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Original Article

Effect of decabromodiphenyl ether (BDE-209) on lung tissues of pregnant maternal and offspring rats

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Abstract: Decabromodiphenyl ether (BDE-209) is the largest contributor to pollution by PBDEs in the atmosphere, sediment, soil and indoor dust because of its wide use. After exposure to BDE-209, it can be detected in the blood, semen, umbilical cord blood and placental tissues of humans. As reported previously, BDE-209 has a biologically toxic effect on neurological development, the immune system, and the endocrine system and is carcinogenic. BDE-209 could enter a fetus through the placental barrier or breast milk and have a significant influence on the health and growth of the fetus or infant. In this study, we established a pregnant rat model and investigated the effect on lung tissues from the pregnant maternal and offspring rats of exposure to different BDE-209 concentrations. We found that BDE-209 exposure could affect the lung tissues, cause inflammation and change inflammation-related factors at low doses. The effect was greater in the pregnant maternal rats than in the offspring, which meant that the placental barrier could not block the effect completely but did reduce it. Therefore, we call for protection for women working in the electronics, paint and other manufacturing industries during pregnancy.

Keywords: Decabromodiphenyl ether (BDE-209), pregnant maternal and offspring rats, lung tissue, inflammation, reactive oxygen species (ROS), cytokines

Introduction

Polybromodiphenyl ethers (PBDEs) are a component of brominated flame-retardants. Over the past few decades, PBDEs have been widely used in the manufacture of petroleum, electronic products and daily life items because of their good flame-retardant effects and low costs. Thus, people often come into contact with them unknowingly because of the presence of PBDEs in the environment [1-3]. In recent years, studies at home and abroad on the impact of human exposure to PBDEs in the environment have confirmed that decabromodiphenyl ether (BDE-209) is the most prevalent PBDE in the atmosphere, sediment, soil and indoor dust because of its wide use [4]. The chemical formula of BDE-209 is $C_{12}Br_{10}O$ (**Figure 1A**). It poorly dissolves in various types of inorganic and organic solvents, and the half-life values in water, soil, sediment, atmosphere and the human body are 180, 360, l600, 460 and 7 d, respectively [5]. After exposure to BDE-209, it can be detected in the blood, semen, umbilical cord blood and placental tissues of humans [6]. Sandholm et al. found that the bioavailability of parental BDE-209 was approximately 26% in rats after the administration of radiolabeled BDE-209 [7]. BDE-209 has the same biological toxicity as other PBDEs, including the toxic effects on neurological development, the immune system and the endocrine system as well as carcinogenicity [8]. Research also has found that exposure to BDE-209 is the main reason that BDE accumulates in the maternal body during pregnancy [9]. BDE-209 not only can cross into the fetus through the placental barrier but also enters the infant directly from milk during breastfeeding. BDE-209 could cause short-term and long-term adverse impacts on the growth and development of children via the placenta, umbilical cord blood and breast milk, which suggests that the exposure to BDE-209 causes serious damage to the mother and infant during the perinatal stage and affects long-term health [10-12].

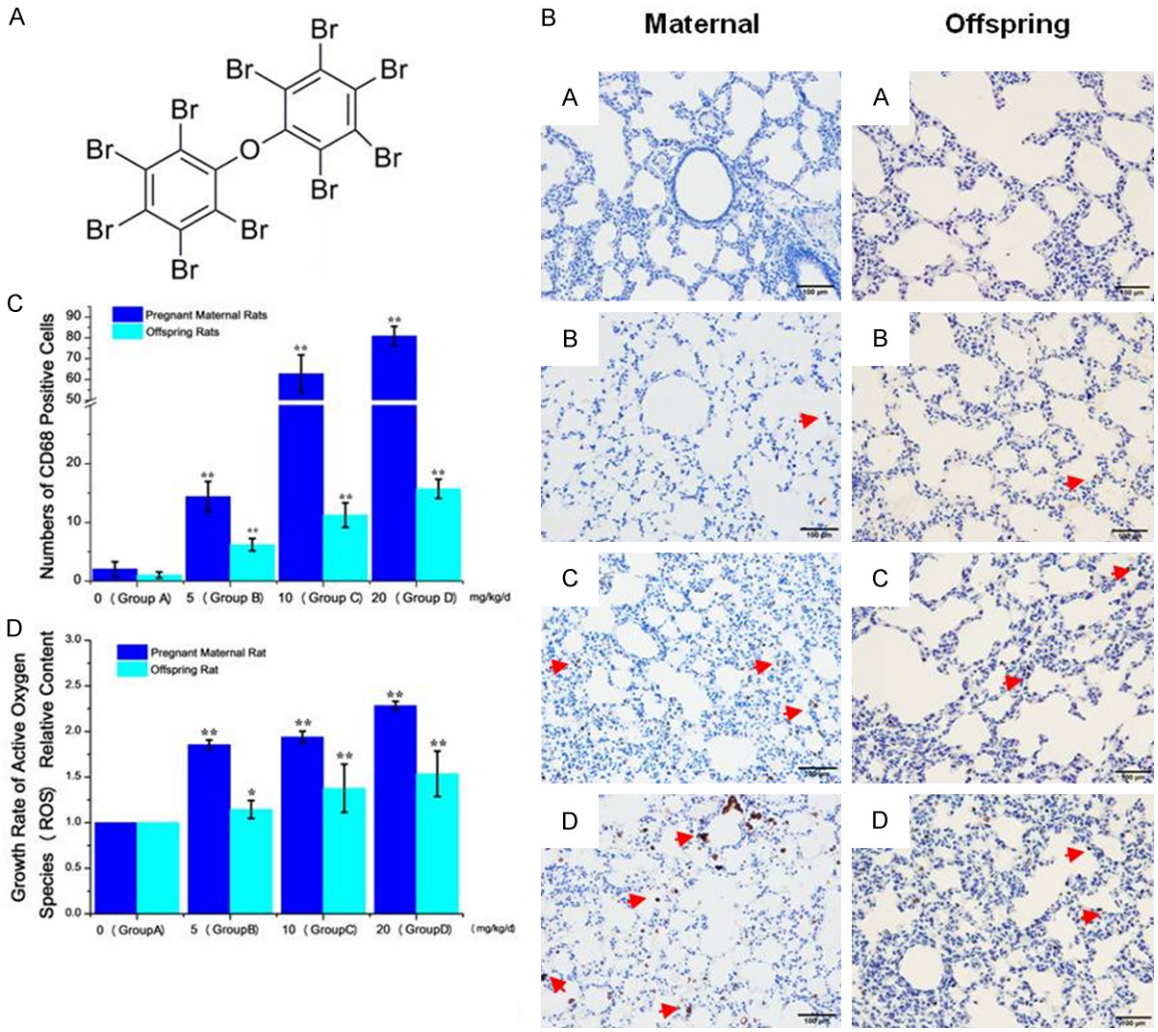

Figure 1. ROS relative content and CD68 staining results of lung tissues of pregnant maternal and offspring rats. A. The chemical structure of BDE-209; B. CD68 staining results of lung tissues of pregnant maternal and offspring rats. The left panels are from pregnant maternal rats while the right panels are from offspring rats. Group A was without BDE-209 exposure, Group B with 5 mg/kg/d BDE-209 exposure, Group C with 10 mg/kg/d BDE-209 exposure and Group D with 20 mg/kg/d BDE-209 exposure. CD68-positive cells were dyed brown and are indicated by the red arrow. C. Numbers of CD68-positive cells in pregnant maternal and offspring rats, respectively. The *indicates a significant difference at $P < 0.05$, and **indicates a significant difference at $P < 0.01$ vs. the control (Group A) within a group. D. Growth rate of ROS relative content of pregnant maternal and offspring rats. Group A served as the control. *indicates a significant difference at $P < 0.05$, and **indicates a significant difference at $P < 0.01$ vs. the control (Group A) within a group.

In the present study, we established a pregnant rat model and investigated the effect on lung tissues from pregnant maternal and offspring rats of different BDE-209 exposure concentrations. We observed by immunohistochemical assay that the pathological changes in lung tissues and inflammatory cell infiltration varied at different degrees and different BDE-209 exposure concentrations both in pregnant maternal and offspring rats. Next, we detected the reactive oxygen species (ROS) relative content in the lung tissues of pregnant maternal and offspring rats, and observed that reactive oxygen species increased in pregnant maternal lung tissues and offspring lung tissues 85.5% and 14.4%, respectively. Finally, we used reverse transcription-polymerase chain reaction (RT-PCR) to study the expression levels of VEGF, TNF-α, IL-1 and IL-6 in the lung tissues of pregnant maternal and offspring rats, an enzyme-linked immuno sorbent assay (ELISA) and western blot assay to detect the corresponding protein

levels in the lung tissues. The expression level of VEGF in pregnant maternal rats decreased, whereas it increased in offspring rats; however, the protein levels of lung tissues for both decreased. The expression levels of other cytokines such as TNF-α, IL-1 and IL-6 all increased in pregnant maternal and offspring rats compared to the levels before exposure. We conclude that BDE-209 exposure had a negative impact on the lung tissues of pregnant maternal and offspring rats at low doses. The situation was more serious in the pregnant maternal rats than the offspring rats, which meant that the placental barrier could not block the effect completely. Therefore, we call for protection for women working in the electronics, paint and other manufacturing industries during pregnancy.

Material and methods

Establishment of pregnant rat models

The animal experiment instrument was approved by the Ethics Committee of First Affiliated Hospital of Sun Yat-sen University. Forty female and twenty male ten-week old SD rats were purchased and were bred in the SPF-level animal room of the animal experiment center at Guangzhou Medical University. Male and female rats were bred in the same cage at a ratio of 2:1, that is, 3 rats per cage. Female and male rats were placed in the same cage at 18:00 on the first day, and the female vaginal discharge was taken for microscopic examination on the next day at 08:00. If sperm were in full view under the microscope, which indicates the first day of pregnancy, the pregnant rat was transferred to a single cage. Non-pregnant female rats were mated with male rats continuously until the female rats became pregnant.

Dosage regimen for the experimental animal groups

Forty female SD rats on the 1st day of pregnancy were randomly divided into five groups, eight in each group. From the first day of pregnancy, maternal rats were given drugs by intragastric administration away from light, once per day. BDE-209 needs to be mixed once every half an hour to promote its full dissolution during intragastric administration. The maternal rats were weighed every two days to adjust the administration dose. Rats in group A were given the same amount of peanut oil to reduce the physiological difference until the 21st day of pregnancy (pregnant 22 days).

Group A (no BDE-209 exposure group): was given refined peanut oil 5 mg/(kg/d); Group B (BDE-209 exposure at a dose of 5 ×): was given 5 mg/(kg/d) decabromodiphenyl ether (dissolved in the same amount of refined peanut oil); Group C (BDE-209 exposure at a dose of 10 ×): was given 10 mg/(kg/d) decabromodiphenyl ether (dissolved in the same amount of refined peanut oil); Group D (BDE-209 exposure at a dose of 20 ×): was given 20 mg/(kg/d) decabromodiphenyl ether (dissolved in the same amount of refined peanut oil).

Sample collection

The lung tissue samples from pregnant maternal rats after childbirth and offspring rats were collected as soon as possible. Lung tissue samples were divided into three parts: one part of the lung tissue was used for pathological immunohistochemical observation, the second part of the lung tissue homogenate was used for cytokine detection and 10 g of lung tissue was left for RT-qPCR detection and ELISA.

Immunohistochemical assay

CD68 dyeing steps were as follows. The process for making tissue paraffin was the same as the hematoxylin-eosin (HE) staining process provided in the S1 file. Samples were dehydrated with xylene and different concentrations of ethanol when the paraffin for lung tissues was prepared. Next, the dehydration section was filled with antigen repair liquid containing EDTA. The paraffin section was put into 3% hydrogen peroxide solution for 25 min at room temperature in the dark. The immunohistochemical assay was performed using anti-CD68 (ab955, abcam) as well as a horseradish peroxidase-conjugated goat anti-mouse secondary antibody, followed by coloring with DAB reagent. In addition, the paraffin was redyed with hematoxylin after washing with water. The samples were dehydrated again and the CD68-positive cells were counted under an optical microscope.

Enzyme-linked immuno sorbent assay- reactive oxygen species relative content

Lung tissues were ground and samples were centrifuged at high speed. 200 μL reactions

Table 1. Primers sequences used in RT-qPCR assay

Names	Sequences (5' to 3')
ACTB A	AACCCTAAGGCCAACCGTGAAAAG
ACTB S	CGACCAGAGGCATACAGGGACAAC
VEGF A	GGCAGCTTGAGTTAAACGAAC
VEGF S	TGGTGACATGGTTAATCGGTC
TNF-α A	CTTCAAGGGACAAGGCTG
TNF-α S	GAGGCTGACTTTCTCCTG
IL-6 A	GTCAACTCCATCTGCCCTTCAG
IL-6 S	GGCAGTGGCTGTCAACAACAT
IL-1 A	CTGAAAGCTCTCCACCTC
IL-1 S	GGTGCTGATGTACCAGTTGG

were set up in 96-well plates containing the enzyme cofactor mixture, nitrate reductase mixture and 80 μL of samples or different diluted concentrations of nitrate standards and 100 μL of diluted testing concentration horseradish peroxidase (HRP)-labeled antibody. The blank control only contained 200 μL of assay buffer. The samples were mixed and incubated for one hour at room temperature, followed by the addition of 90 μL of substrate solution and color development at 37°C for 10 min in the dark. Then, the OD values at 450 nm were determined and the ROS relative content of each sample was determined according to the standard curves. Finally, we use each group untreated as control, calculating the ROS relative content and mapping according to the data.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was obtained from collected lung tissues of pregnant maternal and offspring rats using the classical phenol-chloroform extraction method. The RNA was used as a template for reverse transcription using the following protocol: each 20 μL reaction contained 125 μM dNTPs, 20 pmol of oligo dT18, 25 pmol of random primers, 5 × RT buffer, DTT, RNase inhibitor, DEPC treated water, and 2 μg of total RNA. Briefly, the RNA and oligomer dT18 primer were incubated at 65°C for 10 min, and then immediately placed on ice. Subsequently, the other components were added and incubated at 42°C for 1 h, and then at 70°C for 15 min. Finally, the reaction solution was stored at -20°C. The total volume of 20 μL of quantitative reaction mixtures contained 10 μL of SYBR Green 1 Master (Thermo Fisher Scientific), 0.4 μM each of forward and reverse primers as shown in **Table 1** (the ACTB gene was used as the internal control), 2 μL of cDNA, and nuclease-free water. The program used for all genes consisted of a denaturing cycle of 5 min at 95°C, 40 cycles of PCR (95°C for 10 s, 60°C for 20 s), a melting cycle consisting of 95°C for 5 s, 65°C for 60 s, and a step cycle starting at 60°C with a 0.2°C/s transition rate to 95°C. The specificity of the real-time RT-PCR product was confirmed by melting curve analysis. Three replications were performed, and the target gene mRNA level was normalized to the ACTB mRNA level of each sample. The results of the real-time PCR assay were analyzed using the $2^{-\Delta CT}$ method to compare the transcription levels of the target genes in each sample relative to the untreated control.

Enzyme-linked immuno sorbent assay-cytokine content of lung tissue

Lung tissues were ground and centrifuged at high speed. Samples and gradient diluted concentrations of standard were added to 96-well plates with the test liquid in the following order: biotin labeled antibody (ELR-VEGF-001/EXP: 140628, ELR-IL6-001C/EXP: 140628, ELR-TNF-α-CL/EXP: 140828, ELR-IL lalpha-001/EXP: 140628) incubated at 37°C for 1 h; 100 μL of ABC working liquid incubated at 37°C for 30 s; 100 μL of TMB incubated at 37°C for 25 min in the dark; and 100 μL of TMB termination liquid incubated for 15 min in the dark at room temperature (set up control). Finally, the OD values at 450 nm were determined and the concentrations of the samples were calculated according to the standard curve.

Results

BDE-209 exposure caused pathological changes in lung tissue of pregnant maternal and offspring rats

CD68 specificity staining is the most important sign of apoptosis in histology [13, 14]. In the pregnant maternal rats in Group A (without BDE-209 exposure during pregnancy), CD68-positive cells in lung tissues were rare, with 2.05 ± 1.23 per view. For Groups B, C, and D (with BDE-209 exposure during pregnancy), the CD68-positive cell number increased in the

lung tissues. The CD68 counts were associated with a gradual rise in BDE-209 exposure during pregnancy, increasing from 2.05 ± 1.23 per view in the control group to 80.90 ± 4.62 per view (Group D). In addition, there were signs of gathered groups of CD68-positive cells at 20 mg/kg BDE-209 exposure during pregnancy (group D). For offspring rats in Group A (without BDE-209 exposure during pregnancy), CD68-positive cells in lung tissue were rare, with 1.05 ± 0.51 per view. For Groups B, C, and D (with BDE-209 exposure during pregnancy), CD68 counts for infant rats in groups B, C, and D were higher than the blank control group (group A), exhibiting a gradual rise in BDE-209 exposure increase during pregnancy, increasing gradually from 1.05 ± 0.51 per view in the control group to 15.71 ± 1.63 per view (see **Figure 1B** and **1C**) We also observed changes in lung tissues in the hematoxylin-eosin staining assay results (Figure S1). In the Group A pregnant maternal rats (without BDE-209 exposure during pregnancy), the pathology of the lung tissue was normal. However, for Groups B, C, and D (with BDE-209 exposure during pregnancy), the pathology of the lung tissues demonstrated varying degrees of poor alveolar function: the lung septum was broadened, with congested and expanded capillaries, and we observed infiltration of lymphocytes and other inflammatory cells. With the increase in BDE-209 concentration, the inflammatory response of the alveoli was more serious. These results were the same in offspring rats, but were more serious in maternal rats with the same concentration of BDE-209 exposure.

BDE-209 exposure affected oxidative stress in the lung tissue of pregnant maternal and offspring rats

Exposure of humans to BDE-209 could trigger the oxidative stress injury reaction of a variety of cells in vivo and in vitro, and these oxidative stress injury reactions could increase levels of intracellular reactive oxygen species [15]. Here, we used an enzyme-linked immunosorbent assay to investigate oxidative stress after exposure to different concentrations of BDE-209. Before BDE-209 exposure, the ROS relative value was 3.32 ± 0.075 U/mL. After BDE-209 exposure, the ROS increased to 6.16 ± 0.17 U/mL. The primary increase was 85.5%, gradually increasing to 6.44 ± 0.21 U/mL and finally to 7.58 ± 0.16 U/mL. The largest increase was 128.31%. For offspring rats, before BDE-209 exposure, the ROS relative value was 7.42 ± 1.09 U/mL. After BDE-209 exposure, the primary increase was 14.4%; then it gradually increased to 37.74% and the highest value was 53.64% (see in **Figure 1D**).

BDE-209 exposure had an effect on VEGF, TNF-α, IL-1 and IL-6 in lung tissue from pregnant maternal and offspring rats

We utilized RT-qPCR to detect the gene expression levels of inflammation-associated cytokines such as VEGF, TNF-α, IL-1 and IL-6 in pregnant maternal and offspring rats and ELISA to investigate the content of lung tissues to preliminarily discuss the effects of low-dose BDE-209 exposure on pulmonary injury in pregnant maternal and offspring rats during pregnancy and the related mechanisms.

The gene expression level of VEGF, TNF-α, IL-1 and IL-6 of pregnant maternal and offspring rats exposure to BDE-209

After BDE-209 exposure, the VEGF gene expression level of pregnant maternal rats decreased. The largest decrease was 48.17% (significant differences at all exposure concentrations compared to Group A, $P < 0.01$), while the VEGF gene expression level of offspring rats increased (significant differences at 10 mg/kg and 20 mg/kg exposure concentrations compared to Group A, $P < 0.01$) (**Figure 2Aa**). The TNF-α gene expression level in lung tissues of pregnant maternal rats increased obviously at 20 mg/kg BDE-209 exposure, as well as in offspring rats ($P < 0.01$) (**Figure 2Ab**). IL-1 and IL-6 gene expression levels in pregnant maternal rats and offspring rats both increased, especially in pregnant maternal rats (significant differences at all exposure concentrations compared to Group A, $P < 0.01$) (**Figure 2Ac**, **2Ad**). For the IL-1 gene, the largest increase was 12.90 times for pregnant maternal rats and 0.95 times for offspring. For the IL-6 gene, the largest increase was 4.08 times for pregnant maternal rats and 5.00 times for offspring (See in Table S1).

The lung tissue content of VEGF, TNF-α, IL-1 and IL-6 of pregnant maternal and offspring rats exposed to BDE-209

After BDE-209 exposure during pregnancy, the VEGF content in the lung tissue of Group A was

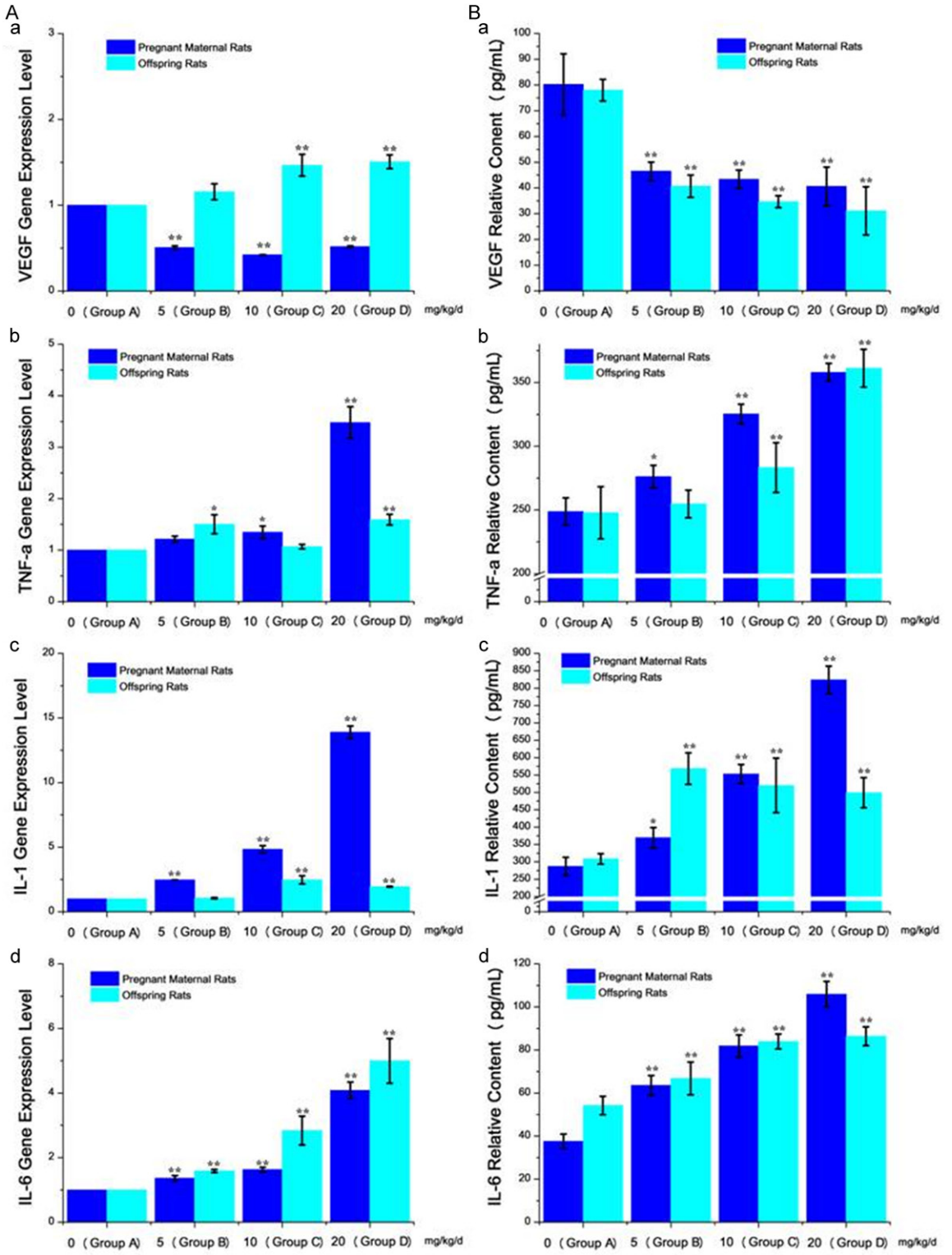

Figure 2. Gene expression level and lung tissue content of VEGF, TNF-α, IL-1 and IL-6 in pregnant maternal and offspring rats. A. Gene expression level results for pregnant maternal and offspring rats. *indicates a significant difference at $P < 0.05$, and **indicates a significant difference at $P < 0.01$ vs. the control (Group A) within a group. B. The lung tissue content results for pregnant maternal and offspring rats. *indicates a significant difference at $P < 0.05$, and **indicates a significant difference at $P < 0.01$ vs. the control (Group A) within a group.

88.22 ± 11.91 pg/mL and decreased to 46.52 ± 3.52 pg/mL, and 43.41 ± 3.52 pg/mL and 40.63 ± 7.51 pg/mL, respectively, in Group B, C and D of pregnant rats (see Table S2). The offspring were lower than that before exposure to BDE-209 and the results were concentration dependent. The VEGF content decreased almost 41.27% at a low dosage of BDE-209 exposure in pregnant maternal rats. The TNF-α content in pregnant maternal and offspring rats increased in the 10 mg/kg and 20 mg/kg samples compared to the content before treatment and were significantly different compared to Group A (both $P < 0.01$). The largest increase was 43.91% for pregnant maternal rats, similar to offspring rats (45.76%). The content of IL-1 and IL-6 both increased as well, especially in pregnant maternal rats. For IL-1, the primary increase was 28.75%; then it rapidly increased to 92.74% and 187.16% in pregnant maternal rats, while the increase was slower in offspring, with the largest increase of 61.75% at 20 mg/kg BDE-209 exposure. For IL-6, the primary increase was 69.07%; then it rapidly increased to 117.74% and 181.76% in pregnant maternal rats, while the increase rate was slower in offspring. In offspring, the primary increase was 23.22%; then it rapidly increased to 54.84% and 59.27% (see in **Figure 2B**).

Discussion

BDE-209 exposure caused pathological changes in lung tissue of pregnant maternal and offspring rats

HE staining studies (provided in the Supporting Information) found that a low dosage of BDE-209 was absorbed by the lung tissues during pregnancy, and a low dosage of BDE-209 exposure could cause a structural disorder of lung tissues, including a decrease in the number of alveoli and the inflammatory response performance such as inflammatory cell infiltration in pregnant maternal and offspring rats. However, the inflammatory response of pregnant maternal rats was more serious than in offspring rats, which meant that pregnant maternal rats could not completely block injury by BDE-209 in lung tissues of offspring rats. These lung tissue changes were consistent with the pathology of traumatic change caused by oxidative stress [16]. Next, we undertook CD68 staining studies to observe the change in inflammation-associated mononuclear and macrophage cells. CD68 staining studies also revealed that a low dosage of BDE-209 exposure in the digestive tract of pregnant maternal rats during pregnancy could move into the lungs of pregnant maternal rats and offspring rats such that CD68-positive cells in the lung tissues of pregnant maternal rats increased gradually from 2.0 per view in the control group to 80.90 per view with the increase in exposure dosage (Group D). CD68-positive cells in the lung tissues of offspring rats increased gradually from 1.05 per view in the control group to 15.71 per view (Group D). Thus, after BDE-209 exposure, the CD68-positive cell number increased nearly 40 times in pregnant maternal rats and 15 times in offspring rats. CD68-specific staining is one of the most important signs of histology in apoptosis [13], and CD68 immunohistochemical staining with monoclonal antibody staining can specifically identify plasmacytoid mononuclear cells. CD4 cells in the lesion areas are mainly the plasmacytoid mononuclear cells of CD68, which are common in respiratory tract inflammatory and infectious diseases, as well as lymphocytoma, indicating that the increase in lymphocytes in tissues affected by lesions were not clonal hyperplasias, but typical morphological changes of apoptotic cells in the involved lung tissues. Additionally, the number of CD68-positive cells was higher in the pregnant maternal rats than in the offspring rats, confirming that pregnant maternal rats were more seriously affected than the offspring rats after BDE-209 exposure.

In general, the studies in this section confirmed that after a low dose of BDE-209 via the digestive tract of pregnant maternal rats during pregnancy, the inflammatory response and the number of CD68-positive cells increased consistent with the increase in BDE-209 exposure. As the dosage rises gradually, there are signs of gathered groups of CD68-positive cells in the lung tissues of pregnant maternal rats after 20 mg/kg BDE-209 exposure during pregnancy (group D), showing the pathological features that indicate a low dosage of BDE-209 exposure during pregnancy had a more severe effect on pregnant maternal rats than offspring rats. These studies suggested that the BDE-209 exposure dosage during pregnancy was relevant to the degree of injury and hypothesized that after BDE-209 exposure via the digestive tract of pregnant maternal rats, there were

inflammatory changes and increases in macrophages in the lung tissues of pregnant maternal and offspring rats, which was one of the pathological mechanisms leading to oxidative stress injury in lung tissues.

BDE-209 exposure affected oxidative stress in lung tissues from pregnant maternal and offspring rats

A comparison of ROS increases in the lung tissues between pregnant maternal and offspring rats before and after BDE-209 exposure revealed that the ROS relative content increased in pregnant maternal and offspring rats. As for pregnant maternal rats, after BDE-209 exposure, the primary ROS increase in pregnant maternal rats was 85.5%, which was significantly different than offspring rats (14.4% ($P < 0.05$)). The highest ROS value for pregnant maternal rats was 128.31%, which was significantly different than offspring rats (53.64% ($P < 0.01$)). This result suggests that the damage to the lung tissue of pregnant maternal rats was more serious than in offspring rats, indicating that after BDE-209 exposure, the pregnant maternal rats were damaged first and the offspring were damaged later. The change in ROS supported the experimental results and conclusions of the immunocytochemistry assay and implied that the oxidative stress injury caused by ROS played an important role in the inflammatory response of the lung tissue. The increase in ROS is thus an index of BDE-209 exposure-damaging degree.

As reported by others, after the human body is exposed to BDE-209, the oxidative stress injury reaction is triggered in a variety of cells in vivo and in vitro, and these oxidative stress injury reactions could increase levels of intracellular ROS, while ROS has a close relationship with cell apoptosis [17]. The capillary endothelial cells of the lung tissue in the human body are extremely sensitive to lipid peroxidation, and ROS can significantly reduce pulmonary surfactants, causing the consumption of a great quantity of anti-oxygen free radicals in the lung tissue, production of a lipid peroxidation chain response, and promotion of the generation of lipid peroxides. These changes cause damage to membrane structures, deactivation of biological functions and membranolysis of vascular endothelial cells and subcellular organelles (for example, microsomes), leading to the extravasation of blood components to cause lung tissue bleeding and damage and/or apoptosis of alveolar epithelial cells. Clinically, acute respiratory distress syndrome, COPD, bronchial asthma, lung cancer, and many other lung diseases all have as a pathogenic basis of oxidative stress damage in the lung caused by the imbalance in the oxidation-anti-oxidation signaling pathways in the lung tissue, leading to the activation and aggregation of inflammatory cells and eventually tissue inflammation and damage [18-20].

BDE-209 exposure had an effect on VEGF, TNF-α, IL-1 and IL-6 in lung tissue from pregnant maternal and offspring rats

Vascular endothelial growth factor (VEGF), originally called vascular permeability factor (VPF), and is a type of important signal protein that stimulates angiogenesis [21]. VEGF is richly expressed in alveolar tissue cells, and VEGF is involved in the process of lung tissue damage caused by oxidative stress through ROS as the downstream messenger intermediates of vascular endothelial growth factor receptor 2. Therefore, VEGF is involved in the development of the lung and the repair of inflammatory damage during embryonic development [22, 23]. Here, we also found that after BDE-209 exposure in pregnant maternal and offspring rats, the content of lung tissue and the gene expression level of VEGF decreased in pregnant maternal rats. The data for offspring rats revealed that the content of lung tissue of VEGF for offspring rats evidently decreased but the gene expression level increased. It was speculated that upon exposure to BDE-209, the damage repair capability of maternal rats decreased while offspring rats were in the growth period, and VEGF gene proliferation caused the VEGF gene expression increase.

Cytokines are small proteins (5-20 kDa) that are primarily released by inflammatory cells and affect the biological functions of other cells. Cytokines include tumor necrosis factor (TNF-α), interleukin I (IL-1), IL-4, IL-6, and transforming growth factor-β (TGF-β) [24]. Among these cytokines, TNF-α is an oligosaccharide protein, and the TNF-α receptor is widely expressed on the cellular surfaces of lung, liver, kidney, intestine, muscle and other tissues and organs. After the formation of TNF-α inflammatory lesion, a rapid rise in the cascading

responses of inflammatory mediators occurs. At the same time, TNF-α can stimulate the release of other cytokines into the blood circulation, and its concentration is positively correlated with the inflammatory injury levels of the body tissues [25]. IL-1 is produced by activated monocytes and is one of the cytokines with the strongest inflammatory effects in the body. After the body is injured, IL-1 initiates the immune response and neutrophils invade injured tissue cells, inducing a series of inflammatory responses via the signal transduction pathway of the IL-1 receptor (IL-1R). Inflammatory responses induced by IL-1 can affect the barrier function of the vascular endothelium in lung tissue and alveolar epithelial cells [26]. Activated macrophages can produce IL-6, and cells in the lung parenchyma can also synthesize and secrete IL-6. IL-6 can induce the fibroblasts of the lung tissues to synthesize and secrete a variety of acute phase proteins that can regulate the body's inflammatory response and immune response. IL-6 can directly affect the vascular endothelial cells, increase the permeability of blood vessels, and promote the infiltration of inflammatory factors; therefore, IL-6 can reflect the severity of the inflammatory lesions [27, 28]. In the present study, combining ELISA with RT-qPCR assay results, we learned that the content and gene expression level of TNF-α, IL-1 and IL-6 all increased after exposure to BDE-209 both in pregnant maternal and offspring rats in a concentration-dependent manner. These data indicated that upon BDE-209 exposure, inflammatory injury and the damage response occurred in pregnant maternal and offspring rats. In addition, BDE-209 lowered the damage repair abilities in maternal and offspring rats.

Conclusion

In this study, we established pregnant rat models and investigated the effect on lung tissues of the pregnant maternal and offspring rats of different BDE-209 exposure concentrations. We observed that BDE-209 exposure could affect the lung tissues and cause inflammation at a low dosage, and these effects were dose-dependent. We also found that the ROS relative content increased under BDE-209 exposure compared with the control in pregnant maternal and offspring rats. However, all conditions were more serious in the pregnant maternal rats than in the offspring rats, which meant that the placental barrier could not block the effect completely, only reduce it. We also found that the gene expression level of VEGF decreased in maternal rats and increased in offspring rats, but the content of VEGF decreased in both. Lastly, we learned that the content and expression levels of inflammation-induced cytokines such as TNF-α, IL-1 and IL-6 both increased, suggesting inflammatory injury to lung tissues of pregnant maternal and offspring rats. Therefore, we call for the protection of women working in the electronics, paint and other manufacturing industries during pregnancy.

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Disclosure of conflict of interest

None.

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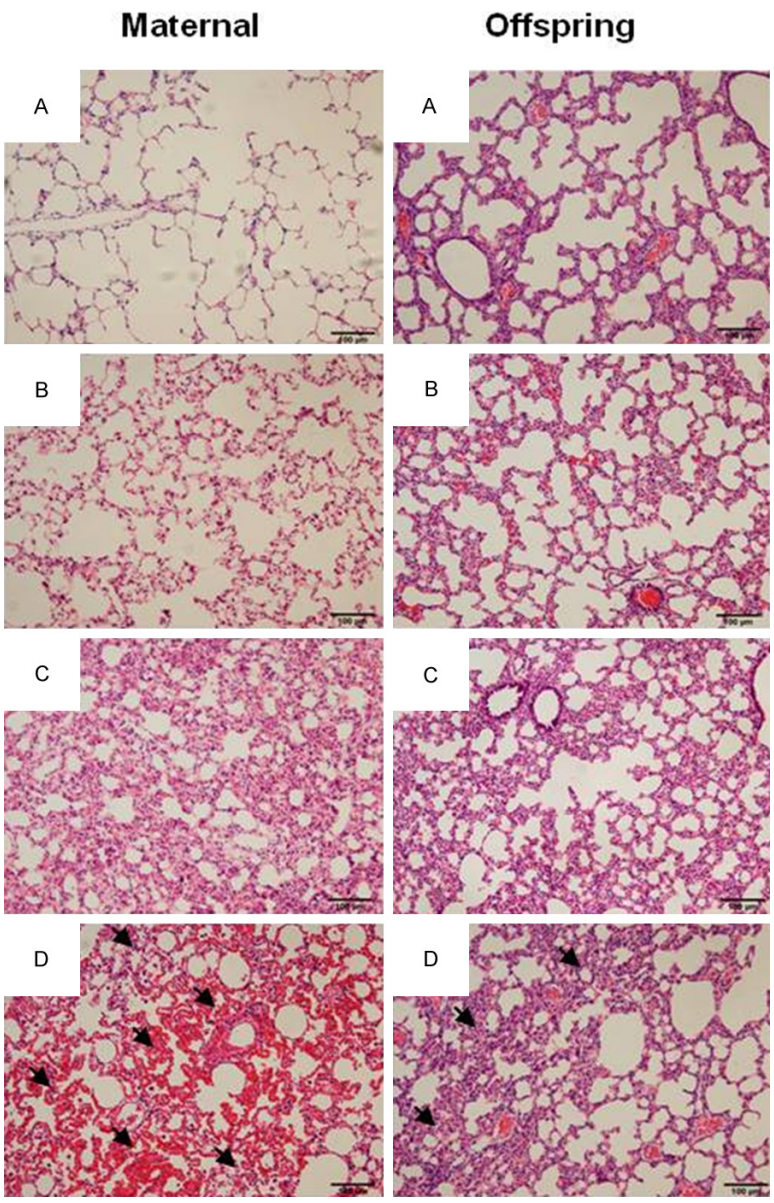

Figure S1. Hematoxylin-eosin (HE) staining results of pregnant maternal rat and offspring rats. Of them, Group A of pregnant maternal and offspring rats were without BDE-209 exposure, the pathology of lung tissue was normal followed by these appearance, the structure of lung tissue was clear; the alveolar walls were complete and the alveolar septum wasn't broaden; with integrated bronchiole mucosa epithelial, cells in neat order, without inflammatory exudate in bronchial lumen and alveolar space; without alveolitis and pulmonary fibrosis changes, and with good alveolar air. But for Groups B-D (with BDE-209 exposure during pregnancy), the pathology of lung tissue had changes as follow, all had varying degrees of bad alveolar air, the lung septum was broaden, with congested and expanded capillary, and the infiltration of lymphocytes and other inflammatory cells. With the increasing of BDE-209 exposure concentration, the inflammatory response of the alveolar was more serious. Lung tissue structure was disorder (directed in black arrow), and part of the alveolar fusion, local small amounts of visible fibroblast proliferation in Group D, especially. These situations were the same to the offspring rats.

Table S1. Expression level of *VEGF*, *TNF-α*, *IL-1* and *IL-6* gene of lung tissues of pregnant maternal and offspring rats

Teams	BDE-209 (mg/kg)	VEGF	TNF-α	IL-1	IL-6
Maternal	0	1.00	1.00	1.00	1.00
	5	0.51 ± 0.02**	1.21 ± 0.06	2.47 ± 0.00**	1.36 ± 0.08**
	10	0.42 ± 0.00**	1.35 ± 0.12*	4.83 ± 0.28**	1.63 ± 0.07**
	20	0.52 ± 0.01**	3.48 ± 0.31**	13.91 ± 0.48**	4.09 ± 0.26**
Offspring	0	1.00	1.00	1.00	1.00
	5	1.16 ± 0.09	1.50 ± 0.18*	1.05 ± 0.05	1.59 ± 0.05**
	10	1.47 ± 0.13**	1.07 ± 0.04	2.47 ± 0.32**	2.84 ± 0.44**
	20	1.51 ± 0.08**	1.59 ± 0.10**	1.95 ± 0.04**	5.00 ± 0.69**

*P < 0.05; **P < 0.01.

Table S2. Content of VEGF, TNF-α, IL-1 and IL-6 of lung tissues of pregnant maternal and offspring rats (pg/mL)

Teams	BDE-209 (mg/kg)	VEGF	TNF-α	IL-1	IL-6
Maternal	0	80.22 ± 11.91	248.73 ± 10.69	286.92 ± 26.21	37.60 ± 3.35
	5	46.52 ± 3.52**	276.16 ± 8.86*	369.40 ± 29.36*	63.57 ± 4.55**
	10	43.41 ± 3.52**	325.31 ± 7.72**	553.01 ± 27.08**	81.87 ± 5.14**
	20	40.63 ± 7.51**	357.94 ± 7.03**	823.93 ± 39.52**	105.94 ± 5.95**
Offspring	0	78.00 ± 4.20	247.76 ± 20.47	308.57 ± 15.16	54.23 ± 4.31
	5	40.69 ± 4.36**	254.72 ± 10.81	568.36 ± 45.23**	66.82 ± 7.56**
	10	34.66 ± 2.31**	283.26 ± 19.53**	520.06 ± 78.44**	83.97 ± 3.54**
	20	31.08 ± 9.35**	361.13 ± 14.79**	499.10 ± 43.20**	86.37 ± 4.35**

*P < 0.05; **P < 0.01.

Supporting Information

Methods and result for hematoxylin-eosin (HE) staining assay

Material and Methods for Hematoxylin-eosin (HE) staining as followed: First of all, right lung tissues from the pregnant maternal rats and offspring rats were fixed in 4% paraformaldehyde for up to 24 hours, then lung tissues were dehydrated with alcohol at different gradient concentrations and xylene until tissues became transparent, processing with paraffin next. After being frozen, paraffin embedding block was cut into 5 μm thick slices, flatting placed in 40°C to dry overnight. Dewaxing the next day, and then put into different concentrations of alcohol, lastly in distilled water for 2 min. Finally, stained with hematoxylin-eosin (HE) and observed under optical microscope. If the cytoplasm was red and the nucleus was blue, meaning staining success.