A rat pup model of cerebral palsy induced by prenatal inflammation and hypoxia

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Abstract
Animal models of cerebral palsy established by simple infection or the hypoxia/ischemia method cannot effectively simulate the brain injury of a premature infant. Healthy 17-day-pregnant Wistar rats were intraperitoneally injected with lipopolysaccharide then subjected to hypoxia. The pups were used for this study at 4 weeks of age. Simultaneously, a hypoxia/ischemia group and a control group were used for comparison. The results of the footprint test, the balance beam test, the water maze test, neuroelectrophysiological examination and neuropathological examination demonstrated that, at 4 weeks after birth, footprint repeat space became larger between the forelimbs and hindlimbs of the rats, the latency period on the balance beam and in the Morris water maze was longer, place navigation and ability were poorer, and the stimulus intensity that induced the maximal wave amplitude of the compound muscle action potential was greater in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. We observed irregular cells around the periventricular area, periventricular leukomalacia and breakage of the nuclear membrane in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. These results indicate that we successfully established a Wistar rat pup model of cerebral palsy by intraperitoneal injection of lipopolysaccharide and hypoxia.

Key Words
neural regeneration; brain injury; hypoxia; lipopolysaccharide; animal models; cerebral palsy; water maze test; neuroelectrophysiology; histopathology; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) This study successfully established a Wistar rat pup model of cerebral palsy by intraperitoneal injection of lipopolysaccharide and hypoxia.
(2) The brain injuries of the rat pup models were evaluated by behavioral, cognitive, neuroelectrophysiological and neuropathological examinations.
INTRODUCTION

Hypoxic-ischemic brain injury causes brain damage in fetuses and newborn infants, and represents a major cause of cerebral palsy, with subsequent lifelong disabilities affecting movement, cognition and behavior. The prevalence of moderate to severe and severe cerebral palsy ranges from 1.5 to 2.5/1 000 live births. In developed countries, advances in perinatal care have enabled nearly 90% of premature newborns to survive. However, many of these infants develop perinatal brain damage, leading to lifelong motor, cognitive and behavioral handicaps.

Because of the unsatisfactory prognosis of cerebral palsy, many animal models have been used in experimental studies of this disease. Commonly used models for perinatal brain damage include postnatal unilateral carotid artery ligation and exposure to hypoxia, postnatal bilateral carotid artery ligation, prenatal inflammation, and prenatal inflammation followed by postnatal unilateral carotid artery ligation and hypoxia. However, these models are not able to accurately imitate perinatal brain damage in humans. A major limitation of most models designed to mimic the perinatal effects of infection or hypoxia-ischemia is the lack of obvious motor deficits reproducing those observed in humans.

Under clinical conditions, intrauterine inflammation is often accompanied by fetal asphyxia. The combination of exposure to inflammation and asphyxia during birth has been linked to a dramatic increase in the risk of spastic cerebral palsy, suggesting that there may be an interaction between the pathophysiological mechanisms induced by inflammation and perinatal hypoxia. Infective-inflammatory mechanisms in combination with subsequent hypoxia could thus underlie developmental brain injury. There are few data on the effect of reproducing this precise order of events for cerebral palsy in human newborns; these events are thought to occur as prenatal intrauterine exposure to infection followed by prenatal hypoxia.

In the present study, we focus our investigations on: (1) designing a relevant new animal model for brain lesions combining the effects of prenatal infection and hypoxia with a demonstrable phenotype, in an attempt to mimic the human conditions of maternal inflammation and hypoxia during a critical period of gestation; and (2) systematically evaluating the behavioral, cognitive, neuroelectrophysiological and neuropathological effects in pups subjected to a combined exposure to prenatal inflammation and hypoxia.

RESULTS

Quantitative analysis of experimental animals

A total of 25 Wistar rats at 7 days of age were randomly assigned to a hypoxia/ischemia group (n = 13, carotid ligation + hypoxia) and a control group (n = 12, normal conditions). Six healthy 17-day-pregnant Wistar rats were administered lipopolysaccharide followed by hypoxia, and 14 immature pups were randomly chosen as the lipopolysaccharide/hypoxia group. All included rats were suitable for final analysis.

Behavioral observations of experimental animals

Footprint analysis showed that the footprint patterns of rats in the control group demonstrated a high degree of coordination of forelimb and hindlimb placement, whereas these parameters were significantly compromised in all injured animals at 4 weeks of age. The footprint repeat distance between the forelimbs and hindlimbs of rats in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was longer than that in the control group at 4 weeks of age (P < 0.05; Figure 1A).

There was no significant difference in performance in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups (P > 0.05). The balance beam test showed that rats in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups displayed hesitation, more exploratory behaviors, stiff joints of the hindlimbs and poor coordination when they were proceeding across the balance beam compared with the control group. The latency period was longer and the number of their hindlimbs slipped was significantly higher in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups than that in the control group (P < 0.05; Figure 1B). However, there were no significant differences in above-mentioned indices between the lipopolysaccharide/hypoxia and hypoxia/ischemia groups (P > 0.05).

Morris water maze test results

The pups in each group showed different behaviors in the Morris water maze test at about 4 weeks of age. During the first training period, they always swam quickly around the wall and tried to escape from the pool. The swimming trajectories did not have a targeted direction. The rats in the control group began to swim in a diagonal line in the maze after a few unsuccessful attempts. They quickly found the platform using a straight line trajectory.
was significantly longer than that of the control group. The escape latency of the hypoxia/ischemia groups. The swimming trajectories the platform in the lipopolysaccharide/hypoxia and noticeably increased, and the pups did not tend to find However, the number of rats swimming around the wall noticeably increased, and the pups did not tend to find the platform in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. The swimming trajectories were mainly random. The escape latency of the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was significantly longer than that of the control group ($P < 0.05$; Figures 2A–C); however, there was no difference between the lipopolysaccharide/hypoxia and hypoxia/ischemia groups ($P > 0.05$).

**Outcomes of the neuroelectrophysiological examination**
Examination of the compound muscle action potential showed that, under the same stimulus intensity, the wave amplitude of the compound muscle action potential in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was lower than that in the control group at 4 weeks of age ($P < 0.05$; Figure 3), and the wave amplitude of the left hindlimb was lower than that of the right hindlimb in the hypoxia/ischemia group ($P < 0.05$). The wave amplitude of the compound muscle action potential was not statistically significant between the lipopolysaccharide/hypoxia and hypoxia/ischemia groups ($P > 0.05$; Figure 3). The latency of the compound muscle action potential was not significantly different

between the three groups ($P > 0.05$; Figure 3).
Outcomes of the neuropathological examination
We did not observe any abnormal findings on serial sections in the control group (Figure 4A). However, white matter lesions extended rostrally from periventricular area to fiber bundles of the striatum in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. The normal architecture in the periventricular area was destroyed, the cells in the periventricular area were irregular, and periventricular leukomalacia was observed (Figures 4B, C). We also observed rarefaction in the subcortical white matter. In the lipopolysaccharide/hypoxia and hypoxia/ischemia groups, we observed small focal cerebral infarctions and small thalamic lesions with some ischemic neurons, but these lesions were only located in the dorsolateral thalamic nucleus adjacent to the internal capsule.

Transmission electron microscopy observations
At 4 weeks of age, we observed regular morphology of neural cells, complete cell membranes and an abundant cytoplasm around the ventricle in the control group. We also observed normal architecture of the mitochondria and endocyttoplasmic reticulum, integrated nuclear membranes and a well-distributed chromoplasm of the cellular nucleus around the ventricle in the control group (Figure 5A).

DISCUSSION
In the present study, we sought to design a new animal model of prenatal brain lesions. We showed that prenatal exposure to lipopolysaccharide followed by hypoxic insult resulted in cerebral white matter changes, which may be clinically relevant to human disease.[20]

Our experimental design was based on the model originally developed by Vanucci's group and subsequently used by other teams[18-20, 22-27]. However, these models have three main disadvantages: (1) they cause extensive and long-term trauma to the common carotid artery; (2) they do not mimic as closely as possible the cause and sequence of prenatal infection and subsequent hypoxia seen in humans, and (3) there is no systematic evaluation of behavioral, cognitive, neuroelectrophysiological and neuropathological effects in pups subjected to a combined exposure to prenatal inflammation and hypoxia. Therefore, our modified protocol entailed hypoxia at the prenatal stage combined with exposure to prenatal infection. Our experimental design aimed to simulate a prenatal infection compounded with a later hypoxic insult by introducing prenatal exposure to lipopolysaccharide at gestational days 16 to 17. Our objective was to closely replicate the cause and sequence of prenatal infection and a subsequent hypoxic insult without causing extensive and long-term trauma to the common carotid artery. This sequence of events is suspected to be one of the main pathophysiological determinants leading to neonatal brain damage and ensuing lifelong disabilities in humans, such as cerebral palsy, and cognitive and behavioral impairment[20].

The cellular/architectural characteristics of perinatal brain lesions are reminiscent of the pattern of selective neuronal necrosis (periventricular leukomalacia) that characterizes some human neonatal encephalopathies occurring in many term and premature newborns[26, 28]. Previous models of periventricular leukomalacia,
including hypoxia/ischemia\cite{10-11} and inflammation involving intrauterine\cite{29-30} or systemic\cite{31-33} lipopolysaccharide injection of pregnant murine animals, have not systematically evaluated the behavioral, cognitive, neuroelectrophysiological and neuropathological effects in pups.

Therefore, this study assessed the neurodevelopmental performance of experimental rats and conducted cognitive and neuroelectrophysiological examinations. In this study, the footprint tests showed that the right limb placement of 28-day-old pups in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was less consistently repeated than that in the control group. The space between the footprints of injured rats was larger and unstable compared with that in controls. On the balance beam, the latency period was longer and the number of the hindlimbs slipped was higher in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups than those in the control group. These results demonstrated that the lipopolysaccharide/hypoxia and the hypoxia/ischemia insults lead to motor impairment of the experimental rats.

To further evaluate the neurodevelopmental performance of the experimental rats, one objective and qualitative evaluation parameter was required. Currently, the most commonly used evaluation parameter is the detection of neuroelectrophysiological function. A neuroelectrophysiological examination (compound muscle action potential) was performed in our study. The results showed that, under the same stimulus intensity, the wave amplitude of the compound muscle action potential of the experimental rats in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was lower than that in the control group in 28-day-old pups. The latency of the compound muscle action potential was not significantly different between the groups. These outcomes were in accordance with those of the neurodevelopmental performance evaluation. These findings further suggested that the lipopolysaccharide/hypoxia and hypoxia/ischemia insults induced abnormal neural development.

It has been reported that damage to cognitive function is one of the main complications of cerebral palsy, and the incidence of mental disability is 60–75%\cite{29}. To verify that our model of neonatal brain injury in the rat reflected these changes in cognition, we performed the Morris water maze test to evaluate cognitive function in this study. The rats in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups tended not to find the platform. The escape latency of the lipopolysaccharide/hypoxia and hypoxia/ischemia groups was significantly longer than that of the control group, but there was no difference between the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. These results are consistent with the outcome of the behavioral tests and the neuroelectrophysiological examination, and they showed that the lipopolysaccharide/hypoxia and hypoxia/ischemia insults induced cognitive abnormality in the experimental rats.

It is critical when evaluating animal models of human disease to assess not only clinically relevant phenotypes (i.e., neonatal developmental behavior, cognition and neuroelectrophysiology), but also morphological changes. We detected visible damage to the white matter of rats in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups (i.e., gross architectural abnormalities, such as cysts or gliosis, characteristic of periventricular leukomalacia). Transmission electron microscopy clearly showed breakdown of the nuclear membrane in some cells of the lipopolysaccharide/hypoxia and hypoxia/ischemia rats. These findings demonstrate that a lipopolysaccharide/hypoxia insult leads to morphological changes (i.e., periventricular leukomalacia), providing further evidence of the validity of our lipopolysaccharide/hypoxia model, together with the alterations in neurodevelopmental performance, cognition and neuroelectrophysiology.

Meanwhile, there were generally identical changes in behavior, cognition, neuroelectrophysiology and morphology in the lipopolysaccharide/hypoxia and hypoxia/ischemia groups. This showed that our lipopolysaccharide hypoxia model was successful and caused alterations in brain function and morphology generally identical to those seen in the hypoxia/ischemia model that is widely accepted by the international academic community.

From the perspective of behavior, cognition, neuroelectrophysiology and morphology, the present experiment suggested that we replicated the potentially deleterious interaction between infectious processes and hypoxic conditions that is believed to play a crucial role in the pathogenesis of human perinatal brain damage. The prenatal exposure to lipopolysaccharide exacerbates the extent of the brain lesions induced by subsequent hypoxia. Further, the sequential order and the timing of the neurobiological effects of the combined infectious and hypoxic insults in our model make it suitable for further studies to investigate the cellular and molecular
basis of many human cerebral lesions inflicted in the neonatal period. These findings lend further support to the concept of a “double hit” on the developing neonatal brain inflicted by compounded infectious and hypoxic insults[34].

In conclusion, this study created an animal model for cerebral palsy that mimics the conditions most likely associated with the occurrence of this disease in humans: a subclinical inflammation and ischemia associated with a phenotype of developmental sequelae and evidence of white matter damage in the brain of the offspring. Additional studies varying the dose or timing of the inflammatory exposure followed by ischemia are needed to create a model with a specific phenotype. Importantly, it is critical when evaluating animal models to assess not only clinically relevant phenotypes, but also biochemical markers for human disease.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
The study was performed at the laboratories of the Xinjiang Center of Disease Prevention and Control from September 2009 to September 2010.

Materials
Six pregnant Wistar rats and 25 Wistar rats, aged 7 days, of both genders were obtained and bred from the animal laboratories of the Xinjiang Center of Disease Prevention and Control (license No. SCXK (Xin) 2003-0002). The mean body weight of the 7-day-old rats on the day of surgery was 13.7 ± 1.5 g. The rats were allowed to acclimate to our animal facility prior to experimental manipulations. They were kept at a constant temperature of 20°C, with a 12-hour day/night cycle, and were maintained on food and drink ad libitum. Animals were handled in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China[35].

Methods
Lipopolysaccharide and hypoxia treatment
The hypoxia/ischemia group (n = 13): there was postnatal exposure to hypoxia (Intensive Care Incubator, Shanghai Pediatric Medical Institute, China) after unilateral ligation of the left common carotid artery[36-37]. The control group (n = 12): animals were treated identically except for the carotid artery ligation and hypoxia exposure. The lipopolysaccharide/hypoxia group: six pregnant Wistar rats (embryonic day 16) were administered lipopolysaccharide by intraperitoneal injection (0.4 mg/kg; Escherichia coli, O55:B5; Sigma, St. Louis, MO, USA) followed by hypoxia, and 14 immature pups were randomly chosen for the subsequent experiments. In each experimental session, the experimental conditions were always simultaneously conducted in the three groups.

Footprint test and balance beam test
At 4 weeks of age, footprint analysis and the balance beam test were performed in the three groups. Footprint analysis was modified from the method of de Medinaceli et al[36-37]. To reduce the risk of selection bias, all of the experimental rats were tested in three groups. Observers blinded to the treatment assessed the footprint test. All timed responses were limited to a maximum of 30 seconds, and a non-responding animal was scored as 30 seconds for continuous variables and with “no” for categorical data. The balance beam test was derived from the method of Carter et al[37-38]. The footprint repeat distances of rats for the forelimbs and hindlimbs were measured in the footprint analysis. The latency period (second) and the number of the hindlimbs slipped were recorded in the balance beam test.

Morris water maze test
At 4 weeks of age, the pups were trained in the Morris water maze on a modified version of the hidden platform task[37-38]. The pups received four trials a day over 5 consecutive days (postnatal days 29, 30, 31, 32 and 33), with every sixth trial being a probe trial. On each training trial, the rats were placed into the water facing the wall, with the start locations varying pseudo-randomly (North, South, East, or West), and permitted to swim until they reached the escape platform. A maximum of 90 seconds was allowed before the rats were assisted to the platform. Once on the platform, rats remained there for 30 seconds before being removed for a 30-second inter-trial interval. The escape latency (second) was recorded as a result of Morris water maze test.

Neuroelectrophysiological examination of experimental animals
To further evaluate functional brain damage in the experimental rats, they were anesthetized intraperitoneally with 10% chloral hydrate (Yangzhou Aoxin, Yangzhou, Jiangsu Province, China) at 4 weeks of age. A neuroelectrophysiological apparatus (Nicolet Viking IV; Nicolet, Madison, WI, USA) was used to detect
the compound muscle action potential of experimental rats. Briefly, two monopolar stimulating electrodes were placed into the subcutaneous tissue of the head. Bipolar recording electrodes were inserted into the ipsilateral quadriceps muscle. Bipolar reference electrodes were inserted into the ipsilateral tibialis anterior muscle, and a grounding electrode was subcutaneously placed into the root segment of the tail. The same stimulus intensity (mA) was used. The wave amplitude (mV) and the latency (ms) of the compound muscle action potential were measured.

Neuropathological examination of experimental animals
At 4 weeks of age, pups were deeply anesthetized with 10% chloral hydrate and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were carefully removed, and fixed in the same fixative for 48 hours. Brain blocks were embedded in paraffin and cut into 6-μm coronal sections. These sections were stained with hematoxylin eosin (Yangzhou Aoxin), and neuropathological changes were investigated (Nikon, Tokyo, Japan). Periventricular leukomalacia was observed.

Transmission electron microscopy observations of experimental animals
The experimental animals were perfused as described above. The brains were carefully removed and placed in 4°C osmic acid for fixation for 2 hours. They were then rinsed with PBS (0.01 M), serially dehydrated with acetone, stained at 4°C in uranyl acetate for 4 hours and embedded in epoxy resin 618 (Tianjin Talents, Tianjin, China). Semi-thin (1 μm) slices of the transverse middle plane were made, stained with toluidine blue, and observed under a light microscope (Olympus, Tokyo, Japan). Ultra-thin slices were then made, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (EM208S, Philip, Amsterdam, Holland). The ultramicro-structures of cells around the ventricle were observed.

Statistical analysis
The data were expressed as mean ± SEM. SPSS for Windows 12.0 statistical software (SPSS, Chicago, IL, USA) was used to process the data. The data were normally distributed, and therefore a two-sample t-test was used to determine the statistical significance of the results. P < 0.05 was considered statistically significant.

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Conflicts of interest: None declared.

Ethical approval: This experiment was approved by the Experimental Animals Ethics Committee of the Fourth People Hospital of Wuxi, and Xinjiang Uygur Autonomous Region People Hospital, China.

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