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Effects of inflammatory stimulation during pregnancy on blood lipid levels and learning and memory abilities of offspring rats

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Abstract

We aimed to evaluate the effects of inflammatory stimulation during pregnancy on blood lipid levels and learning and memory abilities of offspring rats. Twenty-four Sprague-Dawley pregnant rats were randomly divided into control and lipopolysaccharide (LPS) stimulation groups that were intraperitoneally injected with normal saline and LPS (0.79 mg/kg) on the 8th, 10th and 12th days of pregnancy respectively. The offspring rats were weighed 1 day after birth and weekly. The serum levels of triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of 8-week-old male rats were tested. Serum TNF-α level was measured by ELISA. Behavioral changes were detected by the Morris water maze. Morphological changes were observed by using tissue sections. The ultrastructures of liver and thoracic aortas were observed by transmission electron microscopy. 8-OHdG expression in the liver was detected by immunofluorescence assay. Compared with the control group, the progeny of the LPS stimulation group had lower body weights 1 day and 1 week after birth ($P<0.01$), which then increased significantly ($P<0.01$).The memory ability of progeny of LPS stimulation group decreased at 8 weeks of age, and the levels of TG, TC, LDL, AST and TNF-α in the peripheral blood were significantly higher than those of the control group ($P<0.05$). The liver pathological changes were obvious, accompanied by evident mitochondrial damage and elevated expression of 8-OHdG. Inflammatory stimulation during pregnancy leads to mitochondrial damage in offspring, which may be associated with lipid metabolism disorders ultimately inducing atherosclerosis.

Key words: pregnancy, inflammatory stimulation, blood lipid, learning, memory.

1. Introduction

The annual number of deaths due to cardiovascular diseases is 17.3 million which is equivalent to 30% of all deaths worldwide, commonly including coronary heart disease, aneurysm, peripheral vascular occlusion and cardiomyopathy (Thomas, H. et al., 2018). Therefore, it is urgent to prevent and to treat such diseases.

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In recent years, the incidence rate of these diseases has been reduced by 90% through early active preventive measures (Mcgill, H.C. Jr. et al., 2018), such as healthy diet, exercise, smoking cessation and moderate drinking. According to statistics, 13%, 9% and 6% of patients with circulatory system diseases are caused by high blood pressure, smoking and lack of exercise respectively (Rajaraman, P. et al., 2016; Ahmed et al., Alotibi et al., 2019; Kamalpour et al., 2019).

Atherosclerosis (AS) is the common pathological basis and key link of many cardiovascular diseases. Even after primary and secondary preventions,

atherosclerotic cardiovascular disease remains the most common cause for death in the world, and correction of lifestyles can only slightly decrease the incidence rate of AS (Wong, N.D., 2014)(Siemelink, M. et al., 2013; Moore et al., 2019; Yaffe & Seroussi, 2019). Accordingly, it is of great significance to explore the potential pathogenesis of AS from new perspectives and to find novel prevention strategies. It has previously been reported that after inflammatory stimulation using lipopolysaccharide (LPS) during pregnancy, the progeny rats underwent typical AS pathological changes after 12 weeks of normal feeding (Zhao, S. et al., 2014). To this end, we herein intended to assess the effects of inflammatory stimulation using LPS during pregnancy on the blood lipid levels and learning and memory abilities of offspring rats, and to unravel the pathogenesis for AS.

2. Materials and methods

2.1. Experimental animals and materials

Sprague-Dawley pregnant rats weighing (252.9 ± 15) g were provided by the Animal Experimental Center of our hospital. The animals were housed in accordance with laboratory rat feeding standards and kept at the temperature of (25 ± 1)°C. The offspring rats were weaned at 4 weeks of age and separated by gender.

LPS, TMRE and DAPI were purchased from Sigma (USA). 4% Paraformaldehyde was bought from Wuhan Boster Biological Technology, Ltd. (China). 8-OHdG antibody was obtained from Santa Cruz (USA). TNF-α ELISA kit was provided by eBio (USA). Paraffin sections were prepared with Shandon precision cutter (Thermo Fisher Scientific, USA). The other apparatus mainly included E-100 microscope (Nikon, Japan), laser confocal microscope (Carl Zeiss, Germany), transmission electron microscope (TEM; Philips, USA) and multifunctional microplate reader (Thermo Fisher Scientific, USA).

2.2. Establishment of inflammatory stimulation model and grouping

Twenty-four pregnant rats were randomly divided into a control group and an LPS stimulation group which were fed normally, with free access to food and water. The two groups were intraperitoneally injected with 0.5 mL of normal saline and LPS (0.79 mg/kg) on the 8th, 10th and 12th days of pregnancy respectively. The pups were weighed 1 day after birth and at a fixed time per week. The offspring rats were weaned at 4 weeks of age, separated by gender in individual cages and fed normal feed. At 8 weeks of age, 12 male pups were randomly selected from each group for subsequent experiments.

2.3. Behavioral testing

The Morris water maze was used. The training contents are listed below. 1) Positioning navigation: The learning and memory abilities of offspring rats were tested by 6 days of hidden platform test. The rats were trained 4 times a day, with an interval of 30 min. The escape latency, i.e. the time required to find and to climb the platform, was determined with an image tracking system. 2) Spatial search: On the 7th day, the platform was removed, and the time of residence on the original platform quadrant within 120 s was observed.

2.4. Detection of blood lipid and hepatic function indices

After fasting for 12 h, the offspring rats were anesthetized with ether, from which the serum was collected. The serum levels of triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by a biochemical analyzer.

2.5. Detection of serum TNF-α levels in offspring rats

Blood samples were left still at room temperature for 2 h and centrifuged at 3000 rpm for 30 min at room temperature to collect the serum. The serum level of TNF-α was measured by ELISA according to the kit's instructions.

2.6. Observation of liver tissue morphology by HE staining

The liver tissues of offspring rats were cut into 2 mm × 1 mm × 1 mm pieces, immersed in 4% paraformaldehyde overnight, and dehydrated with 50%, 70%, 80% and 100% ethanol solutions. After transparentization with xylene, the samples were embedded in paraffin and cut into 5~8 μm-thick. After deparaffinization and staining, the sections were dehydrated with absolute ethanol, transparentized with xylene, mounted by using resin and observed under a light microscope.

2.7. TEM observation of thoracic aorta and liver microstructures

Liver tissue and aortic arch segment were cut into 2 mm × 1 mm × 1 mm pieces in 2.5% glutaraldehyde respectively, fixed overnight, washed several times with 0.1 mol/L PBS and fixed

with 1% citric acid for 2 h. After dehydration with gradient concentrations of ethanol solutions, the samples were embedded in epoxy resin, sliced by

an ultramicrotome, stained with lead citrate and lead acetate, and observed by TEM.

2.8. Immunofluorescence staining of 8-OHdG in the liver

8-OHdG is currently recognized as a marker of DNA oxidative damage. Under a fluorescence microscope, 8-OHdG-positive cells show red fluorescence, and DAPI, as a nuclear dye, emits blue fluorescence. Therefore, 8-OHdG+/DAPI- is a marker for the oxidative damage of mitochondrial DNA. Liver tissue was OCT-embedded, frozen-sectioned, fixed, blocked, incubated with primary antibody against 8-OHdG (1:100 diluted) and then with CY5 secondary antibody (1:400 diluted), stained by DAPI working solution, mounted and observed by laser confocal microscopy.

2.9. Statistical analysis

All data were analyzed by SPSS 16.0 software and expressed as ($x \pm s$). Intergroup comparisons were conducted by the independent sample t test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Body weight changes of offspring rats

Compared with the control group, the progeny of the LPS stimulation group had lower body weights 1 day and 1 week after birth ($P<0.01$), which then increased significantly ($P<0.01$) (Table 1).

3.2. Behavioral changes of offspring rats

In the positioning navigation experiment, the escape latencies of progeny rats in both LPS stimulation and control groups decreased with increasing training times, but the average escape latency of the LPS stimulation group daily was longer ($P<0.05$).

In the spatial search experiment, the progeny rats of the LPS stimulation group stayed for a shorter time than those of the control group did on the original platform quadrant within 120 s ($P<0.05$) (Table 2).

3.3. Effects of LPS stimulation on blood lipid, ALT, AST and TNF-α levels of offspring rats at 8 weeks of age

The peripheral blood levels of TC, TG, LDL, AST and TNF-α in the progeny rats of the LPS stimulation group were higher than those of the control group at 8 weeks of age ($P<0.05$), but HDL and ALT levels were similar (Table 3).

Table 1: **Body weight changes of offspring rats (n=12)**

	Control group	LPS stimulation group	t	P
1 d	7.42±0.14	6.72±0.12	13.151	<0.001
1 w	13.27±2.58	10.18±1.29	3.711	0.001
2 w	19.52±2.75	24.68±1.32	5.860	<0.001
3 w	30.89±3.12	38.43±3.11	5.929	<0.001
4 w	35.87±3.13	57.92±3.24	16.955	<0.001
5 w	43.26±3.24	83.22±3.45	29.248	<0.001
6 w	58.28±5.02	108.19±6.18	21.745	<0.001
7 w	85.57±5.11	146.18±6.24	26.032	<0.001
8 w	115.77±7.29	194.28±7.03	26.854	<0.001

Table 2: **Behavioral changes of offspring rats (n=12)**

	Control group	LPS stimulation group	t	P
Escape latency (s)	45.17±3.11	56.71±4.18	7.673	<0.001
1 d	28.21±3.51	36.11±3.21	11.312	0.001
2 d	21.87±3.12	28.61±3.34	5.108	<0.001
3 d	16.81±3.34	24.41±3.45	5.483	<0.001
4 d	13.43±3.49	17.98±3.21	3.324	0.003
5 d	10.21±2.16	14.21±2.47	4.223	<0.001
6 d	8.21±2.01	12.11±2.11	4.636	<0.001
Time of residence on original platform	34.51±2.14	18.11±2.21	18.467	<0.001

quadrant within 120 s

Table 3: **Effects of LPS stimulation on blood lipid, ALT, AST and TNF-α levels of offspring rats at 8 weeks of age**

	Control group	LPS stimulation group	t	P
TC (mmol/L)	1.23±0.21	1.65±0.12	6.015	<0.001
TG (mmol/L)	0.39±0.08	0.65±0.09	7.780	0.001
HDL (mmol/L)	0.92±0.11	0.92±0.12	0.000	1.000
LDL (mmol/L)	0.42±0.11	0.59±0.09	4.143	<0.001
ALT (mmol/L)	25.62±2.56	26.12±3.17	0.425	0.675
AST (mmol/L)	72.39±12.12	98.21±12.41	5.188	<0.001
TNF-α (pg/mL)	138.27±12.12	750.29±22.24	83.705	<0.001

3.4. Effects of LPS stimulation on liver histological changes of offspring rats at 8 weeks of age

Under light microscope, the lobular structure of liver tissue of the control group was clear, and liver cells were normal. There was no hepatocyte swelling, steatosis necrosis, no inflammatory cell infiltration or fibrosis in the portal area, and no lipid droplet in the cytoplasm. In the LPS stimulation group, the hepatic lobular structure was disordered, and hepatocytes were enlarged. Fat vacuoles formed, and there was mild inflammatory cell infiltration in the portal area (Figure 1).

3.5. Effects of LPS stimulation on liver ultrastructural changes of offspring rats at 8 weeks of age

There was no abnormality in the liver ultrastructure of the control group. The mitochondria had an elliptical double-layer membrane structure. The inner and outer membranes were intact, and the ridges were arranged in an orderly manner, almost without vacuoles or rupture. In progeny rats of the LPS stimulation at 8 weeks of age, the liver mitochondria became shorter, spherical or irregular from normal filaments, most of which swelled. The ridges were arranged disorderly, even rupturing or dissolving. In severe cases, the mitochondria were entirely vacuolated (Figure 2).

3.6. Immunofluorescence staining results of 8-OHdG in the liver of offspring rats at 8 weeks of age

Compared with the control group, the number and intensity of 8-OHdG+/DAPI- fluorescence significantly increased in offspring rats of the LPS stimulation group at 8 weeks of age (Figure 3).

3.7. Effects of LPS stimulation on liver mitochondrial membrane potentials of offspring rats at 8 weeks of age

The mitochondrial membrane potential of liver tissue of the control group (238.12 ± 9.29) was significantly higher than that of the LPS stimulation group (207.18 ± 10.11; t=7.806, P<0. 001).

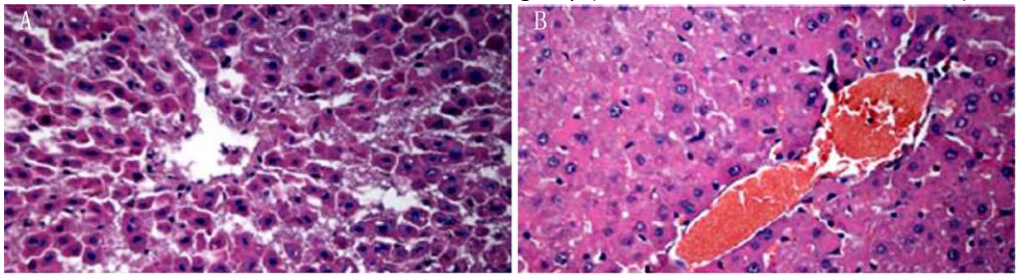

Figure 1: **Liver histological changes of offspring rats at 8 weeks of age. HE staining, 400×.A: Control group; B: LPS stimulation group.**

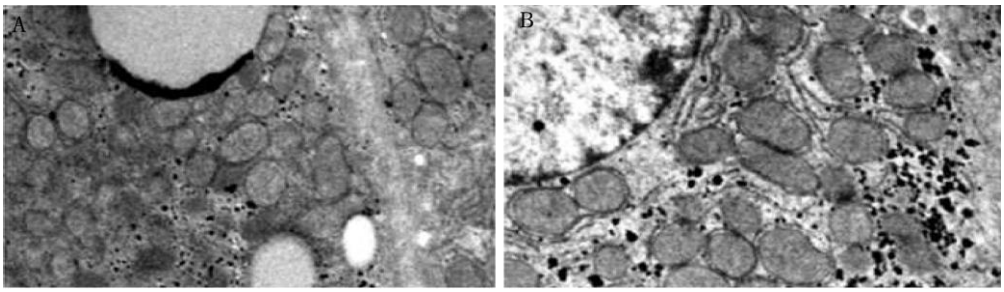

Figure 2: **Liver ultrastructural changes of offspring rats at 8 weeks of age. TEM, 8000×.A: Control group; B: LPS stimulation group.**

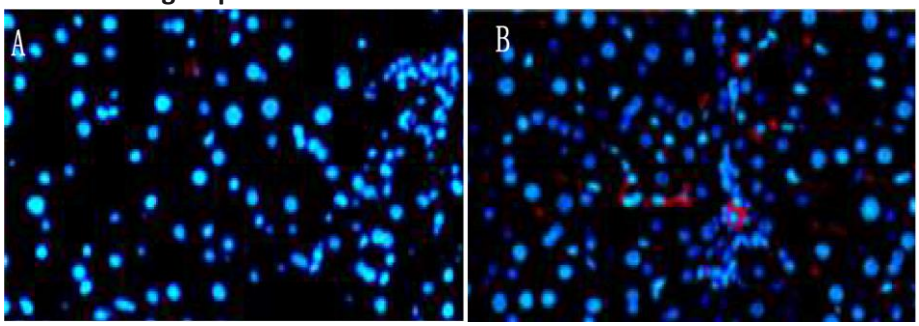

Figure 3: **Immunofluorescence staining results of 8-OHdG+/DAPI- in the liver of offspring rats at 8 weeks of age.**

4. Discussion

AS has already had manifestations during embryonic development (Wang, Y. et al., 2014) A large number of epidemiological investigations and laboratory studies have confirmed the stimulation of adverse environmental conditions in the uterus, such as malnutrition, exposure to ethanol and maternal hyperlipidemia, accelerates the onset and progression of AS in adulthood (Leduc, L. et al., 2010)(Morrison, K.M. et al., 2009)(Harrigan, J. et al., 2017). In the neonatal period after LPS stimulation, there are irregular aortic lumens and endothelial cell shedding (Zhao, S. et al., 2014). With increasing age, vascular injury is gradually aggravated, and the thickness and hardness of the arterial wall increase significantly, as the typical pathological changes of early AS. Meanwhile, the expressions of IL-1β, IL-18, ICAM-1, VCAM-1, TNF-α and other inflammatory factors increase significantly in inflammatory cells in the aorta of progeny, accompanied by enhanced systemic inflammatory responsiveness (Liao, W. et al., 2010). In this study, the peripheral blood TNF-α level of progeny rats in the LPS stimulation group at 8 weeks was higher than that of the control group. Therefore, LPS stimulation during pregnancy caused vascular tissue inflammation of the offspring rats, and abnormally activated inflammatory signal changed the biological characteristics of endothelial cells and smooth muscle cells. Meanwhile, the interactions of inflammatory cells recruited from the circulatory system with cells of the blood vessel wall amplified the inflammatory cascade, forming a vicious circle and finally leading to AS. Inflammation during pregnancy also stimulates progeny animals to have elevated blood pressure and obesity which are risk factors for AS by worsening the systemic/local inflammatory environment (Blasko, I. & Grubeck-Loebenstein, B., 2003).

In recent years, many clinical epidemiological investigations have verified that inflammation may play a crucial role in cardiovascular and neurodegenerative diseases (Wei, Y.L. et al., 2007) In the positioning navigation experiment herein, the escape latencies of progeny rats in both LPS stimulation and control groups decreased with increasing training times, but the average escape latency of the LPS stimulation group daily was longer. In the spatial search experiment, the progeny rats of the LPS stimulation group stayed for a shorter time than those of the control group did on the original platform quadrant within 120 s.

AS is generally considered a multi-factor chronic inflammatory pathological process involving multiple large and medium arteries. Elevated blood lipid level is recognized as an important initiating factor for AS, and serum lipid levels are significantly positively correlated with the branching and

severity of involved blood vessels (Torkan, M. et al., 2015). Herein, the body weight of offspring rats in the LPS stimulation group significantly increased from the 2nd week. At 8 weeks of age, they began to suffer from hyperlipidemia typified by significant increases in TC, TG and LDL levels. Hyperlipidemia can promote the onset and progression of AS by changes the wall structure in the early stage, increasing lipid deposition onto the vascular wall and allowing the invasion of inflammatory factors (Tietge, U.J. , 2014). In combination with the results of this study, we postulated that hyperlipidemia was another pathway for AS induced by LPS stimulation during pregnancy.

The occurrence of hyperlipidemia is closely related to lipid metabolism disorders. The liver is the regulatory center of endogenous and exogenous lipid metabolism pathways. It can synthesize and release various lipoproteins and lipid metabolism enzymes, predominantly maintaining

lipid metabolism balance. In this study, the AST level of offspring rats in the LPS stimulation group was significantly raised, the morphology of hepatocytes was abnormal, and lipid droplets appeared, suggesting that the cells were severely damaged. The ultrastructure of liver tissue revealed that LPS stimulation during pregnancy caused obvious mitochondrial deformation and swelling, showing vacuoles and disordered ridge arrangement. The immunofluorescence assay showed that the 8-OHdG+/GAPI- fluorescence representing mtDNA oxidative damage was significantly enhanced. Moreover, the mitochondrial membrane potential plummeted. Therefore, the progeny rats exhibited liver mitochondrial dysfunction characterized by mtDNA damage.

Such dysfunction is a common mechanism involving various risk factors including obesity, hypertension and hyperglycemia (Oaks, Z. et al., 2016). Studies based on ApoE-/- mice confirmed that mtDNA damage was an early event and independent pathogenic factor of AS (Ballinger, S.W., 2002)(Yu, E. et al., 2014). Upon mitochondrial dysfunction, hepatic mitochondrial TG transporter protein mutates, and the mitochondrial membrane fluidity significantly reduces, thereby considerably producing TG and leading to abnormal liver lipid metabolism (Moffat, C. et al., 2014). By raising the hepatocyte mitochondrial membrane potential, the occurrence and development of hyperlipidemia in ApoE-/- mice can be effectively prevented (Luo, Y. et al., 2015).

5. Conclusion

In summary, inflammatory stimulation during pregnancy leads to mitochondrial damage in offspring, which may be associated with lipid metabolism disorders ultimately inducing AS. Nevertheless, the regulatory effects of mitochondrial dysfunction on liver lipid metabolism still need in-depth studies.

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Compliance with ethical standards

Authors of this study have no conflict of interest. All applicable international, national and institutional principles of handling and using experimental animals for scientific purposes were observed. This study did not involve human subjects as research objects.

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