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A study on the protective role of doxycycline upon dopaminergic neuron of LPS-PD rat model rat

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Abstract. – OBJECTIVE: To investigate the protective role of doxycycline upon the dopaminergic neuron of the lipopolysaccharide-Parkinson disease (LPS-PD) model rat and its mechanism.

MATERIALS AND METHODS: Animals were randomly divided into three groups: normal control group, LPS group and doxycycline intervention. Group; establishing The PD model was created by injecting LPS stereo-tactically into the substantia nigra; observing the changes in the dopaminergic neurons and the major histocompatibility complex II (MHC II) positive microglia before and after the intervention of doxycycline with immunohistochemical staining. Using the HPLC-ED (high performance liquid chromatography-electrochemical detector) to test the changes in the striatal dopamine (DA), and DOPAC (dihydroxy phenyl acetic acid) content; adopting Western blotting was adopted to test the expression of the substantia nigra microglia MHC II (major histocompatibility complex II) protein.

RESULTS: After the intervention of doxycycline, in the LPS group, the surviving dopamine neurons in the substantia nigra rose from 38% ± 5% to 79% ± 4% ($p < 0.01$); striatal DA and DOPAC content of the LPS group increased from 4.89 ± 0.27 and 0.70 ± 0.07 to 7.00 ± 0.34 and 1.10 ± o. 10 respectively ($p < 0.01$). The average number of rotation induced intraperitoneal injection of apomorphine of the animals in the LPS group reduced from (208 ± 14); time/30 min to (80 ± 12) times/30 min ($p < 0.01$); while the number of the MHC II positive cells in the substantia nigra pars compacta in the LPS group reduced from 835 ± 82 to 354 ± 59 ($p < 0.01$); Western blotting of the MHC II protein expression showed a significant reduction.

CONCLUSIONS: Doxycycline can inhibit degeneration of LPS-induced dopaminergic neurons. Its neuroprotective function is achieved by downregulating the microglia MHC II expression.

Key Words:

Doxycycline, Neuroprotection, Microglia, Major histocompatibility complex II, Dopaminergic neurons.

Abbreviations

LPS = lipopolysaccharide; PD = Parkinson disease; HPLC-ED = high performance liquid chromatography-electrochemical detector; MHC = major histocompatibility complex; TH = thyrosine hydroxylase; DOPAC = dihydroxy phenyl acetic acid; PVDF = polyvinylidene fluoride; TBS-T = tris buffered saline-tween; ECL = enhanced chemiluminescence; ROS = Reactive Oxygen Species.

Introduction

Parkinson's disease (PD), which is common among the elderly, is a degenerative disease of the central nervous system. Its main pathological features are degeneration of the dopaminergic neurons in the *substantia nigra*[1-3]. Since its etiology and pathogenesis remain unclear, levodopa replacement serves as the main therapy. However, it cannot prevent the progression of dopamine (DA) neuron degeneration. Therefore, neuroprotection has become a research hotspot. In recent years, evidence has suggested that immune abnormalities and inflammatory responses in the central nervous system are involved in the degeneration of dopaminergic neurons in the substantia nigra. It has been reported that the neuroprotective effect of doxycycline was observed in animal models with cerebral ischemia[4], multiple sclerosis (MS)[5], and familial amyloidotic polyneuropathy (FAP)[6]. Reports of the lipopolysaccharide-Parkinson disease (LPS-PD) animal model is rare. The molecular mechanisms of its neuropro-

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tective role remain unclear. This study has observed the protection of doxycycline in DA neurons and its mechanisms in the LPS-PD rat model, which provides a theoretical basis for the clinical treatment of doxycycline applied to the PD[7-9].

Materials and Methods

Sixty healthy female Sprague Dawley (SD) rats, weighing 250-300 g, were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science Technology, China. They had free access to food and water and were fed in a standard animal room of 12 h light/dark cycle. Before the experiment, the animals were given 7d to adapt to the new environment. The research was approved by Ethics Committee of Tongji Medical College.

The reagents used for this experiment were the following: LPS (lipopolysaccharide, *Escherichia coli*, Serotype 026: B6, Sigma Company, Saint Louis, MO, USA), tyrosine hydroxylase (tyrosine hydroxylase, TH, a monoclonal antibody, Sigma Company), Doxycycline (Doxycycline, Sigma Company), horseradish peroxidase (HRP) labeled secondary antibody from Wuhan Boster Biological Technology Co., Ltd., Wuhan, China, OX6 (MHC II antigen-specific labeled antibody, Abcam Inc., Cambridge, MA, USA), horseradish peroxidase (HRP) labeled secondary antibody for Wuhan Boster Biological Technology Co., Ltd, Wuhan, China, standard DA for detection[3,4], DOPAC were purchased from Sigma.

The animals were randomly divided into three groups: normal control, LPS, and doxycycline intervention (Doxycycline + LPS). Each group included 20 rats. In the LPS group, the rats were stereo-tactically injected into the substantia nigra. In doxycycline intervention group, intraperitoneal injection of doxycycline was conducted 3 d before the LPS modeling (45 mg/kg, one time/d), used for 14 d continuously. Nigra LPS stereotactic injection modeling: Chloral hydrate (300 mg/kg) was injected the abdominal cavity of SD rats for anesthesia, fixed their heads on the stereotaxic instruments, upper incisors below the ear bar 2.3 mm, according to Paxinos (The Rat Brain in Stereotaxic Coordinates) map. Then select the maximum level of the substantia nigra pars compacta (SNpc, coordinates: 5.2 mm behind the anterior fontanelle, 1.8 mm long opening beside dorsal raphe nucleus, 7.8 mm below the dura) using a micro syringe to inject LPS in the right substantia nigra [using sterile phosphate-buffered saline (PBS) to dissolve, final concentration of LPS was 2.5 μg/μl] 4 μl, injection speed was 1 μl/min. Then, we injected 4 μl PBS into the contralateral substantia nigra and the substantia nigra in the control group as a contrast, slowly removed the syringe 10 min after administration, and used bone wax to enclose the burr holes, sutured the skin, and injected 100 000 IU penicillin intramuscularly.

Rotational behavior observation: at second week, injected apomorphine (0.5 mg/g) into the abdominal cavity of the rats. We recorded their start time of rotation behavior, rotation number/30 min, and rotation direction.

Immunohistochemical staining: a week after injecting LPS, the rats under deep anesthesia at room temperature were firstly perfused with 150 ml PBS through the left ventricle, followed by perfusion of 400 ml precooled (4°C) 4% paraformaldehyde (pH 7.4). The perfusion speed should be slow at first and then fast. The perfusion solution should be injected completely within one hour. We decapitated the rats and collected the midbrain, placed the midbrain in the aforementioned solution and continued to get it fixed for 12 hours. Then, we embedded the midbrain in paraffin and underwent consecutive coronal slices, placing the slices on glass slides coated with 2% gelatin. Specific steps: (1) Paraffin sections were deparaffinized through water; (2) 3% H_2O_2: Incubated at room temperature for 5-10 min to eliminate endogenous peroxidase activity, washed with distilled water, soaked in PBS for 5 min. (3) Then, enclosed with 5% to 10% normal goat serum and decanting serum after incubating at room temperature for 10 min; (4) Drop an appropriate proportion of the primary antibody (OX6 work titer 1: 200; TH monoclonal antibody titer of 1 work: 1000), and put it in 4°C refrigerator for the night. In the control group, we replaced the primary antibody with normal serum. Washed it with PBS, 5 min × 3 times; (5) Dropped the biotin-labeled secondary antibody and incubated it for 30 min at 37°C. Then, we washed it with PBS, 5 min × 3 times. (6) DAB (diamino benzidine) color, controlling the color under a micro-

scope, at room temperature for 3-10 min, terminating the coloration after being satisfied, and placing it in an incubator for the night. (7) After dehydration, verification and hematoxylin dyeing, mounting it with neutral resin and observing under a microscope.

Monoamine neurotransmitters test: collecting the right striatum of the rats, weighing them and saving at -80°C. Detecting the content of DA, DOPAC by HPLC with high performance liquid chromatography-electrochemical detector (HPLC-ECD). Test conditions: chromatographic column was octadecyl silane analytical column, the mobile phase was 0.15 mol/L of chloroacetic acid- hydroxide buffer solution (containing 9 mmol/LD-camphor-sulfonic acid, 10% methanol and 0.83. mmoL/L EDTA), detection working voltage was 700 mV, sensitivity 20 nA.

Western blotting detection of nigra microglial MHC II protein: Took four rats in each group and quickly decapitated them to collect the brain. Then, we gently peeled a complete brain under the dissecting microscope, turned it ventral side up, sliced transversely twice along the optic nerve root and the brain stem, separated the substantia nigra with fine anatomical forceps and collected the total protein extraction. For each protein sample, we took 20 μg and added 3 × loading buffer, loaded the sample after boiling it for 3 min, and conducted the vertical electrophoresis in the prepared SDS-polyacrylamide gel for 1.5-2 h. We, then, disassembled the electrophoresis apparatus, cut the needed protein bands gel, and assembled the transferred interlayer. Then, we placed them into a film slot, and then at 4°C 25 V voltage, transferred the protein to PVDF membrane; disassembled the film apparatus, placed the film in tris buffered saline-tween (TBS-T) containing 5% nonfat milk, enclosed the film at room temperature for 2 h; added OX6 antibody (1: 500) 4°C, incubated for the night; the next day, washed the film with TBS-T at room temperature 4 times, 15 min each time, then, added horseradish peroxidase (HRPO) and conjugated IgG corresponding antibody (1: 8000 dilution). Then, gently shook it for 2 h at room temperature; then, in a same manner, washed the film with TBS-T at room temperature four times, 15 min each time; and finally added ECL reagent, conducted X-ray sheet exposure after 2-4 min of incubation at room temperature.

Image Analysis

After scanning TH and MHC II positive signal with HP-IAS21000 medical color image analysis system, count the number of TH-positive cells and MHC II positive cells, and used `x± s to represent the values. Observing the microglia cell morphology under a × 200 times microscope, the 3 phases were divided according to their morphology: the resting phase (small cell body, a plurality of elongated protrusions); active phase (cell body becomes larger, stubby protrusions); excitation phase (ameboid).

Statistical Analysis

Using the SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) for analysis and conducting an analysis of variance on the changes in the TH-positive neurons and DA, DOPAC content before and after the doxycycline treatment. The *t*-test was used to analyze the rest. $p < 0.05$ was considered statistically significant.

Results

The protective effect of doxycycline on dopamine neurons of LPS-PD model rats: after injecting the LPS into the nigra, the TH-positive neurons of the rats in the LPS group reduced significantly (38% ± 5% of the contralateral). In the doxycycline intervention group, the TH-positive neurons in the LPS injection side were 79% ± 4% of the contralateral nigra. The test was statistically significant ($p < 0.01$, Figure 1).

Changes in striatal DA and DOPAC content: a high performance liquid chromatography-electrochemical detector (HPLC-ECD) was used to test the nigra DA and DOPAC content (× ± s, μg/g). The results showed the following: the nigra of the control group were 8.21 ± 0.43 and 1.32 ± 0.09 respectively. After injecting LPS into the substantia nigra of the rats in the LPS group, their DA and DOPAC content decreased, markedly. The figures were 4.90 ± 0.25 and 0.71 ± 0.07 respectively. Compared with the control group, the differences were statistically significant ($p < 0.01$). In the doxycycline intervention group, the DA and DOPAC content were 6.97 ± 0.37 and 1.09 ± 0.09, compared with the LPS group. The differences were statistically significant ($p < 0.01$).

The influence of doxycycline on the behavior of the LPS-PD model rats: two weeks after injecting LPS into the substantia nigra, apomorphine

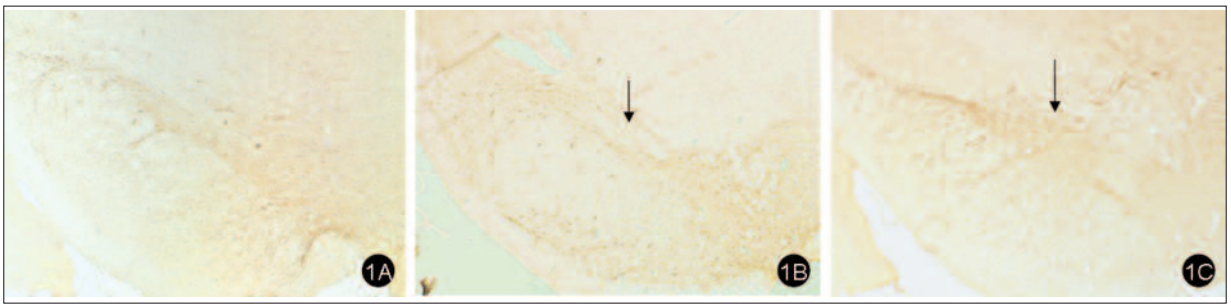

Figure 1. The effect of doxycycline intervention on the TH + neurons in the rat DAB staining ×40; the brown parts are positive cells (↓); ***A,*** Control group. ***B,***. LPS group. ***A,*** Doxycycline intervention group.

was injected into the abdominal cavity to induce the animal to rotate. The average number of rotations of the LPS group was (208 ± 14) times/30 min. The average number of rotations of the doxycycline intervention group was (80 ± 12) times/30 min. The differences were statistically significant (t = 18.73, p < 0.01). The control group did not have any changes in behavior.

The effect of doxycycline intervention on the nigra microglia of the LPS-PD model: no or only a small number of MHC II-positive cells in the nigra were observed in the control group (Figure 2A). After the LPS injection into the nigra, a lot of MHC II positive cells were detected. The cells were round or oval with no protrusions, and were mainly distributed in the substantia nigra pars compacta. They were also scattered in the reticular part (Figure 2B). In the doxycycline intervention group, the MHC II positive microglia cells reduced significantly. The cells were mostly dendroid with long protrusions (Figure 2C), which indicated that the doxycycline inhibited activated microglia, so most of them were in the second phase (according to KreutzbergHl's[4] microglia staging criterion). The smaller cells suggested that the amount of cell antigen expression deduced. Due to the few MHC II positive cells in the normal control group, we compared the number of substantia nigra MHC II positive cells between the LPS group [(835 ± 82)/mm^2] and the doxycycline intervention group [(354 ± 59)/mm^2]. The difference was statistically significant (t = 11.39, p < 0.01).

LPS group; D + LPS. Doxycycline Intervention Group

The effect of the doxycycline intervention on the expression of the MHC II microglia protein: After detecting the MHC II protein expression in the nigra with Western blotting, no or only a very small amount of the MHC II protein expression was observed in the control group. A lot of MHC II expression was observed in the LPS group, and the MHC II protein expression decreased significantly in the doxycycline intervention group (Figure 3).

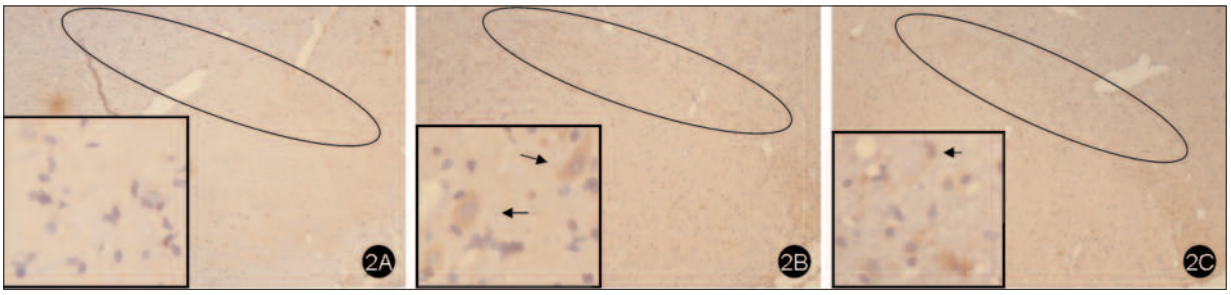

Figure 2. The effect of doxycycline intervention on nigra microglia MHC II expression. Oval laps are substantia nigra pars compacta; the block is for an enlarged view × 400), cytoplasmic brown are positive cells (*arrows)*, ***A,*** Control group. ***B,*** LPS group. ***C,*** Doxycycline intervention group.

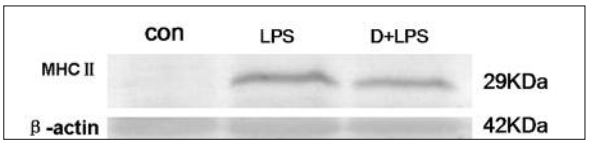

Figure 3. Western blotting detection of MHC II protein, Con. Control; LPS: lipopolysaccharide.

Discussion

Doxycycline[10-12] is a semisynthetic antibiotic among the tetracycline family. In addition to its antibacterial effect, it possesses a strong anti-inflammatory effect. It can go through the blood-brain barrier, so it is more suitable for the treatment of central nervous system diseases. As an effective anti-inflammatory drug, it has exhibited a beneficial role in central nervous system disorders, such as cerebral ischemia, MS, and familial amyloid polyneuropathy (FAP). However, its mechanism of action is still not very clear. LPS is a kind of phospholipid made up of aminoglycoside, which constitutes the main component of the wall of Gram-negative bacteria. It is potent proinflammatory cytokines, when injected into the animal's nigrostriatal can induce microglial activation, and selectively cause the degeneration of DA neurons in the substantial nigra[13,14]. Its pathological changes resemble that of PD, which has become an ideal model for the study of inflammation response and the pathogenesis of PD[5].

In this study of the LPS-PD rat model, the neuroprotective effect of doxycycline was evaluated by testing the behavior of rats in each group, the number of dopamine neurons in the substantia nigra and dopamine levels in striatum. It was found that after injecting LPS into the substantia nigra, the DA neurons decreased, and only amounted to 37.86% of that in contralateral part. The DA levels of striatum also reduced significantly. After the intervention of doxycycline, nigra dopaminergic neurons in LPS injection side rose to 78.57%, striatum DA and DOPAC levels increased significantly compared with LPS group, which showed that doxycycline can inhibit LPS-induced injury in dopaminergic neurons in the substantia nigra. Behavioral tests also showed that after the intervention of doxycycline, the average number of rotation of the animals induced by intraperitoneal injection of apomorphine reduced significantly, suggesting that changes in behavior and the organizational structure were consistent. There are similar studies in other animal models, and patients. Jantzie et al[5] found in studies of animal models of cerebral ischemia that doxycycline could significantly reduce the volume of cerebral infarction under hypoxia. In 15 cases of remission-relapse in MS patients, Minagar et al[14-17] found that, after four months of treatment with doxycycline, the neurological score, disability status (survey) scale score and MRI image analysis of the treatment group, compared with the control group, all reduced. There was only one case of recurrence, and the MRI showed that the damaged area was also reduced. But still rare are reports of its protective effect observed in the LPS-PD model.

It remains unknown on how doxycycline plays a neuroprotective role[18]. Studies have shown that immune abnormalities and inflammation of the central nervous system is involved in the degeneration and necrosis of dopaminergic neurons. Inflammation serves as the first barrier against injury and infection, but the excessive and persistent inflammation can harm host cells. Innate immune cells of the central nervous system-microglia have been proved to be inherent antigen presenting cells of the central nervous system. Under normal circumstances, they express only very low levels of MHC II proteins. When brain tissues are invaded by infection, trauma, ischemic or toxic substances, microglia cells are activated, the cell body becomes larger, functionally-activated microglia cells cause oxidative stress and mitochondrial dysfunction by releasing a variety of toxic substances, such as a large number of proinflammatory cytokines (TNF-α, IL-b), NO, reactive oxygen species, which exerts toxic effects on the DA neurons. Meanwhile, microglia cells start to express a large number of specific membrane-surface molecules related to antigen recognition and presentation, such as the MHC and complement receptor CR3[19-20]. MHC II molecules play an important role in the process of antigen-presenting cells (APC) and present exogenous antigen-presenting to CD4+ Th cells. Imamura et al[21] observed distribution of MHC II-positive microglia cells in the brain autopsy specimens of 12 cases of PD patients, and found that as the degeneration of the substantia nigra DA neuron, the substantia nigra and the striatum of MHC II positive microglia cells also increased. Therefore, MHC II expression is often seen as a sign of inflammatory reaction. This study regarded the microglia MHC II antigens expression in

the LPS-induced substantia nigra as indicators of inflammation to study the neuroprotective mechanism of doxycycline.

Study results found that in LPS group, after LPS injection, a lot of MHC II positive microglia cells could be observed in the substantia nigra, which were round or oval, but with no protrusions, and were mainly distributed in the substantia nigra pars s compacta. They were also scattered in the reticular part; while, in the doxycycline intervention group, the MHC II positive microglia cells reduced significantly. The cells were mostly dendroid with long protrusions, which indicated that doxycycline inhibited activated microglia, so most of them were in the second phase. Meanwhile, after detecting MHC II protein expression in the nigra with Western blotting, an apparent MHC II expression was observed in LPS group. However, the MHC II protein expression reduced significantly after the doxycycline intervention, suggesting that doxycycline can play a neuroprotective role in down regulating the microglia MHC II. In EAE (experimental allergic encephalomyelitis) rat model, Nikodemova et al[22] and others authors[23-24] studied the protective effects of minocycline on spinal cord injury. They found that the down regulation of the MHC II could significantly reduce damage to the spinal cord and clinical symptoms of the EAE, animal model.

It remains unclear how the down regulation of MHC IIplays a neuroprotective role. In a case-control study of 33 cases of PD patients and 34 healthy subjects, Baba et al[25-27] found the presence of immune-related inflammatory response in peripheral blood of PD patients. Compared with the control group, peripheral blood CD4 (+) and CD8 (+) of PD patients decreased. The CD4 + CD25 + regulatory T cells decreased, and the ratio of IFN-γ secreting T cells and IL-4 secreting T cells increased. Suter et al[28] discovered the microglia expression of the MHC II molecules in the EAE animal's CNS and found that the application of the IFN-γ-induced expression of MHCII molecules could aggravate the condition of EAE. The application of IL-4, IL-10, and TGF- to inhibiting MHC II molecule expression helped EAE recovery. Therefore, we could infer that the activated microglia can not only cause oxidative stress and mitochondrial dysfunction by releasing inflammatory cytokines but that in turn lead to degeneration of the DA neuron. They can also express MHCII antigen and present it to the T cells, which starts the T cells to move to the central nervous system. Studies[29,30] have found that T cells can cross the blood-brain barrier and release IFN-γ, which in turn can induce activation of the microglia again, thereby, amplify and prolong the inflammation reaction, resulting in PD progression.

Conclusions

Given that the immune response initiated by the MHC II antigen can advance chronic neurodegeneration in the process of PD, inhibition of the microglial activity and MHC II expression may have important therapeutic significance on the control of PD progression.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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