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Prenatal exposure to TAK242 affects the childhood autism in offspring in animal models of autism spectrum disorder

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ABSTRACT

Objective(s): To evaluate whether prenatal exposure to TAK242 affects childhood autism in the offspring in animal models of autism spectrum disorder (ASD).

Materials and Methods: The pregnant rats were pseudo-randomly divided into three groups, the ASD model group, the TAK242 treatment group, and the control group. The ASD model was constructed by injecting IP with LPS. The blood samples from 1-month-old offspring were collected for cytokine evaluation and the social interaction test was used in the offspring of ASD rats. Rats were killed and the hippocampus, cerebral cortex, and cerebellum were used for the immunohistochemical study.

Results: As compared to the control, the levels of IFN-γ, IL-1β, IL-2, and IL-6 were significantly increased (P<0.05), and the levels of IL-4, IL-10, and TGF-β were significantly decreased (P <0.05) in the offspring of ASD rats; whereas those cytokines were significantly reversed after prenatal exposure to TAK242 (P<0.05). The hesitation time and none-social interaction time were significantly increased as compared to the control (P<0.05); whereas they were both decreased after prenatal exposure to TAK242 (P<0.05). This was contrary to the social interaction time (P<0.05). The expression of GFAP and IBA1 in the cortex, hippocampus, and cerebellum were stronger in the LPS group as compared to control group, and this effect was reversed after prenatal exposure to TAK242.

Conclusion: Prenatal exposure to TAK242 affects serum cytokines levels and the social interaction time in rat offspring in animal models of ASD.

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Introduction

Autism is a collection of chronic, complex neuro-psychiatric diseases, characterized by communication difficulties, social impairments, and repetitive/ stereotyped behaviors. Impaired communication, aberrant repetitive behavior like self-injury, and deficits in social interaction are core symptoms of autism (1). The etiology of autism remains a scientific puzzle and there is no effective treatment for autism yet, with no consensus on the type of medication to prescribe (2). Though a few drugs have been approved, some limitations exist, for example, the limited efficacy, treating part of the symptoms only, and even adverse effects (3). Therefore, the purpose of the present study was to test a treatment for autism by using the rat model of autism.

Immune dysregulation and inflammation, especially neuroinflammation, which generally refers to central nervous (CNS) -specific, chronic glial reactions that may not demonstrate typical peripheral evidence of inflammation, has been implicated in autism (4, 5). Particularly, the family of toll-like receptors (TLRs) is a main agent of the innate immune response. TLRs are pattern recognition receptors that have the ability to circulate pathogen-associated molecular patterns, like the bacterial lipopolysaccharide (LPS), and thus trigger a complex inflammatory cascade, which was characterized by the production of enzymes, cytokines, and other inflammatory mediators. This chain of reactions can cause the activation of oxidative and nitrosative stress pathways, and finally, exert an effect on several aspects of the CNS homeostasis and pathology (6, 7). It is known that TAK-242, a small-molecule antisepsis agent, is an inhibitor of TLR-4-mediated signaling, and has the ability to suppress cytokine levels and has been shown to suppress LPS-induced inflammation (8, 9).

Taking into account this background, the present study aimed to evaluate whether prenatal exposure to TAK242 affects childhood autism in offspring in animal models of ASD.

Materials and Methods

Animals

In this experimental study, 30 female and 6 male Wistar rats at 12 weeks of age (250–300 g), which were obtained from the Beijing Vital River Laboratory Animal Technology Co, Ltd (China), were used. Rats

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were mated overnight (female: male=1:1) in cages and we examined the rats at 7:00 am daily. The presence of a vaginal plug marked that day as embryonic day 0.5 (E0.5). The pregnant rats were then housed in individual cages. The housing room was maintained at 23°C on a 12 hr light/dark cycle, with food and water *ad libitum*. The protocol was approved by the Ethics Committee of Anhui Medical University, Anhui, China.

Groups

The pregnant rats were pseudo-randomly divided into three groups (n=10 in each group), the ASD model group, the TAK242 treatment group and the control group. On E12.5, the pregnant rats were injected intraperitoneally (IP) with 50 micrograms per kilogram (50 μg/kg) of LPS (L2880, Sigma, USA) (LPS group) to construct the ASD model (10). The ASD model rats injected IP with TAK242 (5 mg/kg) were named as the LPS +TAK242 group. The other pregnant rats were injected IP with 2 ml normal saline at E12.5 as a control group. After that, the pregnant rats were housed in individual cages.

Cytokine evaluations

The serum samples of the experimental and control groups were collected. 1 ml blood samples were obtained from the tail vein of 1-month-old offspring rats, followed by sterilization; blood samples were kept at room temperature for 30 min and then centrifuged 4000 r/min for l5 min. The serum was thus separated in the EP tube and frozen at -20 °C. Interleukin (IL) 1-β, IL-4, IL-6, IL-10, interferon-γ (IFN-γ), IL-2, IL-13, IL-17, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) levels were determined using ELISA kits, according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA) (11).

Social interaction test

7 days after the detection of serum cytokines, the offspring rats received the social interaction test. The social interaction test was performed as previously described (12). In brief, behavioral testing was conducted in the colony room in a small, dimly lit (2 lx) area that was enclosed by a curtain. The social interaction time and time to hesitate were recorded by using ANY-MAZE (Stoelting Co, Wood Dale, IL, USA).

Immunohistochemical study

1-month-old offspring rats (n=3) were used and the rats scheduled for death were anesthetized with an IP injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). The brain was removed and the hippocampus, cerebral cortex, and cerebellum were dissected, which were used fresh or kept at -80 °C in our experiment. Immunohistochemistry on formalin-fixed paraffin-embedded sections was performed to determine the immunoreactivity of GFAP and IBA1. Sections were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min, then rinsed in distilled water and washed in PBS for 15 min. Sections were placed in a 10 mmol/l citrate buffer (pH=6.0) and heated in a microwave oven at 95 °C for 30 min. Sections were cooled for 20 min at room temperature and then rinsed in PBS. Non-specific protein binding was blocked by 40 min of incubation in 5% horse serum. Sections were incubated with primary antibodies anti- GFAP (1:200, Santa Cruz Biotechnology, Inc., California, USA) and anti- IBA1 (1:500, Wako Chemicals USA, Inc., Virginia, USA) for 1 hr at room temperature, and then washed in PBS for 15 min. Sections were incubated with horseradish peroxidase (HRP)-conjugated IgG (1:500 dilution, Santa Cruz Biotechnology, Inc., California, USA) for 60 min at room temperature. DAB was used as chromogen and counterstaining was done with hematoxylin. Microscopy of the immunohistochemically stained tissue sections was performed by an experienced pathologist blinded to the experimental condition.

Statistics

All data were analyzed using GraphPad Prism. Data were shown as the mean ± standard deviation (SD). If the data satisfied normality and variance homogeneity, the variance analysis was used and tested using Fisher's Least Significant Difference (LSD) method for compare-son between two groups. If not, Kruskal-Wallis H test was used and Nemenyi method was used for comparison between two groups. $P<0.05$ was considered significant.

Results

Prenatal exposure to TAK242 affects serum cytokines levels in the offspring of ASD rats

As shown in Table 1, the levels of IFN-γ, IL-1β, IL-2, and IL-6 in the offspring of ASD rats were significantly increased as compared to the controls ($P<0.05$). However, after prenatal exposure to TAK242, the levels of IFN-γ, IL-1β, IL-2, and IL-6 in the offspring of ASD rats were significantly decreased ($P<0.05$). Inversely, the levels of IL-4, IL-10, and TGF-β1 in the offspring of ASD rats were significantly decreased as compared to the control ($P<0.05$); whereas, those cytokines were significantly increased after prenatal exposure to TAK242 ($P<0.05$). Besides, the levels of IL-13, IL-17, and TNF-α in the offspring of ASD rats showed no significant differences with the control group ($P>0.05$). Even after prenatal exposure to TAK242, no significant differences were found ($P>0.05$).

Prenatal exposure to TAK242 affects the social interaction time of ASD rats offspring

By the social interaction test (Table 2), we found

Table 1. Serum cytokines levels (mean value)

Groups/Treatments	IFN-γ	IL-1beta	IL-2	IL-6	IL-4	IL-10	IL-13	IL-17	TGF-β1	TNF-a
Control(normal saline)	20.500	12.378	450.137	65.742	78.814	34.258	25.453	16.671	25.767	94.355
LPS	102.244*	25.215*	917.090*	127.430*	50.949*	22.714*	24.856	27.732	43.221*	180.032
LPS+TAK242	27.397#	13.001#	529.667#	70.348#	70.470#	34.770#	29.565	18.104	30.284#	131.131

*P<0.05 *vs.* Control and #P<0.05 *vs.* LPS group. LPS, lipopolysaccharide; Interleukin, IL; interferon-γ, IFN-γ; tumor necrosis factor-α, TNF-α; and transforming growth factor-β, TGF-β

Table 2. Social interaction test (mean value)

Groups/Treatments	Social interaction time	Time to hesitate	None-social interaction time
Control(normal saline)	259.36	12.92	27.46
LPS	31.56*	119.98*	148.00*
LPS+TAK242	245.34#	16.96#	38.20#

*P<0.05 *vs.* Control and #P<0.05 *vs.* LPS group. LPS, lipopolysaccharide

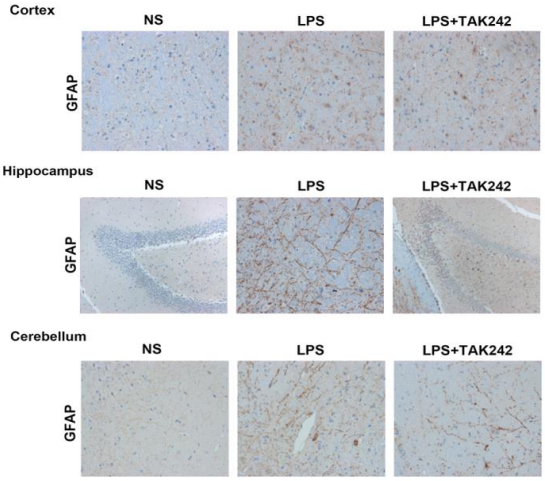

Figure 1. Prenatal exposure to TAK242 affects the expression levels of GFAP in the cortex, hippocampus, and cerebellum in the offspring of ASD rats

Expression of the astroglial marker glial fibrillary acidic protein (GFAP) in the cortex, hippocampus, and cerebellum was stronger in the lipopolysaccharide (LPS) group than in the control group, and this effect was reverted after prenatal exposure to TAK242

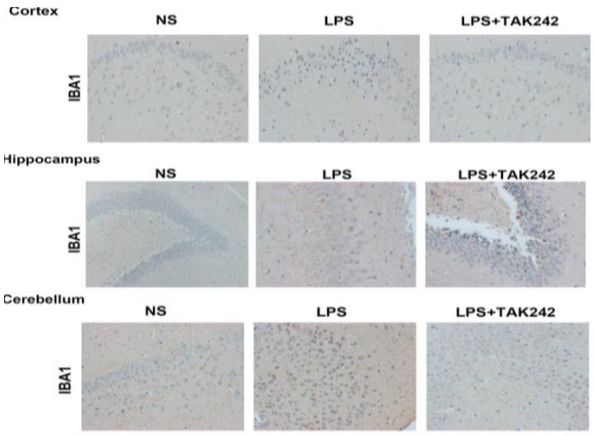

Figure 2. Prenatal exposure to TAK242 affects the expression levels of IBA1 in the cortex, hippocampus, and cerebellum in the offspring of ASD rats

The expression of reactive microglia marker, ionized calcium-binding adaptor molecule 1, in the cortex, hippocampus, and cerebellum were stronger in the lipopolysaccharide (LPS) group than in the control group, and this effect was also reverted after prenatal exposure to TAK242

that the social interaction time of ASD rats offspring was significantly decreased (P<0.05). However, after prenatal exposure to TAK242, the social interaction time was significantly increased (P<0.05). Besides, the hesitation time and none-social interaction time of ASD rats offspring were significantly increased (P<0.05); whereas they were both decreased after prenatal exposure to TAK242 (P<0.05).

Prenatal exposure to TAK242 affects the expression levels of GFAP and IBA1 in the cortex, hippocampus, and cerebellum in the offspring of ASD rats

As shown in Figure 1, the expression of astroglial marker GFAP in the cortex, hippocampus, and cerebellum was stronger in the LPS group than in the control group, and this effect was reverted after prenatal exposure to TAK242. Besides, the expression of reactive microglia marker, IBA1, in the cortex, hippocampus, and cerebellum was stronger in the LPS group than that in the control group, and this effect was also reverted after prenatal exposure to TAK242 (Figure 2).

Discussion

The contribution of neuroimmune functioning to functional dysregulation in ASD was reported in many studies. For example, significantly higher plasma concentrations of IL-1β, IL-5, IL-8, and IL-12 have been reported in individuals with ASD patients compared to the matched normal controls (5). The previous study also indicated the increased proinflammatory cytokines in the brain and cerebrospinal fluid of ASD patients, including higher levels of proinflammatory IL-6 and IFN-γ (13). Consistently, in the offspring of ASD rats, the levels of IFN-γ, IL-1β, IL-2, and IL-6 were significantly increased as compared to the control in our study. Our study also showed that the levels of IL-4, IL-10, and TGF-β1 were significantly decreased as compared to the control, and the levels of IL-13, IL-17, and TNF-α showed no significant differences with the control group. A previous study also showed a reduction in anti-inflammatory TGF-β1 in ASD patients (13). LPS is a cell wall component of Gram-negative bacteria and LPS

administration is a well-characterized and widely accepted model of bacterial infection. It is well-known that systemic LPS administration leads to activation of the innate immune response consisting primarily of cytokine induction, inflammation, complement cascade activation, fever, hypothalamic-pituitary-adrenal axis activation, and sickness behavior (12). It is reported that LPS can bind to toll-like receptor-4 (TLR-4) on macrophages and other immune cells that trigger a signal transduction cascade leading to activation of transcription factors such as nuclear factor kappa B (NFκB) and subsequent transcription of genes coding for pro- and anti-inflammatory mediators such as cytokines, chemokines, and proteins of the complement system (14). In response to LPS, synthesis and release of a family of pro-inflammatory cytokines, most prominently IL-1, IL-6, and TNF-α are elicited.

After prenatal exposure to TAK242, we found the levels of IFN-γ, IL-1β, IL-2, and IL-6 were significantly decreased; whereas the levels of IL-4, IL-10, and TGF-β1 were significantly increased in the offspring of ASD rats. Otherwise, the levels of IL-13, IL-17, and TNF-α in the offspring of ASD rats showed no significant differences after prenatal exposure to TAK242. It seems that prenatal exposure to TAK242 has the ability to reverse the alterations of the serum cytokines levels caused by LPS. Since TAK-242 is an inhibitor of TLR-4-mediated signaling, the effect of TAK-242 on changing cytokines levels and suppressing LPS-induced inflammation may be exerted via the TLR-4-mediated signaling pathway. More molecular mechanisms may be explored in further study.

Social interaction test is a sensitive method for examining autism-related behavioral deficits (15). Through this test, we found that the social interaction time of ASD rats offspring was significantly decreased. However, after prenatal exposure to TAK242, the social interaction time was significantly increased. Besides, the hesitation time and none-social interaction time of the offspring of ASD rats were significantly increased; whereas they were both decreased after prenatal exposure to TAK242. These investigations indicated that LPS caused the social interaction impairment of the ASD rats offspring, and prenatal exposure to TAK242 can alleviate it.

GFAP is a protein expressed in astrocytes and its increase is indicative of astrocyte activation, which is often termed reactive gliosis (16). The IBA-1 protein is a distinct marker of microglia, which are immune specific cells of the central nervous system. Microglia play an important role in protecting the brain tissue from injuries (16). Park *et al.* (17) indicated that the number of activated microglia that showed strong IBA-1 signal and enlarged cell body increased at 24 hr after LPS injection in the cerebral cortex and corpus callosum. The results of a previous study indicated that IBA-1 immunoreactivity of microglia was increaseed in the cortex and hippocampus 24 and 48 hr after LPS challenge, but GFAP immunoreactivity of astrocytes in the hippocampus was unaffected (18). In our study, we found that the expression of astroglial marker GFAP and reactive microglia marker IBA1 in the cortex, hippocampus, and cerebellum were stronger in LPS group than in the control group, and this effect was reversed after prenatal exposure to TAK242. To some extent, it suggests that prenatal exposure to TAK242 inhibited LPS-induced astrocyte activation and microglial activation.

Conclusion

To sum up, the present work shows that prenatal exposure to TAK242 affects serum cytokines levels and affects the social interaction time in rat offspring in animal models of ASD. It may suggest the potential role of TAK-242 in treatment of autism.

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