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Effects of Vitamin D on Infection-Induced Premature Delivery in Rats

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Abstract

Objective: To explore the neuroprotective effects of vitamin D on premature rats with intrauterine infection of white matter damage (WMD).

Methods: A total of 27 pregnant rats were randomly divided into normal group (n=9), model group (n=9) and experimental group (n=9). At 7 d after pregnancy, the rats in experimental group were gavaged with vitamin D (800 IU/d, 7 d in total), while those in normal group and model group were gavaged with an equal amount of normal saline. At 14 d after pregnancy, lipopolysaccharide (LPS, 400 μg/kg) was intraperitoneally injected in model group and experimental group to replicate the infection-induced premature delivery model. Then the content of serum transforming growth factor-β1 (TGF-β1), and amniotic fluid tumor necrosis factor-α (TNF-α) and vitamin D-binding protein (VDBP) was detected by ELISA. At 7 d after birth, neonatal rats in the 3 groups were perfused with formaldehyde to harvest the brain, and the levels of cluster of differentiation 68 (CD68) and glial fibrillary acidic protein (GFAP) in brain tissues were detected using immunofluorescence staining. The neurological behaviors were observed through open field test, suspension test, slope test and anti-capture reaction test at 30 d after birth.

Results: The hippocampal pyramidal cells were arranged neatly and had clear layers in normal group. In model group, the cells had unclear boundaries and disordered layers, and the number of cells declined. In experimental group, the cellular layer was clearer than that in model group. Model group had higher content of amniotic fluid TNF-α and VDBP, and lower content of serum TGF-β1 than normal group ($P<0.05$). In model group and experimental group, the levels of CD68 and GFAP in brain tissues significantly rose compared with those in normal group ($P<0.05$). The levels of CD68 and GFAP in brain tissues significantly declined in experimental group compared with those in model group ($P<0.05$). At 30 d after birth, the neurological behavior score of neonatal rats was significantly lower in model group than that in normal group ($P<0.05$), while it was significantly higher in experimental group than that in model group ($P<0.05$). The content of amniotic fluid TNF-α was lower in experimental group than that in model group ($P<0.05$), while the content of amniotic fluid VDBP and serum TGF-β1 had no statistically significant differences between the two groups ($P>0.05$).

Conclusion: Vitamin D, through inhibiting the pro-inflammatory factor TNF-α, can improve the nervous system development and cognitive function of premature rats with intrauterine infection of WMD.

Keywords: vitamin D; infection-induced premature delivery; CD68; GFAP; TNF-α

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1. Introduction

Premature delivery is one of the common pregnancy complications, and newborns born during this period are called premature infants. In the neonatal period, the death rate of premature infants is high. Some premature infants may suffer from various secondary diseases later due to hypoplasia of organs even if they can survive the neonatal period, affecting the quality of life.

It has been confirmed by pathology and epidemiology that the main type of brain damage in premature infants is white matter damage (WMD). Premature delivery is considered as the most important risk factor for cerebral palsy, and the risk of cerebral palsy will increase by 15 times in premature infants [1,2]. In view of the high incidence and disability rates of WMD in premature infants, it appears to be particularly important and urgent to search for safe and effective treatment methods.

The pathogenic mechanism of WMD in premature infants remains unclear. Recent studies have demonstrated that infection and inflammatory response are important factors for WMD in premature infants. Therefore, the association between perinatal infection and brain damage in premature infants has become a research hotspot for perinatal medical workers. Infection is one of the most important and common causes of premature delivery [3], accounting for approximately 40%. Lipopolysaccharide (LPS) is currently the most commonly-used inducer to establish the premature delivery model, which can activate inflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-12 and IL-6, and induce the inflammatory response at the maternal-fetal interface, namely the placenta, thus leading to premature delivery [4]. In recent years, the Th17/Treg pattern mediating inflammatory response has been paid increasing attention to from experts in immunology and obstetrics and gynecology, making it one of the research hotspots currently [5]. Vitamin D is related to the Th17/Treg axis, and it, jointly with vitamin D-binding protein (VDBP) as a vitamin D carrier, is involved in inflammatory response *in vivo* [6]. In the present study, LPS was intraperitoneally injected into Wistar rats at 15 d after pregnancy to establish the model of intrauterine infection of WMD. The levels of cluster of differentiation 68 (CD68) and glial fibrillary acidic protein (GFAP) in brain tissues were detected using immunofluorescence staining, and the neurological behaviors were observed at 30 d after birth, so as to explore the neuroprotective effect of vitamin D on premature rats with intrauterine infection of WMD.

2 Materials and Methods

Experimental animals

A total of 27 female Wistar rats (about 12 weeks old, 300-350 g) and 10 male SD rats (about 15 weeks old, 450-500 g) were all purchased from Beijing Vital River Laboratory Animal Co., Ltd. [license No. SCXK (Beijing) 2016-0011], and they were ethically reviewed by the Laboratory Animal Center of our hospital. The laboratory animals were processed in strict accordance with the principles of animal ethics.

Main reagents and apparatus

LPS was provided by Shanghai Yanjin Biotechnology Co., Ltd. TGF-β1 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai TW-Reagent Co., Ltd., and VDBP ELISA kits were purchased from Quanzhou Ruixin Biotech Co., Ltd. A microplate fast oscillator QB-9001 was provided by Kylin-bell, a light absorption microplate reader Elx-808 was obtained from BioTek, and an incubator 15AIC was sourced from Sanyo (Japan).

Animal grouping and treatment

Female and male rats cohabited (2:1), and whether the vaginal plug was formed in female rats was checked every day. If so, the day was recorded as 0 d after pregnancy. All female rats were successfully pregnant, and they were randomly divided into normal group (n=9), model group (n=9) and experimental group (n=9). At 7 d after pregnancy, the rats in experimental group were gavaged with vitamin D (800 IU/d) for 7 d in total, while those in normal group and model group were gavaged with an equal amount of normal saline. At 14 d after pregnancy, LPS (400 μg/kg) was intraperitoneally injected in model group and experimental group, while an equal amount of normal saline was intraperitoneally injected in normal group. If vaginal bleeding was found within 24 h after LPS injection, infection-induced premature delivery was confirmed [7]. The infection-induced premature delivery model was successfully established in model group and experimental group.

At 7 d after birth, neonatal rats in the 3 groups were perfused with formaldehyde to harvest the brain, and the levels of CD68 and GFAP in brain tissues were detected using immunofluorescence staining. Moreover, the neurological behaviors were observed through open field test, suspension test,

slope test and anti-capture reaction test at 30 d after birth.

Sample collection

The neonatal rats were randomly selected in each group at 7 d after birth (no treatment after birth), from which 2 mL of orbital venous blood was drawn and centrifuged at 3,500 rpm for 10 min. The upper-layer serum was collected, subpackaged into EP tubes, and stored in a refrigerator at -80°C. Then the rats were anesthetized by oral administration of 10% chloral hydrate (3 mL/kg), and fixed on an operating table. The breastbone was cut off to expose the heart, a 5 mL syringe was inserted into the left ventricle, and the puncture needle was fixed with silk threads after entering the aorta. 4% paraformaldehyde was injected, and the right atrium was cut open, from which the blood flowed out until the effluent became cool. At this moment, the distal limbs of neonatal rats became pale and stiff, indicating satisfactory perfusion, so the perfusion was terminated. Then the rats were decapitated, the skin tissues were peeled off layer by layer to expose the neck bone, and the bone pieces were removed along the collar bone suture to expose the brain tissues. The well-perfused brain tissues had regular appearance, and the cerebral cortex was bloodless. The whole brain tissues were peeled off and fixed in 4% paraformaldehyde solution.

Measurement of serum TGF-β1 and amniotic fluid TNF-α and VDBP levels by ELISA

The levels of serum TGF-β1 and amniotic fluid TNF-α and VDBP were detected by ELISA in strict accordance with the instructions. The absorbance value was measured using a microplate reader, the standard curves were plotted, and the concentration was calculated.

Pathological examination of brain tissues

The morphological changes in brain tissues and brain damage were observed through hematoxylin-eosin (HE) staining, based on which the necrotic and apoptotic cells were determined. HE sections were prepared as follows: The sections were deparaffinized with xylene I for 10 min and with xylene II for 5 min, and then xylene was washed off with absolute ethanol for 1 min × twice. The sections were soaked in 95% ethanol, 90% ethanol and 85% ethanol for 1 min each, rinsed with tap water for 2 min, stained with hematoxylin for 5 min, rinsed with tap water for 1 min, and differentiated with 1% hydrochloric acid alcohol for 20 s. Then the sections were washed with tap water, added with dilute ammonia water for 30 s for blue returning, washed with distilled water for 1 min, stained with eosin for 30 s, washed with tap water for 30 s, dehydrated with 85% ethanol, 90% ethanol, 95% ethanol I, 95% ethanol II, absolute ethanol II and absolute ethanol II for 20 s, 30 s, 1 min, 1 min, 2 min and 2 min, respectively. Finally, the sections were soaked in xylene I, xylene II and xylene III for 2 min each, and sealed with neutral balsam.

Detection of CD68 and GFAP in brain tissues by immunofluorescence assay

The sections were added with a few drops of PBS for 15 min, and washed with PBS for 3 times for hydration. 0.3% Triton-100 was added, and the sections were placed at room temperature for 10 min for transparentization. Then the sections were soaked in PBS for 5 min, washed for 3 times, and incubated with primary antibodies at 37°C for 2 h in a wet box (two primary antibodies were added during double staining), with a negative control (100 μL of PBS was added instead of primary antibodies) set up. The sections were soaked in PBS for 5 min, washed for 3 times, and incubated again with FITC/TRITC-labeled secondary antibodies (1:400) at 37°C for 1 h (two fluorescently-labeled secondary antibodies corresponding to the primary antibodies were added during double staining). After the sections were soaked in PBS for 5 min and washed for 3 times, they were counterstained with DAPI for 5 min, soaked in PBS for 5 min and washed for 3 times, followed by sealing with antifade mounting medium. Then the sections were photographed and observed under a fluorescence microscope. The staining results were quantitatively analyzed using a multimedia color immunofluorescence pathological image analysis system. The green color indicated positive results, and the number of positive reactants on sections was determined by the depth and area of green staining. Several fields of view were randomly selected in each section, and the integrated optical density (IOD) of the green part was measured. Average OD = IOD/green area.

Observation of neurological behaviors

Open field test: An uncapped square box (36 cm × 36 cm × 36 cm) was equally divided into 9 grids at the bottom with chalk lines. The neonatal rats were placed in the central grid of the box, and the movement of them was observed for 30 s. 1 point was given when more than 50% of the rat's body entered the adjacent grid from the grid where it was located, and standing up on its hind limbs was scored as 1 point. The two scores were added up to obtain the total score.

Suspension test: The rats were made to grasp the glass rod 45 cm above the table with forelimbs, and the time of falling of rats was recorded and scored based on the following criteria: <10 s: 1 point, 10-30 s: 2 points, 30 s-2 min: 3 points, 2-5 min: 4 points, and > 5 min: 5 points.

Slope test: The rats were placed upside down on a 45° slope with the head downward, and the time (s) it took for the rat to turn the head up >135° was recorded.

Anti-capture reaction test: The rat was gently lifted with a glove that the rat had never touched, and its reaction was observed and scored based on the following criteria: 0 points: easy to capture the animal, 1 point: scream and evade, 3 points: escape, 4 points: escape and scream, 5 points: bite or try to bite the glove, and 6 points: jump to attack actively.

Statistical analysis

SPSS 23.0 software was used for statistical analysis. Quantitative data were expressed as mean ± standard deviation, and *t* test was used for intergroup comparison. P<0.05 was considered to be statistically significant.

3. Result

HE staining results

The hippocampal pyramidal cells were arranged neatly and had clear layers in normal group. In model group, the cells had unclear boundaries and disordered layers, and the number of cells declined. In experimental group, the cellular layer was clearer than that in model group (Figure 1).

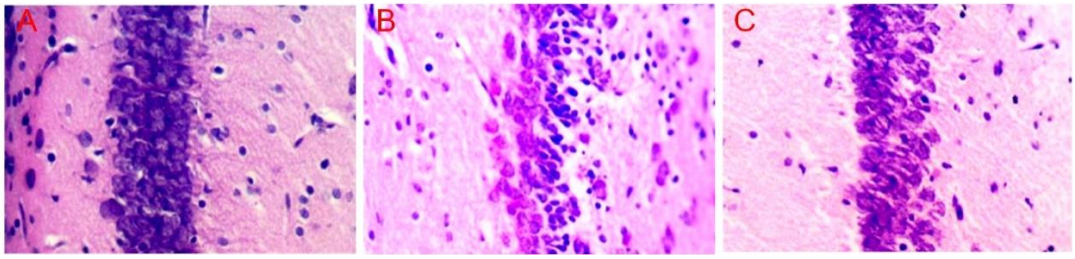

Figure 1. HE staining results of hippocampal pyramidal cells of 7-day-old neonatal rats in each group (×400). A: normal group, B: model group, C: experimental group.

Serum TGF-β1 and amniotic fluid TNF-α and VDBP levels

Model group had higher content of amniotic fluid TNF-α and VDBP, and lower content of serum TGF-β1 than normal group, and the differences were statistically significant (P<0.05). The content of amniotic fluid TNF-α was lower in experimental group than that in model group (P<0.05), while the content of amniotic fluid VDBP and serum TGF-β1 had no statistically significant differences between the two groups (P>0.05) (Table 1).

Table 1. Serum TGF-β1 and amniotic fluid TNF-α and VDBP levels (pg/mL)

Group	TGF-β1	VDBP	TNF-α
Normal	72.97±9.10	184.32±21.22	105.43±18.23
Model	44.38±4.38*	1087.89±123.47*	187.45±23.21*
Experimental	49.27±5.02#	564.39±78.43#	115.32±19.02#

*P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

Immunofluorescence assay results of CD68 and GFAP in brain tissues

In model group and experimental group, the level of CD68 in brain tissues significantly rose compared with that in normal group, and there were statistically significant differences (P<0.05), indicating that intrauterine infection leads to WMD in premature rats. The level of CD68 in brain tissues significantly declined in experimental group compared with that in model group, showing a statistically significant difference (P<0.05), suggesting that early application of vitamin D can suppress the immuno-inflammatory response in the brain of rats to a certain extent.

In model group and experimental group, the level of GFAP in brain tissues significantly rose compared with that in normal group, and there were statistically significant differences (P<0.05), indicating that intrauterine infection leads to WMD in premature rats. The level of GFAP in brain tissues significantly declined in experimental group

compared with that in model group, showing a statistically significant difference (P<0.05), suggesting that early application of vitamin D can inhibit the response of astrocytes in the brain of rats to a certain extent (Table 2).

Table 2. Immunofluorescence assay results of CD68 and GFAP in brain tissues

Group	CD68	GFAP
Normal	46.02±5.43	65.43±6.15
Model	104.32±4.51*	145.38±6.58*
Experimental	85.43±5.37#	77.32±4.39#

*P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

30-day neurological behaviors

At 30 d after birth, the neurological behavior score of neonatal rats was significantly lower in model group than that in normal group, with a statistically significant difference (P<0.05). Premature rats with WMD have development retardation of nervous system and cognitive function. Besides, the neurological behavior score was significantly higher in experimental group than that in model group, with a statistically significant difference (P<0.05), indicating that vitamin D can improve the nervous system development and cognitive function of premature rats with WMD (Table 3).

Table 3. 30-day neurological behaviors

Group	Open field test (point)	Suspension test (point)	Slope test (s)	Anti-capture reaction test (point)
Normal	13.24±1.56	4.99±0.18	2.17±0.21	5.47±0.32
Model	4.76±0.54*	1.74±0.29*	9.32±0.68*	0.95±0.13*
Experimental	11.36±1.43#	3.879±0.21#	4.45±0.57#	3.11±0.67#

*P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

4. Discussion

Premature infants refer to newborns born after 28 weeks of gestation but before 37 weeks of gestation, and they have immature brain development and are vulnerable to damage. With the increasing survival rate of premature infants, the brain damage in them has attracted more and more attention. Brain damage in premature infants during the perinatal period can result in developmental disorders of the nervous system. In severe cases, permanent disability, namely cerebral palsy, can be caused. According to neuropathology, brain damage in premature infants can be classified into WMD, brain non-parenchymal hemorrhage and damage at other sites of the brain. White matter is an important component of the brain parenchyma, formed by massive aggregation of neuroglial cells and nerve fibers, which plays an important role during the transmission of neural signals. WMD in premature infants is a major high-risk factor for cerebral palsy, and it is the most serious brain damage from the perspective of prognosis [8]. At present, the pathogenesis of WMD in premature infants remains unclear. Previously, researchers mostly focused on hypoxia and ischemia. However, infection and inflammatory response are also key factors causing WMD in premature infants, and they exert a sensitizing effect on hypoxic-ischemic brain damage [9].

The establishment of animal models is the basis for experimental research on brain damage in premature infants, and successful modeling can help people study the pathogenesis, treatment and prognosis of brain damage in premature infants more deeply. To study WMD in premature infants, the morphological features and distribution of brain damage model should be similar to those of human WMD. Many rodents, such as rats, have been widely used in basic research because of their low costs, strong fertility and short gestation [10]. As a bacterial product, LPS is often used to establish the immature mouse model of WMD. LPS is the core of Gram-negative bacterial endotoxin, whose mechanism in inducing brain damage may be related to immune response and initiation of systemic inflammatory response, so that hypoglycemia, blood coagulation and insufficient cerebral blood perfusion are caused, and inflammatory cells in the central nervous system are activated. In the present study, LPS was intraperitoneally injected into pregnant rats to successfully establish the intrauterine infection model. It was observed through staining of brain tissues of neonatal rats that in model group, the white matter of neonatal rats had light staining and

loose structure, nerve fibers had uneven thickness and disordered direction, and poorly-differentiated neurons with small nuclei, small volume, deep staining and few processes could be seen. Moreover, the number of glial cells rose, and apoptotic changes such as karyopyknosis, cytoplasmic loosening and cell shrinkage occurred in model group. In normal group, the white matter of neonatal rats had clear staining and normal structure. The cellular layer was clearer in experimental group than that in model group. Besides, at 30 d after birth, the neurological behavior score of neonatal rats was significantly lower in model group than that in normal group, while it was significantly higher in experimental group than that in model group. The above findings demonstrate that vitamin D can improve the nervous system development and cognitive function of premature rats with intrauterine infection of WMD.

CD68 is a highly glycosylated type I transmembrane protein distributed on the cell surface. It has been determined that CD68 can serve as a specific molecular marker for macrophages. Microglia, as macrophages in the nervous system, are the most important and earliest inflammatory cells involved in the inflammatory response. Astrocytes, oligodendrocytes and axons are the main components of the white matter [11,12]. The central link in the process of infection leading to WMD in premature infants is the damage of oligodendrocytes, especially the rapidly differentiating ones. Due to the dysfunction and decreased number of oligodendrocytes, developmental disorders occur in myelin sheath, thus leading to decline in the volume of white matter and ventricular expansion. GFAP is a component of intermediate filament associated protein of astrocytes in the brain, which is recognized as a characteristic marker for astrocytes [13]. In this study, the levels of CD68 and GFAP in brain tissues significantly rose in model group and experimental group compared with those in normal group, and there were statistically significant differences ($P<0.05$). The levels of CD68 and GFAP in brain tissues significantly declined in experimental group compared with those in model group, showing statistically significant differences ($P<0.05$), indicating that early application of vitamin D can inhibit the response of astrocytes in the brain of rats to a certain extent.

During normal pregnancy, the fetus can grow in the uterus until birth, which relies on the unique immune microenvironment balance at the maternal-fetal interface. Imbalance will cause inflammatory response, thus leading to pregnancy failure, such as miscarriage, fetal growth restriction, premature delivery, preeclampsia and other major diseases, seriously affecting the maternal and fetal health. Many positive results have been obtained by scholars in the research on the balance of Th1/Th2 axis at the maternal-fetal interface, for example, the transformation of cytokines into Th2 is conducive to the maintenance of pregnancy. The balance of Th17/Treg axis playing an important role in maternal-fetal immunity has become a research hotspot [14]. Th17 cells, a $CD4^+$ T cell subset, can secrete cytokines including TNF-α, IL-17, IL-22, IL-21 and IL-6, and participate in the body's inflammatory response and immune response. The abnormal increase in Th17 cells may cause chronic inflammatory response at the maternal-fetal interface. It has been found that activating the inflammatory signaling pathway in the uterus can lead to premature delivery, and inflammatory factors such as TNF-α and IL-6 can induce inflammatory response at the site of infection. VDBP is an alpha globulin with a variety of physiological functions, whose main biological function is to bind to and transport vitamin D and its metabolites, and also enhance the chemotactic activity of C5 towards neutrophils [15]. Moreover, in research on liver cancer, VDBP is negatively correlated with the risk of liver cancer. In this study, the results manifested that model group had higher content of amniotic fluid TNF-α and VDBP, and lower content of serum TGF-β1 than normal group. The content of amniotic fluid TNF-α was lower in experimental group than that in model group, while the content of amniotic fluid VDBP and serum TGF-β1 had no statistically significant differences between the two groups.

In conclusion, vitamin D, through inhibiting the pro-inflammatory factor TNF-α, can improve the nervous system development and cognitive function of premature rats with intrauterine infection of WMD.

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