

Establishment and identification of a hypoxia-ischemia brain damage model in neonatal rats

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Received December 1, 2015; Accepted January 25, 2016

DOI: 10.3892/br.2016.610

Abstract. The present study was designed to set up a reliable model of severe hypoxia-ischemia brain damage (HIBD) in neonatal rats and several methods were used to identify whether the model was successful. A total of 40 healthy 7-day-old Sprague-Dawley rats were randomly divided into 2 groups: The sham-surgery group (n=18) and the HIBD model group (n=22). The HIBD model was produced according to the traditional Rice method. The rats were anesthetized with ethyl ether. The left common carotid artery (CCA) was exposed, ligated and cut. Following this, the rats were exposed to hypoxia in a normobaric chamber filled with 8% oxygen and 92% nitrogen for 2 h. In the sham-surgery group, the left CCA was exposed but was not ligated, cut or exposed to hypoxia. The neurobehavioral changes of the rats were observed in the 24 h after HIBD. The brains were collected after 72 h to observe the pathological morphological changes of the brain tissue. The behavioral ability and neurobehavioral changes were studied in each group. The water maze test was used for evaluating the learning-memory ability when the rats were 28 days old. Compared with the sham-surgery group, all the HIBD model rats had a lag of motor development. The rats had evident changes in anatomy and Nissl staining, and cognitive impairment was shown through the result of the water maze. Therefore, the model of HIBD in neonatal rats is feasible and provides a reliable model for subsequent studies.

Introduction

Hypoxia-ischemic brain damage (HIBD) refers to the fetal/neonatal brain damage caused by partial or complete cerebral hypoxia, cerebral blood flow reduction or

suspension (1,2). HIBD is a major cause of acute mortality and subsequent central nervous system sequelae. These serious complications include learning disability, epilepsy and even cerebral palsy and mental retardation (3,4). Regardless of the improvements in obstetric and neonatal care, HIBD with severe neurological disability remains a clinical issue. Presently, all the clinically available therapies are ineffective at reducing the neurodevelopmental disorders identified in the surviving infants. Recently, therapeutic measures of HIBD have been investigated in clinical as well as animal studies (5). In demonstrating the mechanisms underlying the HI injury in the neonatal brain to devise effective therapeutic strategies, several methods have been used to establish HIBD models, such as unilateral common carotid artery (CCA) ligation with hypoxia (6,7), transient cerebral ischemia/reperfusion (8) and intrauterine hypoxia and ischemia (9,10). Among those methods, the 'Rice method' is the most popular. The present study was designed to set up a reliable model of severe HIBD in neonatal rats and several methods were used to identify whether this was successful. Neonatal hypoxia-ischemia brain injury models were generated by referring to the Rice method (6) and adopting the methods of improved arterial ligation by Nakajima *et al* (11). The influence of the recent and long-term neurological pathology and the behavioral changes were observed in the perinatal hypoxic ischemia of newborn rats, and cognitive functions were studied in each group.

Materials and methods

Animals. A total of 40 healthy 7-day-old Sprague-Dawley rats (weighing 12-20 g) were obtained from the Experimental Animal Centre of Zhejiang University (Zhejiang, China) along with their mother for breastfeeding. They were randomly divided into 2 groups: The sham-surgery group (n=18) and the HIBD model group (n=22). The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang University.

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Key words: hypoxia-ischemic brain damage, model, Morris water maze, neonatal rat

Improvement of the HIBD animal model. The experiment was approved by the University Animal Ethics Committee according to the local government legislation. All the animals

were weighed prior to surgery at room temperature ($20\pm 5^{\circ}\text{C}$). To generate the HIBD model, the Rice method (6) was referred to and the methods of improved arterial ligation were adopted from the study by Nakajima *et al.* (11) (the left CCA was isolated, double-ligated and cut between the ligatures). After anesthesia with 0.2 ml of ether in an airtight box for 1 min, the rat limbs were fixed on the minor board in the supine position. The skin was disinfected with 75% alcohol, and following the anterior portion of the incision, the left CCA underwent sterile separation, and was double-ligated with a 7-0 surgical silk suture and subsequently cut in the middle. The skin incision was subsequently sutured and disinfected again. The pups were permitted to recover for 2 h in a temperature-controlled incubator (32.5°C). The rats were subsequently placed in a 2-litre volume airtight chamber with soda lime at the bottom (to absorb the CO_2 and water vapor), which was maintained at 37°C in a thermostatic water bath. HIBD was generated by exposure to a rate of 1-2 l/min of 8% O_2 +92% N_2 for 2 h. The oxygen concentration was monitored with an oxygen monitor and maintained at $\sim 8\%$ (7-9%). At the end of the 2 h of hypoxia, the pups were returned to their mother for nursing. The surgery lasted <15 min, avoiding an anesthesia time that was too long. The surgery appeared to minimize bleeding and stimulate the surrounding tissues. Bloodstains following the surgery were removed to minimize the refusal of the mother to feed the pups due to any changes in odor. The control group rats only received the ether anesthesia and exposure of the left CCA without ligation, cutting or hypoxia following skin suture. All the pups were returned to their cages following the surgery.

Behavioral observation. A state of consciousness, general behavior and physical activity in the HIBD-model and sham-surgery groups were observed prior to the experiment, following anesthesia and surgery, in the process of anaerobic and at 1, 4, 12, 24 and 72 h after anoxia, respectively.

Collection and processing of brain tissue. The rats were ether anesthetized and their limbs fixed on the minor board in the supine position 7 days after the HI insult. The chest was opened, and 20 ml of a medical sterile syringe needle was carefully inserted into the left ventricle, the right auricle was simultaneously cut and sterilized saline was injected. Until the fluid was clear, 10-20 ml of 4% paraformaldehyde was injected. The brains were removed from the skulls and it was observed by eye that the limbs and liver of the rats were white. The brains were post-fixed in the 4% paraformaldehyde for 24-48 h. Subsequently, the brains were placed in 10, 20 and 30% sucrose solution in turn until they sank.

Preparation of the frozen section. For the slide processing, the slides were soaked in concentrated sulfuric acid for 24 h, and were subsequently rinsed gently with running water, and dried in a temperature-controlled incubator (37°C). Slides were dried briefly to remove excess liquid. Following this, the slides were immersed in a mixed liquor [10 ml mix of 3-aminopropyltriethoxysilane (APES) with 500 ml acetone] for 2 min. Finally, unbound APES was washed with acetone and dried in the 37°C temperature-controlled incubator for use.

The dehydrated brain specimens were embedded in optimal cutting temperature compound. Coronal serials

sections ($20\text{-}\mu\text{m}$) containing the hippocampus were cut by freezing microtome, and washed with phosphate-buffered saline. Tissue sections were mounted on the treated glass slides with a brush. The group and slice number were marked on the frosted side of the slides, and Nissl staining continued following drying.

Nissl staining. Slides were placed in distilled water with gentle agitation for 2 min. The slides were subsequently placed in Nissl staining fluid for 5-10 min. Following this the slides were washed with distilled water twice (for several sec each time). The slides were dehydrated in 95% ethanol for 2 min twice, and subsequently they were cleared in xylene for 5 min twice. Finally, the slides were mounted with neutral gum. The tissue structures and the morphology and arrangement of neurons were observed in the cerebral cortex and hippocampus by light microscopy (CX-21 DIN; Olympus, Tokyo, Japan).

Learning and memory ability assessment. The Morris water maze test was used to examine the long-term learning and memory abilities at the age of 28 days (12,13).

The experimental apparatus consisted of a circular water pool (diameter, 120 cm; height, 60 cm) filled with carbon ink-clouded water and the temperature was controlled at $22\pm 0.5^{\circ}\text{C}$. The maze was divided geographically into four equal quadrants and included release points in each quadrant (marked as N, E, S and W). A plexiglas hidden platform (10×50 cm) situated in the center of the target quadrant was submerged 1-cm below the surface of the water (thereby making it invisible) for testing of spatial learning. A camera was mounted above the center of the maze. The motion of the animals could be recorded and sent to a computer. A tracking system was used to measure the escape latency (EL), traveled path and swimming speed (14,15). Throughout the tests, the investigator was always placed in the same position. Care was taken to maintain the location of the water maze relative to other objects in the laboratory for consistency and so that prominent visual clues would not be disturbed during the test.

Maze adaptation training and swimming ability test. One day before the spatial training in the water maze was initiated, the platform was removed and the rat was placed into the pool from a fixed starting point and was allowed to swim freely for a duration of 2 min. This was the first contact with the water (water adaptation trial) for the animal. The aim was for the rats to adapt to the environment, and to allow a preliminary inspection of the swimming ability of rats, and to eliminate evident abnormalities.

Acquisition trials. Acquisition trials were used to the testing the object recognition memory of the animals. All the rats performed a block of four trials during four daily sessions. The hidden platform position remained stable during the 4 days of the assessment. Each rat was placed in the water gently while facing the wall, at every quadrant each day, and rats were allowed a ≤ 120 sec at each trial to find the hidden platform. The duration to find the platform was called the EL. When the rat succeeded to escape, it was allowed to stay on the platform for an additional 30 sec before starting the next

trial, in order to help the rat to recognize its orientation cues. If the rat failed to find and reach the platform within 120 sec, it was gently guided onto the platform by the investigator with 120 sec scored, and allowed to remain there for 30 sec. Subsequently, the rat was removed from the platform and the next trial was initiated. Following completion of the fourth trial, the rats were gently dried with a towel, kept warm for 1 h, and returned to their home cage. The mean data of EL from four trials each day was taken as the daily average of the aforementioned parameters and used to form cognitive function-time curves.

Retrieval trial. A retrieval trial was used to measure the accurate memory of the platform space position subsequent to the rats learning to search platform, namely memory retention. The platform was removed following the place navigation (day 5 of the water maze). The rat was placed into the pool facing the wall from a optional fixed starting point and was allowed to swim freely for a duration of 120 sec. The traveled path and the number of times crossing the former platform location were recorded as an index of retrieval.

Retention trial. The rats had a rest of 3 days after the retrieval trial. Subsequently, on day 8, the Morris water maze assessment was repeated to obtain retention memory data. The method was similar with the acquisition trials, in order to assess their long-term memory retention.

Statistical analysis. Experimental data are presented as mean \pm standard deviation (SD), using SPSS 20.0 statistical software (IBM Corp., Armonk, NY, USA). Repeated measures analyses of variance [one-way repeated measures analysis of variance (ANOVA)] were used to compare the EL of the experiment. The process of multifactor ANOVA made comparisons between the groups at each time point. Statistical differences between multiple groups were compared using one-way ANOVA and with least SD post hoc tests used for pairwise comparison. Comparison between the two groups used independent sample t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Behavior observation. All the neonatal rats had normal behaviors prior to the experiment. The rats gradually ceased activity and their respiratory rate decreased following ether anesthesia, and became sober quickly following surgery. During hypoxia, all the animals exhibited different levels of behavioral change, including behaving with intermittent agitation, rolling for 5-10 min or even 3 min as the earliest following exposure in the hypoxic device. Following this, those animals exhibited shortness of breath, gradual cyanoderma was observed in their faces, ears, limbs and tails, their muscle twitched, they were restless, exhibited head tremors, stay downed and exhibited disturbances in their consciousness (such as drowsiness, lethargy, confusion, and in certain cases, even coma). Those symptoms were alternating, and fatality occurred in the low oxygen tank in severe cases. Following hypoxia, 90% (18/20) showed turning to the left spontaneously or when the tail was clamped the tail, 85% (17/20) showed

a poor turn over ability, 55% (11/20) exhibited limb shaking and 45% (9/20) showed convulsion. When exposed to normal oxygen, the animals awoke gradually, resumed sucking ability, and increased the activity of the limbs. Activity gradually increased after 1 h, and the majority of the neurobehavioral abnormalities disappeared 4 h later. However, features such as turning to the left spontaneously or when the tail was clamped remained for >1 day (fixed rotation to the left is due to cerebral cortex motor center damage caused by HI, leading to imbalance. Paralysis of the ligated limbs is the typical behavioral changes of the model). Two rats succumbed to hypoxia during the modeling, and the survival rate was 90.91%. In the latter feeding process, HIBD rats showed slow weight gain, and the weight of certain individuals remained stable or even reduced. The opening of the left eye was delayed and eye fissure was smaller compared with the opposite side.

Among the 18 rats in the sham-surgery group, 1 succumbed due to over-medication of the anesthesia and was excluded from the study. There were no significantly different findings in the other animals at any time-point.

Gross anatomy observation. A total of 9 pups from each group were sacrificed and their brains were removed after 7 days of HI exposure. The general features of the brain in the HIBD group were as follows: Atrophy of the left brain, 89% (8/9); softening or liquefaction of the left brain, 67% (6/9); and even cavum brain (the walls were smooth-filled with liquid but there was no colloid filling in the cavity), 33% (3/9). There was no evident abnormality identified in the right brain, brain stem or cerebellum of the HIBD group and the cerebral hemispheres of the sham-surgery group (Fig. 1).

Pathological examination of brain tissue (Nissl staining by light microscopy). Normal hippocampal cells were arranged in neat rows of 3-4 layers, and were large and round. Nissl bodies were deeply stained. There was no evident neuronal damage in the cerebrals of the sham-surgery group and the contralateral cerebral hemisphere of the HIBD group. Morphology and the levels of the cells were clear and complete with clearly visible nucleoli. Nissl bodies were equidistributed around the nucleus, with rules and without cavitation.

The pathological alterations were prominent in the left hemisphere (modeling side) of the HIBD group, characterized by a large number of nuclear pyknosis and nuclear fragmentation. The Nissl body was blurred or disappeared, with vacuolation, a disordered arrangement and formation of a network. The morphology of pyramidal cells of the HIBD group changed significantly compared with the sham-surgery group. Morphological features included a disordered arrangement of the cells, significant loss in volume, light staining of the Nissl body, deformation or disappearance of the nucleus. There was even apparent cell death and cell loss, and the normal pyramidal cells were scattered within the background of the dead cells (Fig. 2).

Swimming ability test. All the rats passed the swimming ability test on day 1, and the swimming ability in all rats was normal.

Acquisition trials. Repeated measure ANOVA results were as follows: The tests of within-subjects effects showed that the

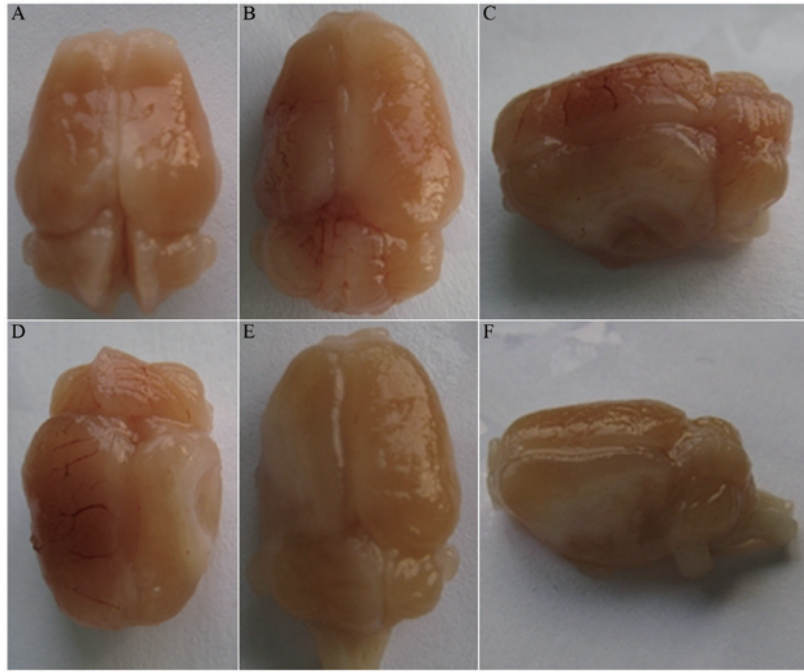


Figure 1. Brain of 7-day old rats. (A) The sham-surgery group: Both cerebral hemispheres had a similar appearance without bleeding or swelling; hypoxia-ischemia brain damage group: (B) Left brain atrophy; (C and D) softening and a slight liquefaction of the left brain; (E and F) ‘Cavum brain’ caused by most of the liquefaction of the left brain.

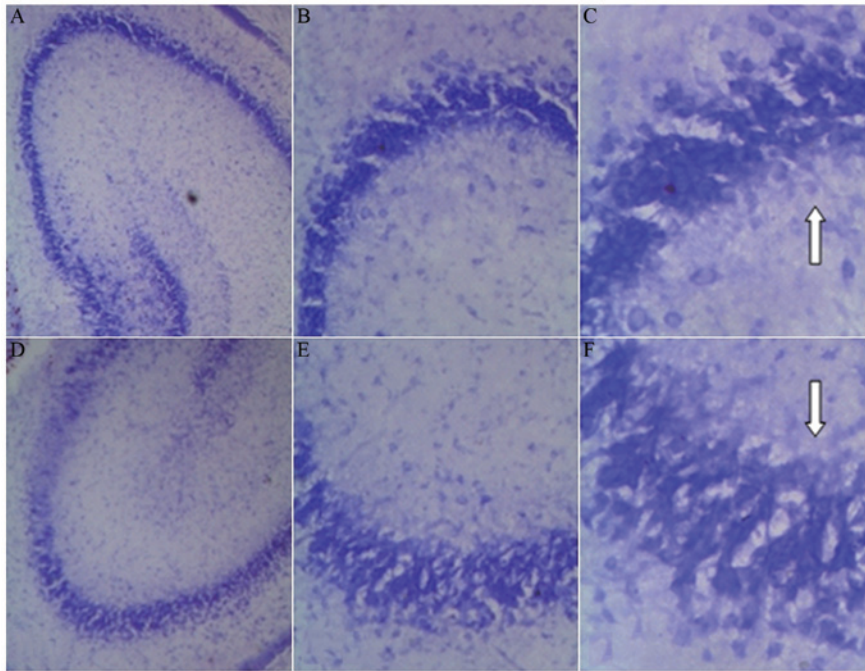


Figure 2. Nissl staining of the hippocampus of 7-day-old rats. hypoxia-ischemia brain damage (A-C) right and (D-F) hippocampus (modeling side).

time factor (day) was statistically significant (HIBD group: $F=0.057, P<0.01$; sham group: $F=3.043, P=0.046$). This denoted that the EL of the rats in each group had a variation trend over the time, but was more evident in the sham group. The day and group interaction (day x group) was also statistically significant ($F=3.410, P=0.025$). This indicated that the role of the time factor varies within the groups. After 4 days of the hidden platform training, the EL curves showed a tendency to decrease in each group of rats; however, the EL values of the sham-surgery

group exhibited a more evident reduction, and showed the ability to maintain a good memory by day 8 (Fig. 3).

Between-group comparison at the same time were as follows: There was no significant difference between the average EL in the groups on day 1 ($t=0.169; P=0.868$). From day 2, the EL value of the HIBD group was higher compared to the sham-surgery group, and the gap increased with time, which indicated that spatial learning and short-term memory ability were damaged in the rats following HI. On day 8, the

Table I. Average escape latency at the different time-points in each group.

Group	Total, n	Escape latency, mean \pm standard deviation				
		Day 1	Day 2	Day 3	Day 4	Day 8
Sham-operation	8	85.25 \pm 22.19	55.19 \pm 33.47	26.64 \pm 28.24	20.93 \pm 22.14	10.93 \pm 5.52
HIBD	10	87.46 \pm 31.02	80.69 \pm 12.86	65.72 \pm 35.76	59.41 \pm 35.59	68.25 \pm 31.14
t-test		0.169	2.227	2.521	2.676	5.111
P-value		0.868	0.041	0.023	0.017	<0.001

HIBD, hypoxia-ischemia brain damage.

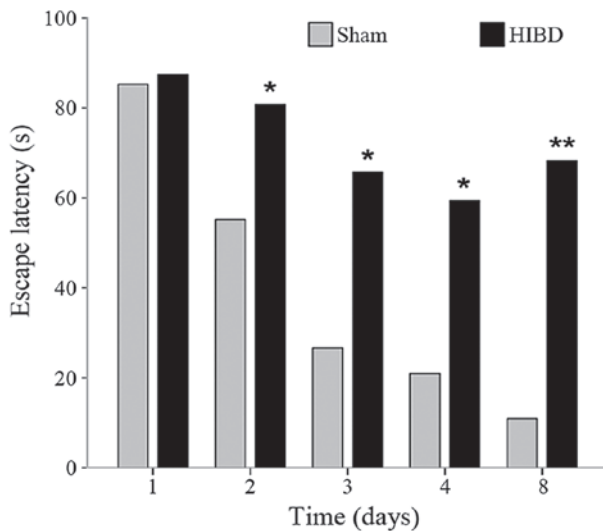


Figure 3. Mean change trend chart of the escape latency repeated measures on the first 4 days and the day 8 for the two group rats. Means statistical differences between the two groups at the same time (* P <0.05; ** P <0.001). HIBD, hypoxia-ischemia brain damage.

difference of the EL values between groups were the most evident ($t=5.111$, $P<0.001$) (Table I).

On day 8, the sham-surgery group rats were able to rapidly identify the hidden platform, and the swimming path was short and targeted. By contrast, the EL time increased rather than decreased for the HIBD group, indicating the loss of spatial memory ability and positioning capabilities following HI injury, and long-term memory defects (Fig. 4).

Spatial probe. On day 5 of the water maze, there were significant differences in the number of times for crossing the former platform location between the two groups ($t=2.756$, $P=0.022$). This indicated that the sham-surgery group rats had an improved spatial memory ability and positioning capabilities compared to that of the HIBD group (Fig. 5).

Discussion

In experimental animals, structural brain damage from HI has been produced in immature rats, rabbits, sheep and monkeys. These models have provided the basis for investigations to clarify not only the physiological and biochemical mechanisms of tissue injury, but also the efficacy of specific management

strategies. Among those models, the fetal and newborn rhesus monkey and immature rat have been studied most extensively due to their similarities to humans in respect to the physiology of reproduction and their neuroanatomy. Newborn rats have more advantages, such as easiness to obtain, short pregnancy and fast breeding, and a nest of rats can produce 8-15 newborn rats. The advantages also include the low cost, and the favorable control of the experimental conditions. The sources of newborn pigs, monkeys and sheep are more difficult, and the mortality and costs are higher. Therefore, newborn rats are the preferred experimental animals for neonatal HIBD research by medical workers from domestic and foreign medical institutions. Studies have shown that the degrees of the rat brains are different at distinct ages. The 7-day postnatal rat was originally chosen for study as at this stage of development its brain is histologically similar to that of a 32- to 34-week gestation human fetus or newborn infant (16,17). The characteristic features (18) include completion of cerebral cortical layering, involution of germinal matrix, the presence of little myelinated white matter, neurotransmitters and immune inflammatory changes. Cerebral blood flow and metabolic correlates have been fully characterized. The immature rat model has been proved and applied for studies of HIBD (19). Consequently, at present, the 7-day postnatal rat was originally utilized to produce the HIBD model, of which the most popular method is based on the Rice method (6). The specific surgical procedure is as follows: The left carotid artery was exposed and permanently ligated under the mixture of halothane anesthesia. The rat pups were returned to their home cage for 4 h followed by exposure to hypoxia (92% N_2 +8% O_2) for 3.5 h by placing them in an airtight chamber partially submersed in a 37°C water bath. The oxygen concentration was maintained at 8 \pm 0.1%. This model of HIBD was successful and is cheap, easily duplicated and has a higher successful rate. Additionally, there were significant differences in terms of long-term behavioral and morphological changes in the HIBD group compared with the control group. This study demonstrated that it is the ideal model for the research of HI brain injury on athletic ability, the brain maturation process and other mechanisms of short- and long-term effects, and is therefore a good model of HIBD. However, there are numerous defects in the conventional Rice method. First, the use of halothane during anesthesia can inhibit the respiration and circulation, while the neonatal rat is small in size and lightweight, and prone to overdose fatalities. Second, only the left CCA was ligated instead of being cut

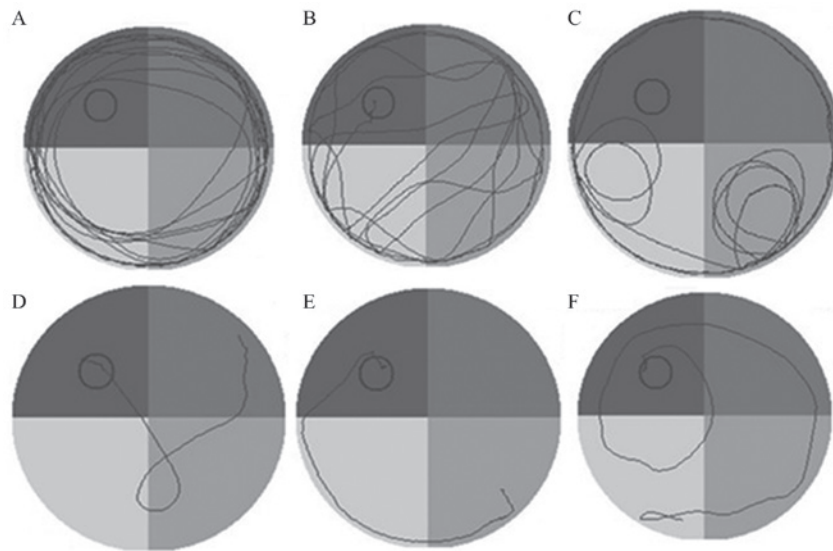


Figure 4. Swimming trajectories of the two groups on day 8. (A-C) Hypoxia-ischemia brain damage group; (D-F) the sham-surgery group.

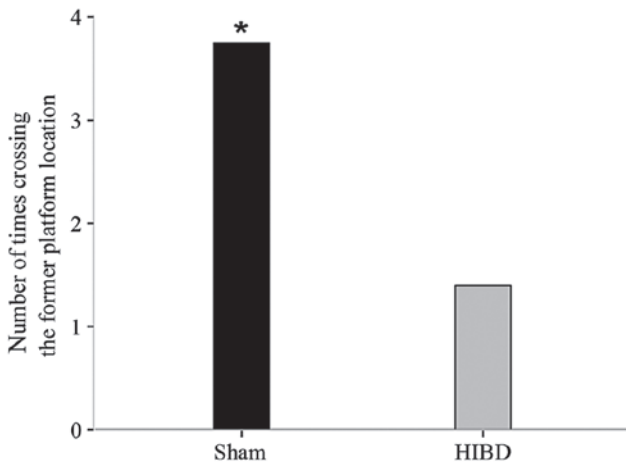


Figure 5. Schematic diagram shows the number of times the rats crossed the platform in the two groups. Means statistical differences between the two groups (*P<0.05). HIBD, hypoxia-ischemia brain damage.

completely. Therefore, the ligated artery may require recanalization and bypass formation with the growth of animals to influence the degree of brain injury.

Therefore, certain minor modifications of the Rice method were performed as described previously. First, anesthesia was improved. In the present study, the anesthesia ether was selected instead of halothane. Although it takes a longer time compared to halothane in the induction of anesthesia, muscle relaxation is good, and relatively safe, avoiding inhibition of the circulation and respiration. Second, arterial ligation was improved. The present study improved artery ligation using the study by Nakajima *et al* (11). The left CCA was isolated, double-ligated and cut between the ligatures, to avoid the formation of blood reperfusion. One key to success lies in the accuracy of unilateral carotid artery ligation. In order to avoid mistaking the neck muscles for vascular, the blood flow must be identified and the arterial pulse can be observed prior to making a permanent ligation and cut. The second was that the reasonable ambient temperature in the airtight chamber should

be maintained at 37±0.5°C. If the temperature is too high or too low it can increase the mortality rate in rats. Additionally, low or moderate hypothermia has a protective effect on HIBD to impact model preparation effect. Therefore, it is crucial to control the temperature with a water bath to ensure the modeling effort.

Indicators of successful HIBD model preparation are as follows: Slow weight gain, neurobehavioral abnormalities, general and microscopic abnormalities of the lesion side, increased brain water content and decreased brain blood volume. The present study identified that all the animals have different levels of behavioral change during hypoxia, mainly as irritability, head tremor, intermittent agitation, rolling, convulsions, sleepiness, skin bruising and urine and feces incontinence. Following hypoxia, the animals awoke gradually, resumed sucking and the activity of their limbs increased. The minority exhibited head tremor, convulsion and could not turnover. Certain rats showed a spontaneously turn round to the left and right limb movement disorder. Mortality in the HIBD group rats was 9.1%, and 90% had abnormal neurological behavior, particularly during the course of hypoxia, which disappeared 4 h after oxygen was supplied. In the latter feeding process, the HIBD group rats showed slow weight gain, and the weight of certain individuals stayed constant or even reduced. The opening of the left eye was delayed and the eye fissure was smaller compared with the contralateral side. It showed that compared with the sham-surgery group, behaviors were significantly different in the HIBD group, which indicates HI induced behavioral changes in rats. On this basis, the general and microscopic pathology of the brain tissues were observed. The pathological features in the HIBD group were as follows: Atrophy, softening and liquefaction of left brain, and even cavum brain in severe cases. Analysis by light microscopy observed cellular swelling and degeneration, nuclear condensation and fragmentation, the Nissl body was blurred or disappeared, cytoplasm was vacuolated and the endoplasmic reticulum was disarranged. Morphological alterations were more prominent in the cerebral cortex and hippocampus neurons. Further validation from morphological

and histological analysis indicated that this method successfully established the model of HIBD in the neonatal rats.

Learning-memory is an advanced feature of the brain. Learning-memory disabilities, cognitive function deficit or even loss are important signs of brain injury causing mental decline, and may also be the core symptoms of HIBD. Manifestations are as follows: Memory and cognitive decline, decrease in spatial orientation and learning ability, and even cerebral palsy. The integrity of the hippocampus is extremely important for normal development of spatial learning and memory. Other regions cannot compensate the hippocampal spatial learning and memory ability following neonatal injury. There were pathological impairments in the hippocampus of HIBD rats. The Morris water maze was also adopted to assess the learning and memory performance. The HIBD rats were significantly worse in the learning/memory acquisition and memory retention ability compared to the sham-surgery group. These findings demonstrate that HI brain injury can cause learning/memory and cognitive function defects.

Therefore, according to the aforementioned method using 7-day-old Sprague-Dawley rats, the HIBD animal model can be established with morphological structure and function injury of the brain. This experiment established the neonatal rat HIBD model that is similar to a clinical environment, and thus can contribute to perform future associated studies.

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