Original Article
Different extent of hypoxic-ischemic brain damage in newborn rats: histopathology, hemodynamic, virtual touch tissue quantification and neurobehavioral observation

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Abstract: Objective: To explore the correlation between pathological and ultrasound changes applying conventional ultrasound, Color Doppler ultrasound andVirtual Touch Tissue Quantification (VTQ) technique in newborn hypoxic-ischemic brain damage (HIBD) rat models. To provide theoretical basis for early diagnosis and treatment of HIBD neonatal. Methods: A total of 90 newborn Wistar rats were divided into ischemia, asphyxia and control group according to different HIBD molding methods. Conventional ultrasound, Color Doppler ultrasound and VTQ were applied on 3 h, 12 h, 24 h, 48 h and 72 h postoperative. After the observation of 72 h, 10 rats in each group were randomly selected for pathological specimens production. The rest rats were raised for 30 days for neuroethology detection. Results: In ischemia group and asphyxia group, there were 4 deaths and 6 deaths in the modeling process; the mortality rate was 13.33% (4/30) and 20.00% (6/30) respectively. For ischemia group, the systoli velocity (Vs), diastolic velocity (Vd) and resistance index (RI) of right middle cerebral artery (MCA) were significantly decreased after operation (P<0.05). For asphyxia group, the Vs and RI of right MCA were significantly decreased after operation (P<0.05), while the Vd of right MCA was significantly increased after operation (P<0.05), which lead to the postoperative RI value in each time point was all significantly lower than that in ischemia group (P<0.05). For ischemia group and asphyxia group, the VTQ results increased significantly postoperative (P<0.05), and compared with ischemia group and control group, the postoperative VTQ value in each time point was all significantly higher in asphyxia group (P<0.05). The neuroethology results were significantly lower in the ischemia group and asphyxia group (P<0.05), and the results in ischemia group were significantly higher than those of asphyxia group (P<0.05). And the results are consistent with the pathological findings. Conclusion: There is a consistent correlation among histopathological changes, hemodynamic changes, VTQ values and neuroethology results in HIBD animal models. As noninvasive quantitative ultrasound elastography methods, Color Doppler ultrasound and VTQ can assess the extent of HIBD damages in newborn rats with specific values. This study provides basic research and theory to early diagnosis and early treatment of neonatal hypoxic-ischemic brain damage.

Keywords: Hypoxic-ischemic brain damage, histopathology, hemodynamic, virtual touch tissue quantification, neuroethology

Introduction

Hypoxic-ischemic brain damage (HIBD) is hypoxia-induced perinatal hypoxic-ischemic brain injury. Some scholars believe HIBD is caused by asphyxia in utero or at birth, which leads to metabolic disorders, cell damages, vascular regulation disorders, and cerebral flow reduction [1, 2]. When the condition is serious,

HIBD can be combined with brain hemorrhage, and finally brain tissue will form liquefaction necrosis and cysts [3].

With the development of imaging technology, imaging detection methods can be applied for dynamic monitoring of HIBD in different stages and different severities. The ultrasound examination has been widely applied in neonatal

monitoring because its non-invasive, inexpensive, and bedside-operated features. Color Doppler flow imaging (CDFI) can dynamically observe the changes of intracranial vessels [4] and cerebral hemodynamics changes of early neonatal HIBD, which is beneficial in HIBD diagnosis and prognosis. Several studies show that newborn asphyxia will perform in cerebral hemodynamics changes, especially those associated with HIBD [5]. The disorder extent is directly related to the degree of HIBD. Asphyxia within 72 h can lead to significant changes in cerebral blood flow, if the blood flow fails to restore normality in 72 h may prompt to serious brain injury, and is likely to cause severe HIBD [6]. The dynamic monitoring of neonatal cerebral blood flow between 48 h to 72 h after asphyxia can provide important information for early clinical diagnosis of HIBD [7]. In terms of the relationship between pathology and hemodynamic changes, it is always a difficult point in research. But whether the findings are consistent remains basic research of animal models to prove.

Nowadays, the technology of HIBD animal model production has been more mature. Seven days newborn rats are equivalent to 32 to 36 weeks premature human beings, ten days newborn rats are equivalent to fully mature neonates [8, 9]. In addition, researches on brain structure of 3 d, 7 d, 10 d and 21 d newborn rats shows that 3 d and 7 d are the peak period of periventricular white matter damages [10, 11]. In rodents, the brain vascular structures are similar to humanbeings; Willis ring is composed of the internal carotid artery and basilar artery trunk [12]. Therefore, seven days newborn rats are more preferred for observation in studies. Recently, there is one study demonstrated that the blood flow velocity can easily be detected by non-invasive vascular ultrasound imaging and pulsed wave Doppler (PW) technique within small animal skulls, and Color Doppler ultrasound can dynamically observe the parameter changes in cerebral blood flow during cerebral ischemia [13]. Therefore, Color Doppler ultrasound can monitor cerebral hemodynamics changes, and accurately assess varying degrees of damages caused by cerebral perfusion. It is a useful and repeatable tool in dynamic monitoring of cerebral blood flow for HIBD patients.

Acoustic radiation force impulse (ARFI) is a new imaging technique on the basis of ultrasound elastography, including Virtual Touch Tissue Imaging (VTI) and Virtual Touch Tissue Quantification (VTQ) technologies. Compare to other elastic strain imaging methods, ARFI technology has significant advantages. VTQ is an absolutely organization quantitative indicator, it can make up the defects of semi-quantitative assessment by conventional elastography [14-16]. Currently, this technology has been widely applied to breast, thyroid, liver, and muscle tissue, et al [17, 18]. At present, there are domestic scholars [19, 20] applied this technology in the diagnosis of neonatal HIBD, studies have shown that children with different levels of HIBD showed various VTQ values, the more severe the disease, the higher the VTQ value. And for mild HIBD in the early stage, it does not show on conventional two-dimensional ultrasound (2DUS), but in VTQ mode, the extent of damages can be determinedspecifically [21, 22].

Therefore, in this study, we establish HIBD models in newborn rats, observe lateral ventricles and brain parenchyma lesions with high-frequency ultrasound, monitor arteriaecerebri media (MCA) blood flow changes through Doppler ultrasound, and detecte VTQ value changes by ARFI technique, then compare the results with pathological changes. To explore the correlation between pathological and ultrasound changes applying conventional ultrasound combined with VTQ technique in newborn HIBD rat models. And we aim to provide theoretical basis for early diagnosis and treatment of neonatal HIBD.

Materials and methods

Experimental animals

Labor Wistar rats were bought from Guangxi Medical University Experimental Animal Center (Certificate of Conformity: SCXK Gui 2009-0002), and housed separately waiting for delivery. The Animal Ethics Committee of Guangxi Medical University approved all animal experiments. "Experimental Animal Regulations" (National Science & Technology Committee of People's Republic of China, Order No. 2, 1988) and "Experimental Animal License Management Approach (Trial)" ([1997] No. 593) were carried out in this study.

Material

Materials and equipment necessary for the production of animal model are plexiglass hypoxia cabin, 100% oxygen, 100% nitrogen and 8%
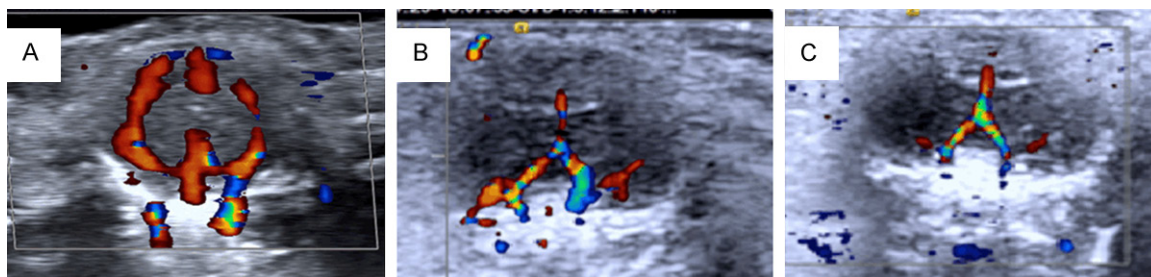
Figure 1. A. CDFI can display Wills ring and bilateral MCA in rat brain. B. 72 h of ischemia group, bilateral Wills ring displays color flow signals of different directions. C. 72 h ofasphyxia group, the blood flow was thinner.

gas mixture of oxygen and nitrogen, simple artificial respirator, ophthalmic surgical instruments, 5.0 silk, medical cotton, sterile gauze, normal saline, 1% amobarbital sodium, 10% neutral formalin, infusion pumps, intravenous catheter, sterile syringes, blood oxygen monitors, blood pressure meter, and recovery medication.

HIBD model making

A total of 90 7-day newborn rats with weight ≥10 g were selected as experimental subjects. Room temperature for feeding and operating the animals were kept at 28~30°C. After intraperitoneal injection anesthesia with 1% amobarbital sodium (100 mg/kg), the newborn rat was fixed on the operating plate on spinal position. Then we made an incision on the middle neck skin, and isolated the right common carotid artery. The newborn rats were randomly divided into three groups according to treatment:

Ischemia group: a total of 30 rats were directly ligatured the right common carotid artery with 5.0 silk, then we sutured the wound.

Asphyxia group: a total of 30 rats were ligatured the right common carotid artery with 5.0 silk. After we sutured the wound and the rats recovered for 1 hour, we put the rats in a 60 cm × 40 cm × 40 cm sized plexiglass hypoxia cabin, the cabin was filled with 8% gas mixture of oxygen and nitrogen, the oxygen concentration was monitored by oxygenanalyzer, hypoxia duration was last for 2 hours.

Control group: a total of 30 rats were sutured the wound directly without ligation of right common carotid artery.

Equipment

Siemens ACUSON S2000 Doppler ultrasound diagnostic equipment was applied. The equipment was built with Acoustic radiation force impulse (ARFI) technology. The probe was 9L4 with a frequency of 7~9 MHz.

Four-color upright fluorescence microscope (model: DX53, Olympus, Japan) and Olympus digital imaging systems (model: DP72) were used for pathological examination.

Cranial ultrasound examination

Three groups were undergone cranial ultrasound examination preoperative and 3 h, 12 h, 24 h, 48 h and 72 h respectively after surgery. First, the rat was fixed to the operating table and ultrasound probe was placed on the midline of the head bone, and weobserved the lateral ventricles and brain parenchyma lesions with 2DUS. Second, we activated CDFI mode, and used PW technology to determine systoli velocity (Vs), diastolic velocity (Vd) and resistance index (RI) of bilateral MCA. PW sample volume was set for 1 mm, the angle between sample line and MCA was set for 0~10°. Finally, we activated VTQ function and sampled the regions of interest (sized 5 mm × 5 mm) inside the brain tissue (we placed a relatively large amount coupling agent on the head of the rats, and floated wrist to avoid the pressure applied to the probe), then measured shear wave velocity therein. All measurements were conducted three times to obtain average values. One professional examiner accomplished all operations.

Pathology specimen processing

After the observation of 72 h, 10 rats in each group were randomly selected for pathological

Table 1. Vs of MCA preoperative and postoperative in three groups

Index	Preoperative		3 h Postoperative		12 h Postoperative		24 h Postoperative		48 h Postoperative		72 h Postoperative	
Vs	L	R	L	R	L	R	L	R	L	R	L	R
Ischemia group	9.96±1.27	10.62±1.28	9.97±1.94	6.80±1.62▲☆	10.55±2.30▲	7.28±1.73▲☆	9.83±2.01▲	6.92±1.68▲☆	11.75±2.13☆◆	7.29±1.51▲☆◆	11.74±1.98☆	6.15±1.51▲☆
Asphyxia group	10.16±1.33	10.77±1.30	10.03±2.08	7.01±1.55▲	10.61±2.34▲★	5.97±0.60▲☆	11.22±2.06★☆	6.32±1.24▲☆	12.69±1.43▲☆◆	6.50±0.76▲☆	11.37±1.47	6.82±0.82▲☆
Control group	10.73±1.46	10.23±0.83	10.51±1.29	10.63±1.22	11.63±1.68	10.70±1.83	11.75±1.72★	10.83±1.63★	11.06±1.32	10.90±1.02★△	10.88±2.03	11.47±1.38△★

Note: ▲: compare with control group, P<0.05; ★: compare with asphyxia group, P<0.05; ☆: compare with preoperative results, P<0.05; ◆: compare with postoperative previous time point, P<0.05.

Table 2. Vd of MCA preoperative and postoperative in three groups

Index	Preoperative		3 h Postoperative		12 h Postoperative		24 h Postoperative		48 h Postoperative		72 h Postoperative	
Vd	L	R	L	R	L	R	L	R	L	R	L	R
Ischemia group	3.12±0.55	3.17±0.69	2.93±0.57	3.00±0.46☆	3.46±0.89	3.44±0.65◆	3.23±0.74	3.28±0.55	3.65±0.96▲☆◆	3.42±0.85▲	3.68±1.14☆	3.23±1.01
Asphyxia group	3.09±0.61	3.2±0.77	3.42±1.08	4.20±0.97▲	3.43±0.75	3.31±0.56◆	3.53±1.10	3.59±0.96☆	3.92±0.94▲☆	3.72±0.65▲△	3.44±0.76	3.62±0.73☆
Control group	3.28±0.62	3.19±0.69	3.06±0.57	2.95±0.60	3.65±0.78	3.19±0.83	3.65±0.69	3.05±0.72	3.05±0.52★	3.58±0.49★	3.58±0.88	3.58±0.80

Note: ▲: compare with control group, P<0.05; ★: compare with asphyxia group, P<0.05; ☆: compare with preoperative results, P<0.05; ◆: compare with postoperative previous time point, P<0.05.

Table 3. RI of MCA preoperative and postoperative in three groups

Index	Preoperative		3 h Postoperative		12 h Postoperative		24 h Postoperative		48 h Postoperative		72 h Postoperative	
RI	L	R	L	R	L	R	L	R	L	R	L	R
Ischemia group	0.69±0.03	0.69±0.05	0.70±0.05☆	0.55±0.07▲☆	0.67±0.06☆◆	0.52±0.07▲☆◆	0.67±0.05☆	0.52±0.06▲☆	0.69±0.06▲☆	0.53±0.06▲☆	0.69±0.06☆◆	0.48±0.10▲☆◆
Asphyxia group	0.70±0.04	0.71±0.03	0.66±0.05▲★	0.40±0.05▲★☆	0.67±0.05	0.45±0.64▲★☆◆	0.69±0.06	0.44±0.08▲★☆	0.70±0.06	0.42±0.08▲★☆	0.70±0.06	0.47±0.06▲☆◆
Control group	0.69±0.04	0.68±0.05	0.71±0.04	0.72±0.05	0.69±0.03	0.70±0.04	0.69±0.03	0.70±0.04	0.72±0.06	0.72±0.05	0.67±0.03	0.69±0.04★△

Note: ▲: compare with control group, P<0.05; ★: compare with asphyxia group, P<0.05; ☆: compare with preoperative results, P<0.05; ◆: compare with postoperative previous time point, P<0.05.
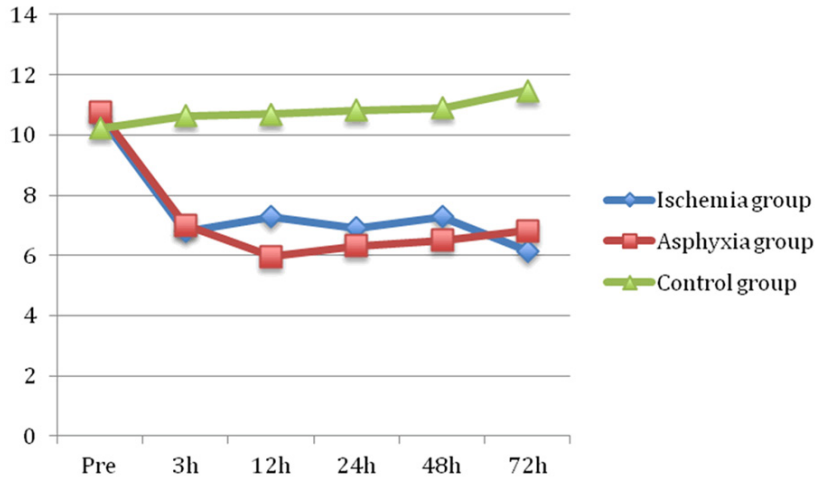
Figure 2. Right Vs of MCA preoperative and postoperative in three groups.
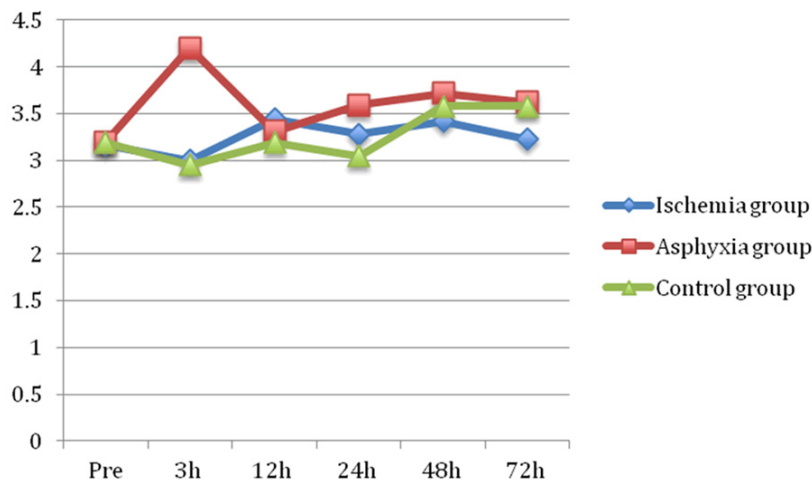

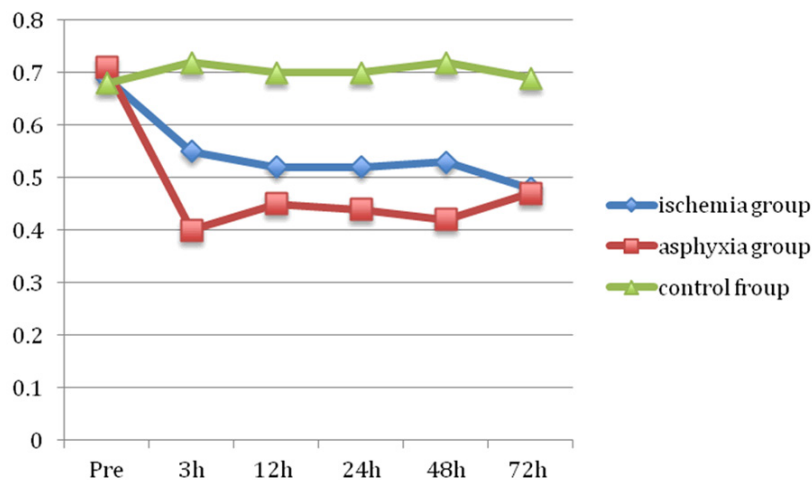**Figure 3.** Right Vd of MCA preoperative and postoperative in three groups.

Figure 4. Right RI of MCA preoperative and postoperative in three groups.

specimens production. The steps of sample procession including perfusion fixation, brain tissue fixation, paraffin blocks making, stained sections, and sealing, et al.

We observed the HE staining slices under microscope to see the edema range, nerve cells, glial cell state and hemorrhage location and extent of the bilateral brain tissue.

Neuroethology detection method

The rest rats were raised for 30 days for neuroethology detection by the following methods:

Open-field test: The rat was placed in the middle of the grid in an open container, and the activities of the rat were observed for 30 s. The device was a 36 cm × 36 cm × 36 cm topless square box, the bottom was lined into 9 aliquot cells by ink. The rat was place in the middle cell, and the activities of the rat were observed for 30 s. We scored 1 point when 1/2 or more of the rat body got into the adjacent cell. And 1 point was scored when the rat stood on hind legs. Both parts were adding up for total score.

Suspension test: The rats were made to catch a glass rod (diameter of 0.5 cm) with the forelimbs 45 cm high from the desktop, the fall off time of the rats were record. Grading: 1 point for <10 s, 2 points for 10-30 s, 3 points for

Table 4. VTQ value preoperative and postoperative of the three groups

VTQ	Preoperative	3 h Postoperative	12 h Postoperative	24 h Postoperative	48 h Postoperative	72 h Postoperative
Ischemia group	0.86±0.22	1.34±0.12▲,☆	1.39±0.12▲,☆	1.36±0.14▲,☆	1.41±0.12▲,☆,◆	1.38±0.13▲,☆
Asphyxia group	0.83±0.20	1.61±0.08▲,★,☆	1.54±0.20▲,★,☆	1.65±0.08▲,★,☆,◆	1.76±0.15▲,★,☆,◆	1.64±0.18▲,★,☆,◆
Control group	0.82±0.21	0.81±0.18	0.78±0.17	0.83±0.21	0.71±0.21	0.80±0.20

Note: ▲: compare with control group, P<0.05; ★: compare with asphyxia group, P<0.05; ☆: compare with preoperative results, P<0.05; ◆: compare with postoperative previous time point, P<0.05.
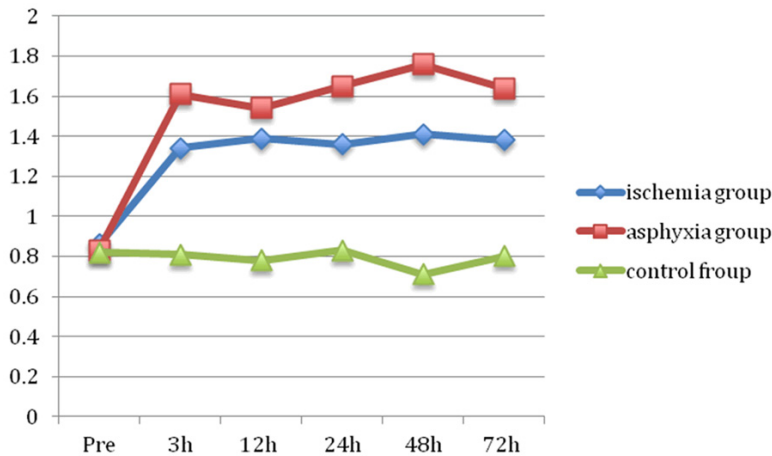

Figure 5. VTQ values preoperative and postoperative in three groups.

30 s-2 min, 4 points for 2-5 min, 5 points for >5 min.

Slope test: The rat was put on a 45-degree slope head down. The time for the head of the rat turned upwards into >135 degrees was recorded. The time was recorded in seconds for the unit.

Capture experiment: We lightly scratched the rats with gloves they never had any contact with. Grading: 0 point for rats being easily grabbed; 1 point for rats screaming or avoiding; 2 points for rats screaming and avoiding; 3 points for rats escaping; 4 points for rats escaping and screaming; 5 points for rats biting or attempting to bite gloves; 6 points for rats attacking actively.

Statistical analysis

Count data are expressed as means ± standard deviation. For measurement data in normal distribution, completely randomized design analysis of variance (ANOVA) was applied for comparison. For not normally distributed data, rank sum test was applied for comparison. A value of P<0.05 was considered statistically significant.

Results

Results of HIBD animal model producing

In ischemia group, there were 4 deaths in the modeling process, the mortality rate was 13.33% (4/30), in which 2 rats died within 12 h after operation, 2 rats died within 12 h to 24 h after the operation, modeling success rate was 86.67% (26/30); in asphyxia group, there were 6 deaths in the modeling process, the mortality rate was 20.00% (6/30), in which 3 died during suffocation, 2 died within 12 h to 24 h after suffocation, 1 died within 48 h to 72 h after suffocation, modeling success rate was 80.00% (24/30). In control group, modeling success rate was 100.00% (30/30).

Ultrasound results

2DUS results: There were no significant differences in echo intensity among ischemia group, asphyxia group and control group.

CDFI results: CDFI can display Wills ring and bilateral MCA in rat brain (**Figure 1A**). Since we ligatured the right common carotid artery of the rats, it was compensated by Wills ring, the Wills ring on the ligation side showedblue signal back to probe (**Figure 1B**). In asphyxia group after 72 h, the blood flow in MCA was significantly reduced, and therefore, the blood flow was thinner (**Figure 1C**).

PW results: The Vs, Vdand RI value before and after surgery of three groups are detailed in **Tables 1-3**. For control group, no significant difference was seen in VsVs, Vd and RI results pre and postoperative (P>0.05).

The Vs, Vd and RI value of the right MCA (ligation side) of the two experimental groups are shown as following:
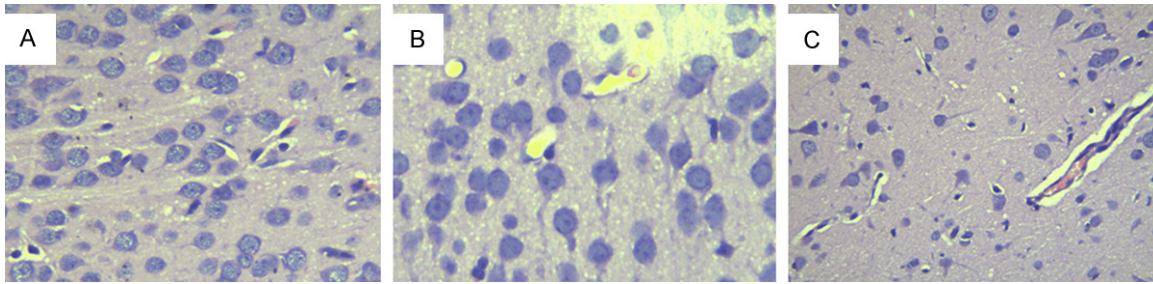
Figure 6. HE staining results: A. For control group, neuronal cell nucleuses was visible with clear nucleoli, abundant cytoplasm and Nissl's Body. B. For ischemia group, cell nucleuses were visible with occasional karyopyknosis. The perivascular cellswere shown with edema. C. For asphyxia group, the number of nerve cells was reduce, accumulation of a large number of red blood cells were visible in small blood vessels.

Table 5. Theneuroethology results of three groups

	Ischemia group	Asphyxia group	Control group	X^2
Open-field test	44.57▲	18.07▲,★	73.87	69.62
Suspension test	50.00▲	19.55▲,★	66.95	53.71
Slope test	37.93▲	74.33▲,★	24.23	61.12
Capture experiment	49.13	15.87	71.5	71.25

Note: ▲: compare with control group, $P<0.05$; ★: compare with asphyxia group, $P<0.05$.

For ischemia group, the Vs of right MCA was significantly decreased after operation, from 10.62±1.28 (preoperative) to6.80±1.62 (3 h postoperative) ($P<0.05$), the changes of 12 h and 24 h were not significant, and after 48 h, Vs increased slightly. The Vd of right MCA was significantly decreased after operation, from 3.17±0.6 (preoperative) to 3.00±0.46 (3 h postoperative) ($P<0.05$), then increased to 3.44±0.65 (12 h postoperative), the changes after 12 h were not significant. The RI of right MCA was kept decreasing significantly after operation, from 0.69±0.05 (preoperative) to 0.52±0.07 (12 h postoperative) ($P<0.05$), the changes of 24 h and 48 h were not significant, and in 72 h, RI decreased to 0.48±0.10 ($P<0.05$) (**Tables 1**-**3** and **Figures 2**-**4**).

For asphyxia group, the Vs of right MCA was significantly decreased after operation, from 10.77±1.30 (preoperative) to 5.97±0.60 (12 h postoperative) ($P<0.05$), the changes after 12 h were not significant. The Vd of right MCA was significantly increased after operation, from 3.2±0.77 (preoperative) to 4.2±0.97 (3 h postoperative) ($P<0.05$), it dropped back to 3.31 ±0.75 ($P<0.05$) in 12 h postoperative, the changes after 12 h were not significant. The RI of right MCA was kept decreasing significantly after operation ($P<0.05$), from 0.71±0.03 (pre-

operative) to 0.45±0.64 (12 h postoperative), the changes after 12 h were not significant, but compared with ischemia group, the postoperative RI value in each time point was all significantly lower ($P<0.05$) (**Tables 1**-**3** and **Figures 2**-**4**).

VTQ results: The VTQ results before and after surgery of three groups are detailed in **Table 4** and **Figure 5**.

For control group, there was no significant difference in the VTQ resultspreoperative and postoperative ($P>0.05$).

For ischemia group, the VTQ results increased significantly postoperative, from 0.86±0.22 (preoperative) to 1.34±0.12 (3 h postoperative) ($P<0.05$), the changes after 3 h were not significant (**Table 4**; **Figure 5**).

For asphyxia group, the VTQ results were significantly increased after operation ($P<0.05$), from 0.83±0.20 (preoperative) to 1.61±0.08 (3 h postoperative) ($P<0.05$), and the value kept raising 12 h to 48 h, in 72 h, the value dropped slightly, but compared with ischemia group and control group, the postoperative VTQ value in each time point was all significantly higher ($P<0.05$) (**Table 4**; **Figure 5**).

Pathological changes of brain tissue for newborn rats

HE staining results: For control group, hierarchical structure of the brain tissue was clear; the boundaries of cortex and medulla were distinct; neuronal cell nucleuses were visible with clear

nucleoli, abundant cytoplasm and Nissl's Body (**Figure 6A**). For ischemia group, hierarchical structure of the brain tissue was still clear; the boundaries of cortex and medulla could be identified; neuronal cell nucleuses were visible with occasional karyopyknosis. The perivascular cells were shown with edema (**Figure 6B**). For asphyxia group, hierarchical structure of the brain tissue was less distinct; the boundaries of cortex and medulla were unclear; neuronal cell nucleuses were visible with occasional karyopyknosis, unclear nucleuses and less Nissl's Body; clear nucleoli, abundant cytoplasm and Nissl's Body; accumulation of a large number of red blood cells were visible in small blood vessels (**Figure 6C**).

Neuroethology results

The neuroethology results of three groups are detailed in **Table 5**.

Compared with control group, the neuroethology results were significantly lower in the ischemia group and asphyxia group (P<0.05), which suggesting that HIBD rats would occur neurobehavioral abnormalities, mainly in random movement limitation and poor emotional behavior. The neuroethology results of ischemia group were significantly higher than those of asphyxia group (P<0.05), which prompting that ischemia in combination with asphyxia would have a greater impact in HIBD rats' neurobehavioral findings.

Discussion

Neonatal asphyxia is common in critically ill newborn patients, and it is an important pathogenic factor of HIBD. HIBD is an important cause of neonatal death and legacy of neurological sequela, such as cerebral palsy, epilepsy, mental retardation, visual and hearing impairments, it will seriously affect the quality of the ill children's life [23-25]. Therefore, early diagnosis, active prevention and appropriate treatment in the neonatal period are the key to reducing the incidence of children with disabilities. The basic research of HIBD will allow us a clearer understanding of the pathogenesis of the disease, and will provide a theoretical basis for early clinical diagnosis.

Animal Model is an important research tool to understand the pathogenesis of HIBD andto assess the efficacy of treatment. In 1994, Yager et al [26] made statistics in more than 200 kinds of HIBD animal model experiments, and they found that the proportion of neonatal rats was about 29%. In which, embryonic or immature rats were the most widely studied, because these two newborn rats are similar to human in neuroanatomy and physiology. Therefore, domestic and foreign medical workers preferred to use newborn rats as HIBD experimental animals. Seven days newborn rats are equivalent to 32 to 36 weeks premature, their hemodynamics, protein metabolism, energy metabolism, neurotransmitter and immune inflammatory changes can be well characterized [12, 27]. Therefore, the researchers mostly use 7 d newborn rats as HIBD animal model.

However, in the production process of HIBD animal model, we still encountered with some difficulties. Experimental results showed that in ischemia group, there were 4 deaths in the modeling process, the mortality rate was 13.33% (4/30), modeling success rate was 86.67% (26/30); in asphyxia group, were 6 deaths in the modeling process, the mortality rate was 20.00% (6/30), modeling success rate was 80.00% (24/30). So in order to ensure the success rate of the experimental model making, we should pay attention to several issues. In the modeling process, we should pay attention to the standardization of the operation; the action should be gentle in the separation of the common carotid artery to avoid ligation of the nerves, especially the recurrent laryngeal nerve, which may affect the survival of the animals. And we also found that special attention should be paid in environmental temperature of the hypoxic cabin, if the temperature is too low, brain damages may become mild, if the temperature is too high, the mortality of the animal models may increase. Therefore, in the process of carotid artery ligation and hypoxia, the environmental temperature should be kept at 36 to 37°C, which is an important factor in the success of HIBD model making [28]. In addition, since the neonatal rats are light in weight, small in size, and poor in tolerance, so in order to reduce mortality, the rats should have a 1 h recovery after the ligation operation.

In this study, HE staining results showed that for ischemia group, hierarchical structure of the brain tissue was still clear; the boundaries of cortex and medulla could be identified; neuronal cell nucleuses were visible with occasional

karyopyknosis. The perivascular cells were shown with edema. Because we only ligated unilateral carotid artery, on histology, nerve cells were only mildly damaged. And the perivascular cells were only shown with edema. The reason is the particularity of the vascular structure of newborn rats. Cerebral blood supply of rats is similar to humans [12], internal carotid and vertebral arteries form willis ring on the bottom of the brain, which can communicate and compensate on both side, carotid artery ligation on one side alone cannot cause severe brain damages [29]. Previous animal studies also confirmed that if the newborn pig only underwent unilateral carotid artery ligation, without hypoxia process, there were no changes in MRI signals and values [30, 31]. Therefore, the ischemia group in this study was equivalent to mild HIBD patients with no obvious clinical symptoms. It also indicated that HIBD is the result of the joint action of both ischemia and hypoxia. And for asphyxia group, we not only ligated the unilateral carotid artery, and also put the rats in the hypoxia cabin, so that the damages were more severe. In HE staining of asphyxia group, hierarchical structure of the brain tissue was less distinct; the boundaries of cortex and medulla were unclear; neuronal cell nucleuses were visible with occasional karyopyknosis, unclear nucleuses and less Nissl's Body; clear nucleoli, abundant cytoplasm and Nissl's Body; accumulation of a large number of red blood cells were visible in small blood vessels.

The combination of newborn rat models and ultrasound examination may provide basic research data of neonatal HIBD brain damage. Applying 2DUS in HIBD judgment mainly rely on the echo strength of brain tissue, the echo strength judgment is based on the reference of the echo of choroid plexus, and each doctor will have different determination on the echo changes of localized or diffuse brain echogenic. HIBD diagnosis by 2DUS has greater subjectivity; the credibility in clinical applications is not very high. So a more intuitive outcome measurement should be established.

Lacking of oxygen will lead to the reduction of cerebral blood flow and necrosis in part of the brain tissue. Newborns, especially prematures, the autoregulation of cerebral blood flow was inadequate or absent, and was susceptible to a variety of factors. When autoregulation was absent, the fluctuation of blood pressure would form passive-dependent blood pressure, and the cerebral hemodynamics changes are closely related to the occurrence and severity of HIBD [32]. Therefore, the dynamic monitoring of cerebral blood flow changes in neonatal HIBD patients will provide information in early diagnosis and appropriate treatment. In this study, we found that for ischemia group, the Vs, Vd and RI of right MCA were significantly decreased after operation ($P<0.05$). For asphyxia group, the Vs and RI of right MCA were significantly decreased after operation ($P<0.05$), while the Vd of right MCA was significantly increased after operation ($P<0.05$), which lead to the postoperative RI value in each time point was all significantly lower than that in ischemia group ($P<0.05$). The results are consistent with the pathological findings. Color Doppler ultrasound can monitor cerebral hemodynamics changes with specific values, may give early detection of varying degrees of brain damage. It is a useful and repeatable tool in dynamic monitoring of cerebral blood flow for HIBD patients.

The latest means of elastography can explore the tissue hardness sensitively, and can provide information about the illness extent of the tissue from the other hand. In this study, the velocity values can be sensitively detected under ARFI mode. Ultrasound elastography concept was firstly proposed by the Ophi et al in 1991 [33]. In recent years, ultrasound elastography technology has been rapid developed. This technology can make up the defects of semi-quantitative assessment by conventional elastography, and it has advances in tissue hardness evaluation [15]. In this study, we found that there was no significant difference in the VTQ results in control group preoperative and postoperative ($P>0.05$). For ischemia group and asphyxia group, the VTQ results increased significantly postoperative ($P<0.05$), and compared with ischemia group and control group, the postoperative VTQ value in each time point was all significantly higher in asphyxia group ($P<0.05$). This shows that with the aggravation of hypoxic-ischemic, the damages of brain tissue increased gradually, and this would decrease its elasticity. And this is consistent with the pathological findings.

It was reported that neurodevelopment of 30 days newborn rats were similar as 2-3 years human beings, it is equivalent to typical clinical symptom stage of cerebral palsy [34]. So to

choose the 30 days newborn rats has clinically significant in neuroethology detection. In this study, we found that the neuroethology results were significantly lower in the ischemia group and asphyxia group (P<0.05), and suggesting that the neurobehavioral abnormalities of HIBD rats mainly occured in random movement limitation and poor emotional behavior. The neuroethology results of ischemia group were significantly higher than those of asphyxia group (P<0.05), which prompting that ischemia in combination with asphyxia would have a greater impact in HIBD rats' neurobehavioral findings. And the results are consistent with the pathological findings.

Conclusion

In this study, we find that there is a consistent correlation among histopathological changes, hemodynamic changes, VTQ values and neuroethology results in HIBD animal models. As noninvasive quantitative ultrasound elastography methods, Color Doppler ultrasound and VTQ can assess the extent of HIBD damages in newborn rats with specific values. This study provides basic research and theory to early diagnosis and early treatment of neonatal hypoxic-ischemic brain damage.

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Disclosure of conflict of interest

None.

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References

[1] Li ZH and Chen C. [Analysis of 58 neonatal cases with cerebral infarction]. Zhonghua Er Ke Za Zhi 2013; 51: 16-20.

[2] Spasojevic SD, Stojanovic VD, Barisic NA, Doronjski AR, Zikic DR and Babovic SM. Neuroprotective effects of hypothermia and erythropoietin after perinatal asphyxia in newborn rats. J Matern Fetal Neonatal Med 2013; 26: 1506-1509.

[3] Zhan ZH, Ou LP and Chen ZB. CT diagnosis and related factors analysis of hypoxic-ischemic encephalopathy with intracranial hemorrhage. China Foreign Medical Treatment 2011; 30: 66.

[4] Van Bel F, Schipper J, Guit GL and Visser MO. The contribution of colour Doppler flow imaging to the study of cerebral haemodynamics in the neonate. Neuroradiology 1993; 35: 300-306.

[5] Lee JY, Kim HS, Jung E, Kim ES, Shim GH, Lee HJ, Lee JA, Choi CW, Kim EK, Kim BI and Choi JH. Risk factors for periventricular-intraventricular hemorrhage in premature infants. J Korean Med Sci 2010; 25: 418-424.

[6] Victor S, Appleton RE, Beirne M, Marson AG and Weindling AM. Effect of carbon dioxide on background cerebral electrical activity and fractional oxygen extraction in very low birth weight infants just after birth. Pediatr Res 2005; 58: 579-585.

[7] Lee JA, Kim BI, Jo CH, Choi CW, Kim EK, Kim HS, Yoon KS and Choi JH. Mesenchymal stem-cell transplantation for hypoxic-ischemic brain injury in neonatal rat model. Pediatr Res 2010; 67: 42-46.

[8] Hagberg H, Peebles D and Mallard C. Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. Ment Retard Dev Disabil Res Rev 2002; 8: 30-38.

[9] Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H and Blomgren K. The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. Cell Death Differ 2005; 12: 162-176.

[10] Tai WC, Burke KA, Dominguez JF, Gundamraj L and Turman JE Jr. Growth deficits in a postnatal day 3 rat model of hypoxic-ischemic brain injury. Behav Brain Res 2009; 202: 40-49.

[11] Stadlin A, James A, Fiscus R, Wong YF, Rogers M and Haines C. Development of a postnatal 3-day-old rat model of mild hypoxic-ischemic brain injury. Brain Res 2003; 993: 101-110.

[12] Bonnin P, Debbabi H, Mariani J, Charriaut-Marlangue C and Renolleau S. Ultrasonic assessment of cerebral blood flow changes during ischemia-reperfusion in 7-day-old rats. Ultrasound Med Biol 2008; 34: 913-922.

[13] Fischer JS, Edelman SV and Schwartz SL. United States patient preference and usability for the new disposable insulin device Solostar versus other disposable pens. J Diabetes Sci Technol 2008; 2: 1157-1160.

[14] Nightingale KR, Palmeri ML, Nightingale RW and Trahey GE. On the feasibility of remote pal-

pation using acoustic radiation force. J Acoust Soc Am 2001; 110: 625-634.

[15] Rotemberg V, Palmeri M, Rosenzweig S, Grant S, Macleod D and Nightingale K. Acoustic Radiation Force Impulse (ARFI) imaging-based needle visualization. Ultrason Imaging 2011; 33: 1-16.

[16] Zhai L, Palmeri ML, Bouchard RR, Nightingale RW and Nightingale KR. An integrated indenter-ARFI imaging system for tissue stiffness quantification. Ultrason Imaging 2008; 30: 95-111.

[17] Mansour N, Stock KF, Chaker A, Bas M and Knopf A. Evaluation of parotid gland lesions with standard ultrasound, color duplex sonography, sonoelastography, and acoustic radiation force impulse imaging - a pilot study. Ultraschall Med 2012; 33: 283-288.

[18] Sporea I, Sirli R, Bota S, Vlad M, Popescu A and Zosin I. ARFI elastography for the evaluation of diffuse thyroid gland pathology: Preliminary results. World J Radiol 2012; 4: 174-178.

[19] Zhao XP and Cao CN. Advances of acoustic radiation force impulse imaging in ultrasound diagnosis. China Medical Engineering 2012; 20: 185-186.

[20] Xia SW, Chen XL, Fu CH and Huang JH. Ultrasound acoustic radiation force impulse imaging evaluation of early brain injury in neonatal hypoxic-ischemic encephalopathy. Chinese Journal of Neonatology 2012; 27: 10-13.

[21] Lu D and Zhang BH. The application of ARFI in hypoxic-ischemic encephalopathy. Chinese Journal of Microcirculation 2012; 22: 34-36.

[22] Chen XL, Xia SW, Song F and Huang JH. Primary study on brain injury in neonate hypoxic-ischemic encephalopathy by ultrasound acoustic radiation force impulse imaging. Chinese Journal of Medical Ultrasound (Electronic Version) 2009; 6: 1016-1023.

[23] Mu DZ and Xia B. Current research in diagnosis and treatment of neonatal hypoxic-ischemic encephalopathy. Chinese Journal of Contemporary Pediatrics 2007; 9: 289-292.

[24] Shao XM. Advances and associated problems in diagnosis and treatment of neonatal hypoxic-ischemic encephalopathy. Journal of Clinical Pediatrics 2007; 25: 179-182.

[25] Zhang YL. Ganglioside, hyperbaric oxygen, curative effect observation of cryogenic treatment of neonatal hypoxic ischemic encephalopathy. Journal of Clinical and Experimental Medicine 2014; 0: 1265-1268.

[26] Yager J, Greden J, Abrams M and Riba M. The Institute of Medicine's report on Research Training in Psychiatry Residency: Strategies for Reform--background, results, and follow up. Acad Psychiatry 2004; 28: 267-274.

[27] Hagberg H, Ichord R, Palmer C, Yager JY and Vannucci SJ. Animal models of developmental brain injury: relevance to human disease. A summary of the panel discussion from the Third Hershey Conference on Developmental Cerebral Blood Flow and Metabolism. Dev Neurosci 2002; 24: 364-366.

[28] Kong WG, Wu XM, Zhang KN, Huang G, Zhang HL and Liu SM. Establishment of Local Cerebral Ischemia/Reperfusion Model in Rats and Experience. Practical Clinical Medicine 2010; 11: 1-15.

[29] Ma J, Yang M, Yang JH and Jiang F. A model of hypoxic-ischemic encephalopathy in neonatal mice. Chinese Journal of Neurosurgery 2007; 23: 713-715.

[30] Huang NC, Yongbi MN and Helpern JA. The influence of preischemic hyperglycemia on acute changes in the apparent diffusion coefficient of brain water following global ischemia in rats. Brain Res 1997; 757: 139-145.

[31] Wang XM, Guo QY, Lin N, Ding CW, Wang SX, Chen LY, Lv QJ and Jiang WG. Histopathological correlation with temporal diffusion changes in neonatal piglet model of hypoxic-ischemic encephalopathy. Chinese Journal of Medical Imaging Technology 2005; 21: 18-22.

[32] Deeg KH and Rupprecht T. [Pulsed Doppler sonographic determination of normal values of blood flow velocities in the anterior cerebral artery in premature and newborn infants]. Klin Padiatr 1988; 200: 307-315.

[33] Ophir J, Cespedes I, Ponnekanti H, Yazdi Y and Li X. Elastography: a quantitative method for imaging the elasticity of biological tissues. Ultrason Imaging 1991; 13: 111-134.

[34] Li XJ, Gao J and Sun ZR. A study on establishing a new preterm animal model with cerebral palsy and identifying the animal model. Chinese Journal of Rehabilitaiion Medicine 2004; 19: 885-889.