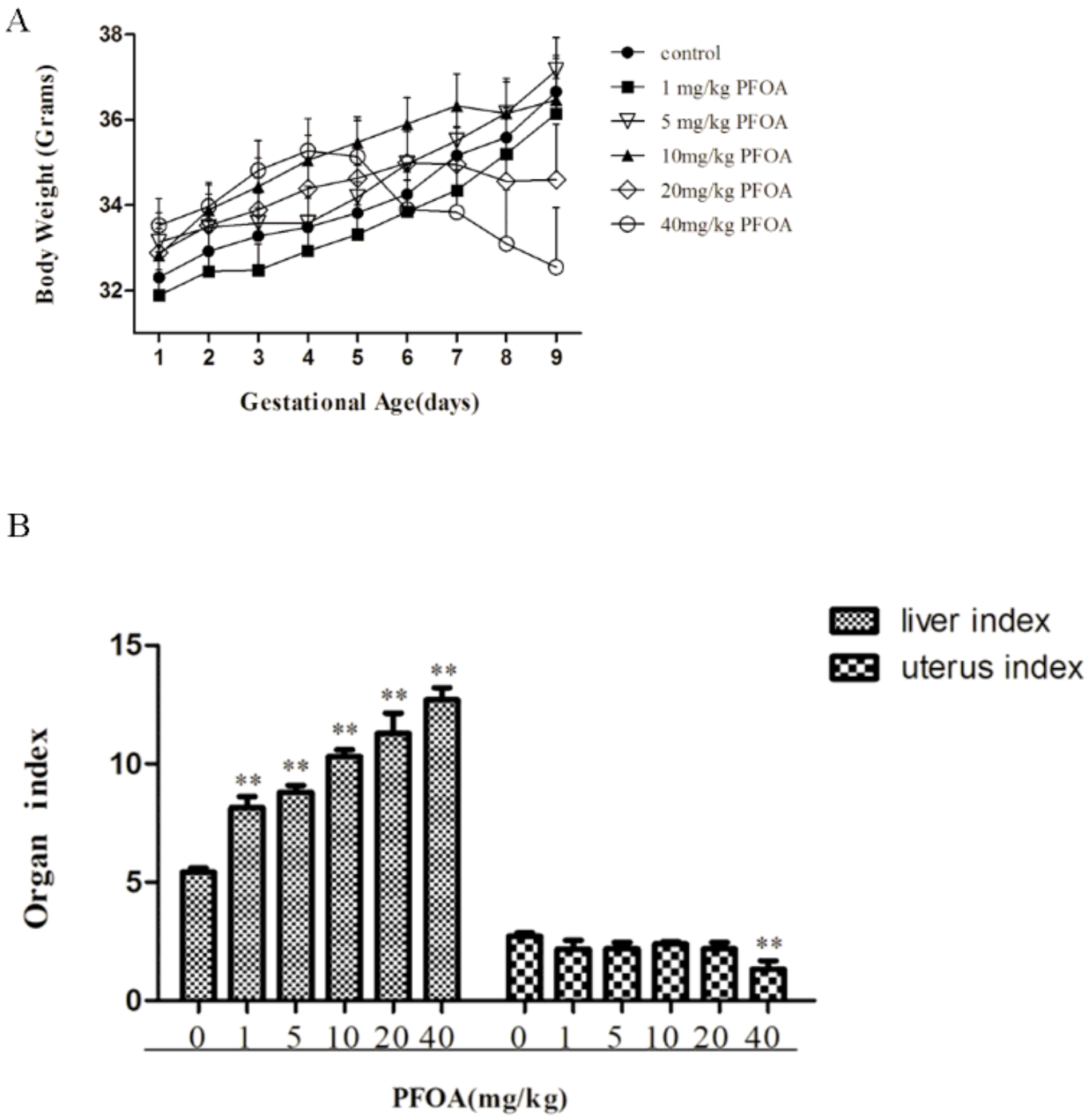

Figure 1

Body weight of the pregnant mice in each group(A). Effect of PFOA on liver index and uterus index(B). * P< 0.05, **P< 0.01, ***P< 0.001, compared with control group.

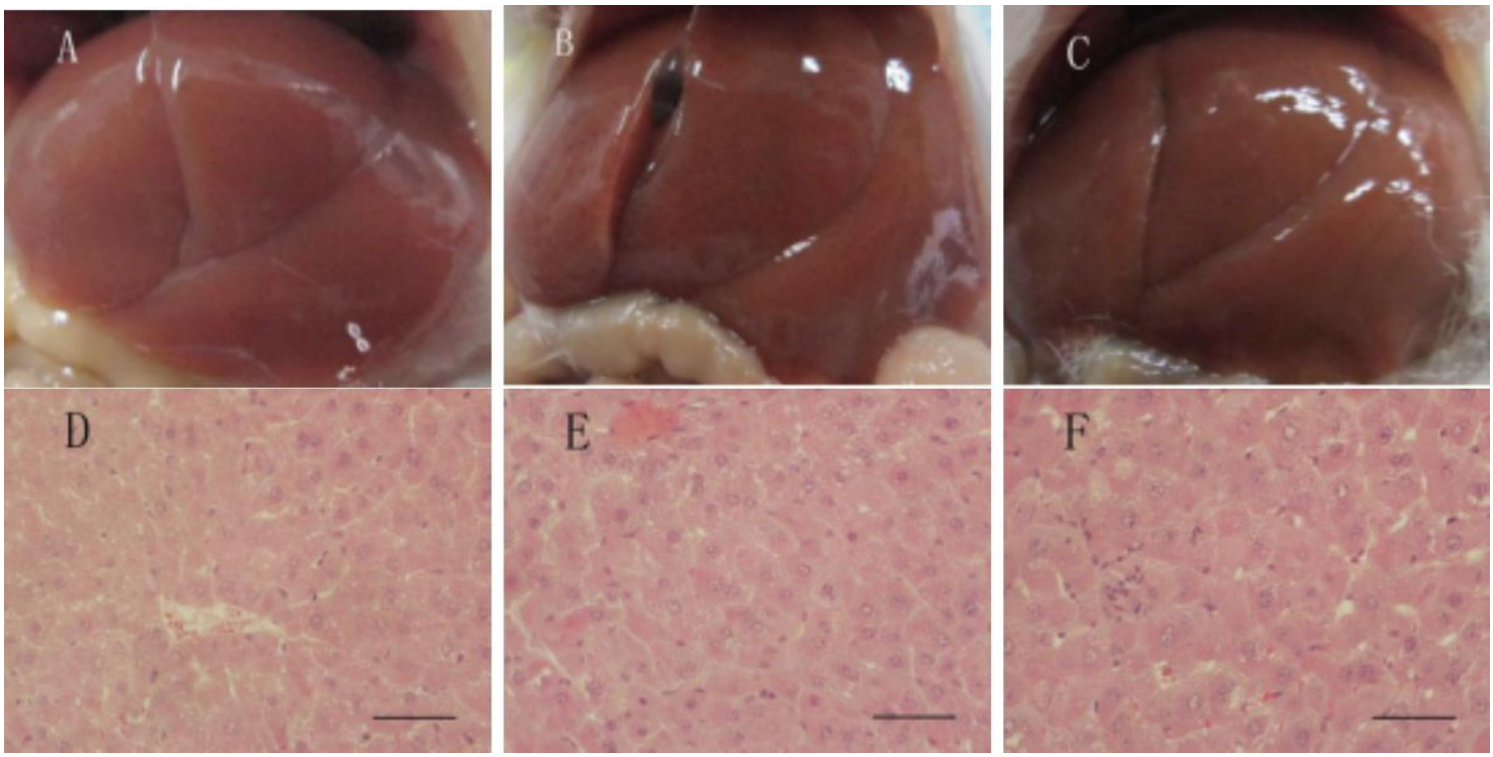

Figure 2

Histopathological and pathological changes in the liver of mice. A is liver in the control group, B is liver in the group treated with 20 mg/kg PFOA, C is liver in the group treated with 40mg/kg PFOA. Histopathological changes in the liver, under microscope of 400 times. Bar=20μm. D is the liver tissue structure in the control group, E is the liver tissue structure in group treated with 20mg/kg PFOA, F is the liver tissue structure in group treated with 40mg/kg PFOA.

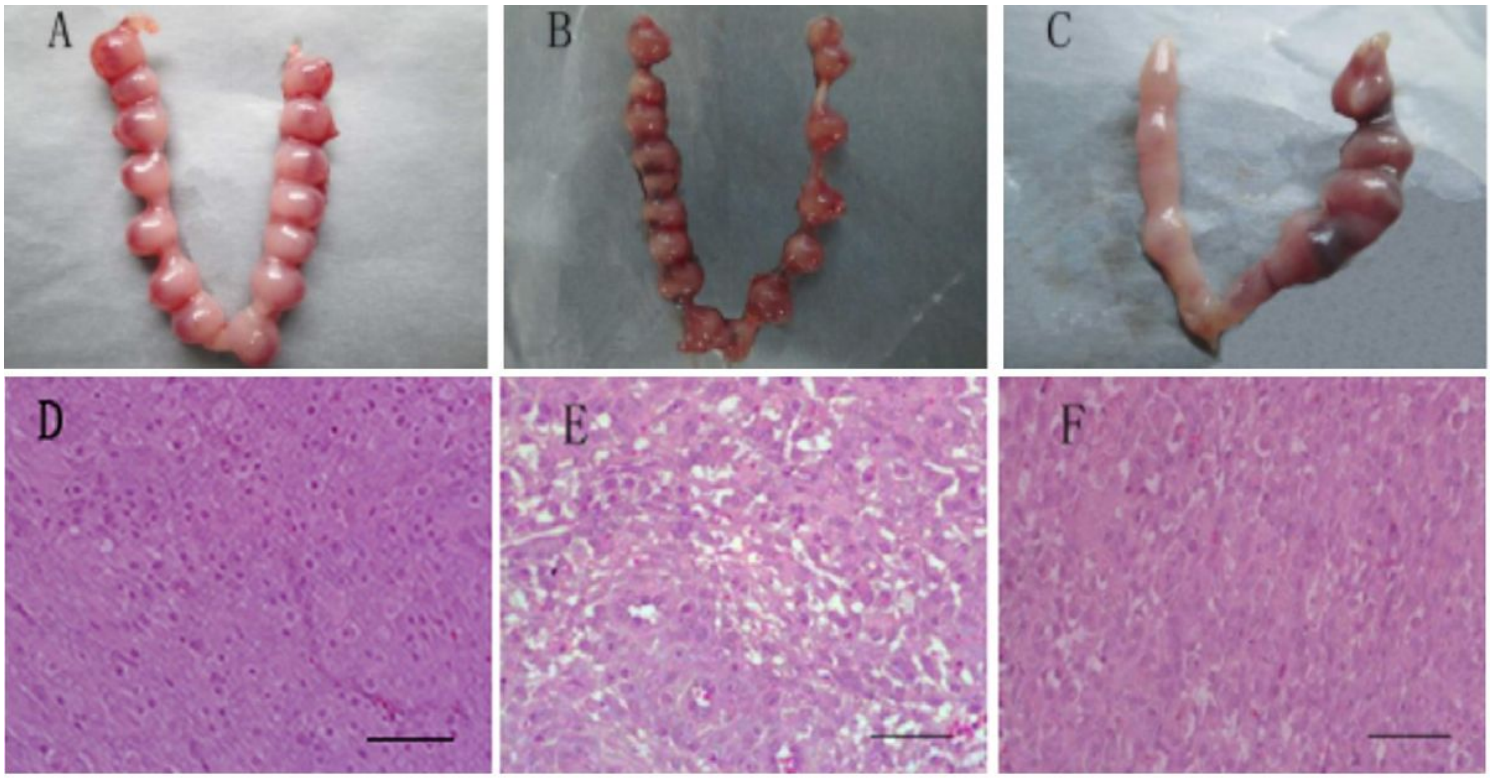

Figure 3

Histopathological and pathological changes in the uterus of mice. A is the uterus in the control group, B is uterus in the group treated with 20mg/kg PFOA, C is uterus in the group treated with 40mg/kg PFOA. D is the uterus tissue structure in control group, E is uterus tissue structure in group treated with 20mg/kg PFOA, F is uterus tissue structure in group treated with 40mg/kg PFOA. Histopathological changes in the uterus, under microscope of 400 times. Bar=20μm.

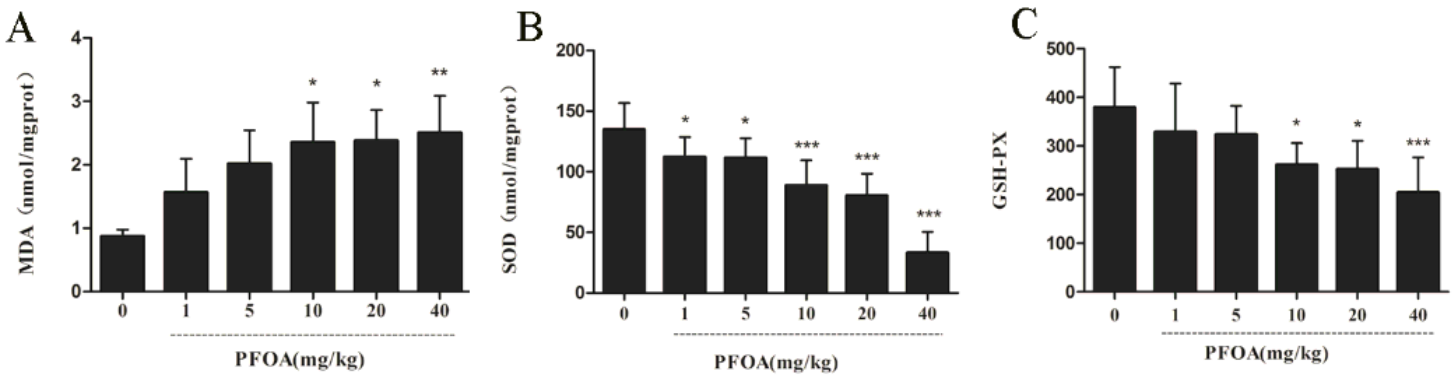

Figure 4

Effects of PFOA on liver of early pregnant mice. Levels of MDA (A), SOD (B), and GSH-Px (C) were detected. * P< 0.05, **P< 0.01, ***P< 0.001, compared with control group.

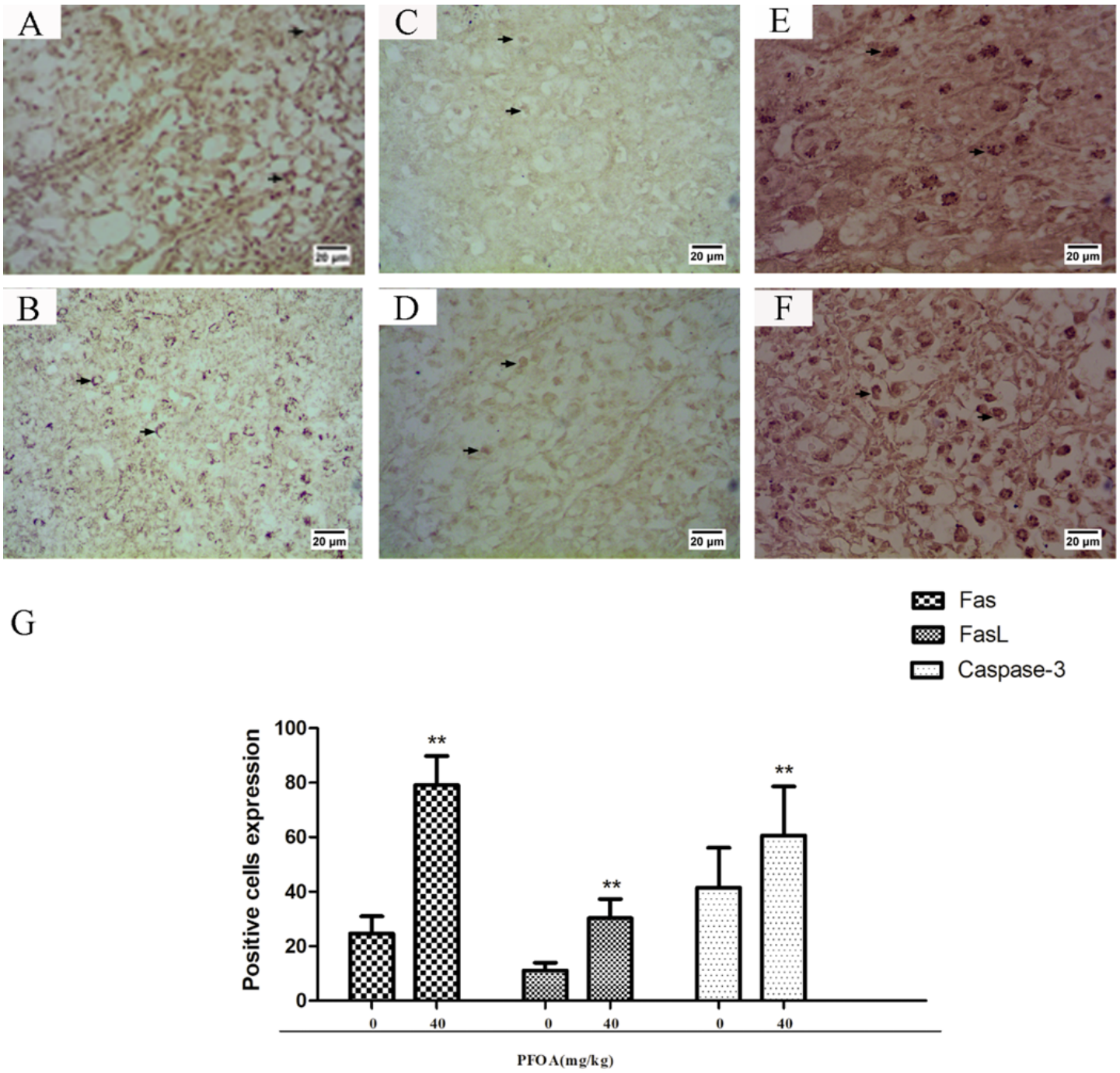

Figure 5

Positive cells expression of FAS in the control group(A) and in the 40mg/kg PFOA group(B) in the mice uterus; Positive cells expression of FASL in the control group(C) and in the 40mg/kg PFOA group(D) in the mice uterus; Positive cells expression of Capase-3 in the control group(E) and in the 40mg/kg PFOA group(F) in the mice uterus; Effect of PFOA on the expression of FAS, FASL, Caspase-3 in the uterus of mice in early pregnancy(G). Distribution of positive cells of FAS, FASL, Capase-3 expression in the uterus under microscope of 400 times, as shown by the arrows, Bar=20μm. * P< 0.05, **P< 0.01, ***P< 0.001, compared with control group.

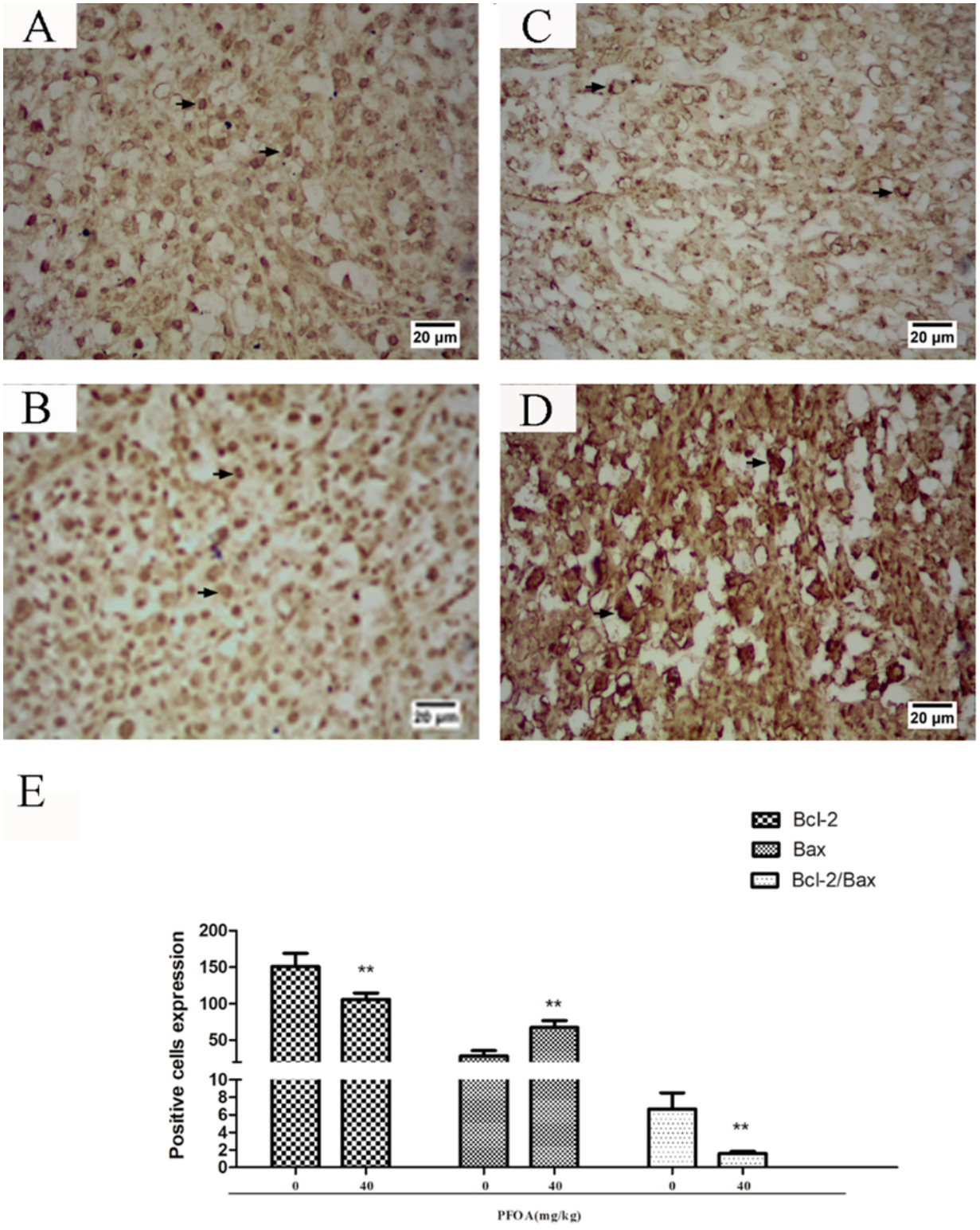

Figure 6

Positive cells expression of Bcl-2 in the control group(A) and in the 40mg/kg PFOA group(B) in the mice uterus; Positive cells expression of Bax in the control group(C) and in the 40mg/kg PFOA group(D) in the mice uterus; Effect of PFOA on the expression of Bcl-2, Bax in the uterus of mice in early pregnancy(E). Distribution of positive cells of Bcl-2, Bax expression in the uterus under microscope of 400 times, as shown by the arrows, Bar=20μm. * P< 0.05, **P< 0.01, ***P< 0.001, compared with control group.

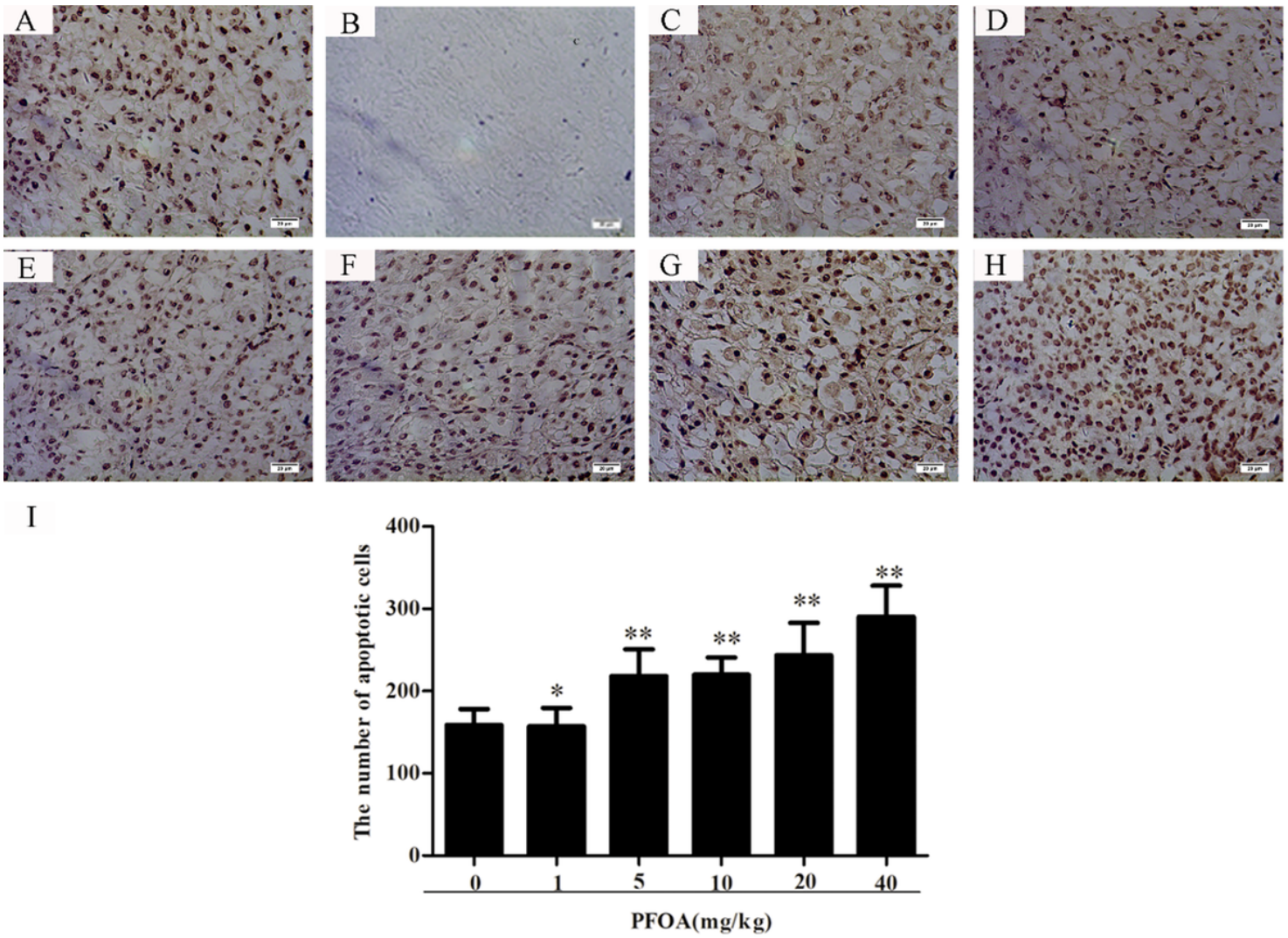

Figure 7

Cell apoptosis in uterus of mice on gestational day 9. Apoptosis of uterine cells of mice on gestational day 9, Bar =20 μm. A is the positive control, E is the negative control, B is the control group, C is the 1 mg/kg PFOA group, D is the 5 mg/kg PFOA group, F is the 10 mg/kg PFOA group, G is the 20 mg/kg PFOA group, and H is the 40 mg/kg PFOA group. Bar = 20 μm. * P< 0.05, **P< 0.01, ***P< 0.001, compared with control group.