Regulatory Mechanism of Circadian Clock in Cerebral Ischemia Reperfusion

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ABSTRACT
This paper aims to study the regulatory mechanism of circadian clock in cerebral ischemia reperfusion. It is investigated how well the sleeps of child patients with cerebral ischemia have been treated with neonates as study subjects. The core clock genes during the regulation of the circadian clock get explored by the bioinformatics methods. Here also analyzes pineal gland expression by combination with relevant technologies. The results reveal that the patients’ sleep quality has a stake in circadian rhythm and respiratory problems; the pineal gland can regulate the core clock genes in the circadian clock during the regulation. It is concluded that in relation to the treatment against ischemic diseases of neonates, sleep disorder and pineal function lesion require lucubrating to seek appropriate therapeutic regimen.

Key Words: Circadian Clock, Cerebral Ischemia Reperfusion, Regulatory Mechanism

DOI Number: 10.14704/nq.2018.16.6.1672

Introduction
Hypoxic-ischemic encephalopathy (Fig. 1) (HIE) that neonates suffer from is serious complications from perinatal asphyxia, that is, it refers to hypoxic-ischemic brain lesion caused by neonatal hypoxia asphyxia in the perinatal period, including characteristic neuropathology and pathophysiology changes, and a range of clinical encephalopathy manifestations. Some child patients can leave neurological sequelae in different degrees. HIE plays a dominant role in neurological diseases in perinatal period. According to statistics, the incidence of neonate asphyxiation in different regions of China is between 1.14% and 11.7%; the mortality rate of birth asphyxia of children under 5 years old reaches 221.3/10; the mental disability rate among children aged 0 - 17 is 0.9%, while some are caused by neonate asphyxia as the third disability etiology, accounting for 8.6% (Golubnitschaja et al., 2011). Among children with hearing disabilities aged 0-6 years, 6.34% are caused by neonatal asphyxia. This disease has become a major issue that jeopardizes the health and living quality of children in China. As it is prevalent in the world, it has won widely concern of scholars at home and abroad over many years.

Figure 1. Hypoxic ischemia in new-born perinatal period

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 7 March 2018; Accepted: 3 May 2018
The consequences of perinatal hypoxia-ischemia (HI) may vary according to the severity of the disease. Severe cases may cause death, while mild patients may have relatively normal neurological function P-S1 (Takenouchi et al., 2011).

Although moderate to severe HI often causes serious motor impairments such as cerebral pain (CP), cognitive deficits, etc., child patients with mild HI are often found no obvious difference in both functions from normal and full-term neonates. However, recent studies have found that child patients with such mild HI have a high risk W of low IQ, but mostly mitigate due to the relatively normal motor function some child patients have. In recent years, the launch of the neonatal resuscitation program has greatly reduced perinatal mortality from hypoxia, while the prognosis for child patients after their resuscitation has aroused wide concern (de Vries and Jongmans, 2010). Despite the "three-support and three-symptomatic treatment" program in pediatrics and staged and sequential treatments after neonatal period have significantly alleviated the mortality of child patients with moderate-severe HI and also greatly improved their prognosis, these treatments still have problems such as long-term prognosis of mild HI syndrome is under-evaluated. Some scholars studied skulls of child patients with HI at different levels and found that most of the cerebral lesions lie in the basal ganglia, between the thalamus and the frontal lobe. Consequently, in addition to cognition and dyskinesia, multiple types of neurological impairments can occur in child patients with HI in perinatal period. For example, moderate-to-severe HIE can cause a delay in the initiation of the sleep-wake cycle (SWC). For child patients with cerebral ischemia, somniphathy often occurs due to visual and airway abnormalities. By far, the relationship between HI and the biological rhythm disorder has not yet been concerned by medical circle.

Up to now, no literature has not reported systematic study on the specific sleep disorders of child patients with HI in different degrees. For this purpose, this study aims to analyze 216 child patients in attempt to identify whether the increased risk of sleep disorders during development is associated with perinatal HI in child patients (especially for those with mild HI easily overlooked). This study will expose the health hazards from hypoxic-ischemic impairment to the human body in a naval and broad sense, and provide scientific basis for a more profound recognition of HI and its treatment.

**Methods**

**Study subjects**

In this study, some neonates, less than one week, born from May 2010 to August 2013, in the Renmin Hospital of Wuhan University and Wuhan children's Hospital, are chosen as primary screening subjects. Inclusion criteria: (1) There is a history of abnormal obstetric diseases that can cause fetal distress in the uterus and the symptom of fetal intrauterine distress (fetus < 100 beats/min, last for more than 5 Fmin); those with amniotic fluid grade III contaminated areas have clear history of asphyxia during delivery; (2) there is history of asphyxia at birth, i.e. the Apgar is rated 1 Filing; (3) Neurological symptoms occur soon after birth and continue to 24 FhW or above, such as altered state of consciousness (excessive excitement, drowsiness), change in muscle tone (high or slightly weak), abnormality of primitive reflexes (normal or weak embrace reflexes); (4) it is exclusive of diseases caused by intracranial hemorrhage and birth injuries, as well as brain injuries caused by intrauterine infection, genetic metabolic diseases and other congenital disorders. Exclusion criteria include:

- (1) severe HE;
- (2) acquired brain injury such as trauma;
- (3) other major lesions such as neurological and psychiatric disorders after birth;
- (4) those whose parents/siblings are unable to provide population demography data and medical information and/or complete questionnaires;
- (5) those who often took anticonvulsant drugs, sedatives, and neuroleptic drugs during the past few months;
- (6) those who have congenital development malformation that can cause upper airway obstruction (Perlman et al., 2010). The control group includes those born in that period, 35 weeks ≤ gestational weeks ≤ 42 weeks, 2500 grams ≤ birth weight ≤ 4000 grams, and the metaplasia weight is between the average weight P10-P90 at gestational ages; 5-minute Apgar score> 8; the medical records and surveys in clinical practices show that the perinatal children and infants have normal development (The basic profiles of the study subjects are shown in Table 1 below).
Table 1. Basic information of the subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Moderate HIE (n = 44)</th>
<th>Mild HIE (n = 84)</th>
<th>Normal (n = 88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>3.3±1.65</td>
<td>3.3±1.15</td>
<td>3.2±1.28</td>
</tr>
<tr>
<td>Gender</td>
<td>80% of men</td>
<td>74% of men</td>
<td>71% of men</td>
</tr>
<tr>
<td>Gestational age</td>
<td>37.41±6.24*</td>
<td>38.45±4.02*</td>
<td>40.22±4.38</td>
</tr>
<tr>
<td>Birth weight (Kg)</td>
<td>2.97±1.77</td>
<td>3.04±1.32</td>
<td>3.21±1.51</td>
</tr>
<tr>
<td>Cerebral palsy (case)</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Study method
We collect clinical data of child patients, including ages, genders, gestational ages, birth weights, rescue history after birth, iconography data, and diagnosis and treatment process; mild HIE: patients present alternate excitement and suppression, normal or slightly increased muscle tone, active embrace reflex, normal sucking reflex, visible myoclonus, no central respiratory failure, normal or enlarged pupil diameter; EEG examines normal; the clinical symptoms can disappear within 72 hours, and the prognosis is good; moderate HIE: patients may emerge drowsiness, low muscle tone, weak cuddle and sucking reflexes and common convulsions, visible central respiratory failure, shrink pupils, low EEG voltage, visible epileptiform discharge, clinical symptoms disappeared within 14 days, and sequelae may occur (Shilane et al., 2004).

The Sleep Questionnaire form is developed with reference to the Brief Infant Sleep Questionnaire (BISQ) and the Sleep Disorders Scale for Children (SDSC). Questionnaire variables include:
1) unfixed start time of nightly sleep (clock time when the child patients fall asleep at night);
2) Longer sleep latency (sleep time after going to bed at night> 20 min);
3) lack of sleep (<11 hours)
4) changes in daily bedtime routine (changes in start time and duration of siesta, prolonged sleep at night);
5) loud snore;
6) poor sleep breath (including shortness of breath, intermittent apneas during night sleep); 7) awakening frequently at night (>2 times/night). Each variable has an option of 0-2 levels: 0, never occurred in the past 6 months; 1, occurred≤3 times/week in the past 6 months; 2, occurred>3 times/week in the past 6 months. All 7 variables are added to get a sleep quality score. In addition, sleep relevant respiratory problems include the following symptoms: marked snore, shortness of breath, intermittent apnea. The physical examination excludes the cases with congenital development malformation, if possible, that can cause upper airway obstruction (Robertson et al., 1989).

The role of miRNAs in pineal gland after incidence of HIE in neonates
Surgical operations on all animals must be approved by IACUC, the Medical College of Soochow University. The new-born HIBD animal model is built as reported by SUN et al. Choose neonatal SDs, born for 7 days, let them inhaled with ether for anesthesia, secure them on a surgical table in a supine position, make a longitudinal incision of approximately 1 cm in the center of the neck, and separate the subcutaneous tissues and muscles. Find and freed the left common carotid artery from between the deep side of the left sternocleidomastoid muscle and the trachea, permanently ligate it with a 4-0 silk doubly and suture the incision. Each animal is operated for 5-10 minutes at room temperature 24-26°C. After the surgery, place the SD in a 37°C incubator for 1 h and then move it into a closed, atmospheric hypoxia chamber previously placed in a 32°C incubator, where the humidified 8% nitrogen-oxygen mixture is supplied for 2 h at a flow rate of 1.5 L/min. There should be an oxygen meter that can monitor the oxygen concentration. After the model is built, the SD should be fed back to the mother rats. For the control group, only the left common carotid artery should be isolated, but not ligated after suture. The skin should be sutured. (The production of a new D HIBD SD model is shown in Fig. 2)

Figure 2. The production of the new D HIBD rat model

SDs in the experimental group (n=7) and the control group (n=6) are randomized. All SDs in the same litter are randomly assigned to the following two groups, and put to death by HIBD or in 12 h after sham operation. The Kuszak method is referred to remove the pineal gland. The animal
lies prostrate on a homemade ice bench soon after ether anesthesia. Cut skin between the ears of the head to expose sagittal and lambdoidal sutures. Shear apart the skull sagittal bone and endocranium with the sagittal suture as the vertical axis, use an eyelid forceps to gently lift up the joint sinus and sagittal sinus veins and make them form a 45-60° angle to the brain plane, and another eyelid forceps to remove pineal gland from below joint sinus with another eyelid, pulverize and place it in an 1 ml Eppendorf tube added with TRIzol. In the process, the pipette repeatedly beats it evenly, and then store it in a -20°C freezer.

**Results and discussion**

**Various sleep disorder of child patients with HIE**

As compared to child patients with mild HIE, those with moderate HIE have a higher risk of breathe relevant sleep disorders, including apparent snoring and other symptoms. These findings coincide with the conclusion previously reported that there are positive correlation between the occurrence of cerebral palsy and sleep relevant respiratory problems, possibly because: as compared to those mild HIE, moderate HIE can cause extensive pallium and subcortical injuries which may have a significant impact on the neuromuscular control of the upper respiratory tract. Child patients with mild HIE present changes in daily sleep schedules, which implies that these child patients’ sleep rhythms have been disturbed more or less. Although previous studies have suggested that the mild hypothermia therapy may delay the formation of the SWC of child patients with moderate-severe HIE, the relationship between the maintenances of HIE and SWC remains mystery. Therefore, this study has discovered for the first time that the maintenance of regular SWCs during childhood development may also be vulnerable to the history of HI disease. Just as what the previous studies have concluded, we also find that the incidence of pineal gland cysts in the child patients with mild HIE may be higher (Robertson et al., 1993). These pineal gland cysts may be involved in large mass melatonin secreted, pineal gland swelling stress-induced in the development of mild HIE, as well as subsequent melatonin secretion in quantity. It is proved that melatonin can play a protective role in HIE pathophysiology (See Table 2 for details).

**miR-182 binds the 3′-UTR of the CLOCK gene in vitro**

In this experiment, luciferase gene detection can validate the interaction between miR-182 and the CLOCK gene. Hela cells are transfected with a reporter gene carrying the predlicative miR-182 targeting sequence in the CLOCK gene 3′-UTR, and then the miR-182 mimics are added, it is found that the luciferase activity declines by 55% (the luciferase activity in the target sequence is shown in Fig. 3). However, when the targeting sequence is substituted by a mutant sequence, this activity decline phenomenon disappears. Conversely, if a negative, non-targeted miRNA is added, neither predictive sequence of transfected cell nor the miR-182 targeting sequence of mutant miR-182 has any effect on luciferase activity (Odd et al., 2009). Thus, it is known that miR-182 interacts with the targeting sequence specificity in the CLOCK gene 3′-UTR. (The luciferase activity in the mutant sequence is shown in Figure. 4)

**Table 2. Various sleep problems in children with HIE**

<table>
<thead>
<tr>
<th>Sleep disorders</th>
<th>Moderate the HI(n = 44)</th>
<th>Mild HI(n = 84)</th>
<th>normal(n = 88)</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>The starting time of a fixed night sleep</td>
<td>20 (12) 12 (12)</td>
<td>15 (37) 32 (2) 72 (14)</td>
<td>2</td>
<td>0.92</td>
</tr>
<tr>
<td>Sleep duration (&lt; 20 min)</td>
<td>13 (9) 19 (12)</td>
<td>54 (26) 4 (4) 81 (6)</td>
<td>1</td>
<td>0.85</td>
</tr>
<tr>
<td>Sleep time is less than 11h</td>
<td>21 (18) 5 (6)</td>
<td>48 (28) 8 (7) 78 (8)</td>
<td>2</td>
<td>0.95</td>
</tr>
<tr>
<td>Change your routine</td>
<td>23 (13) 8 (8)</td>
<td>25 (41) 18 (8) 68 (14)</td>
<td>2</td>
<td>0.83</td>
</tr>
<tr>
<td>Obvious snoring</td>
<td>9 (23) 12 (12)</td>
<td>50 (25) 9 (7) 76 (9)</td>
<td>3</td>
<td>0.92</td>
</tr>
<tr>
<td>Sleep apnea</td>
<td>28 (9) 7 (7)</td>
<td>70 (10) 4 (4) 82 (6)</td>
<td>0</td>
<td>0.81</td>
</tr>
<tr>
<td>Frequent night awakenings</td>
<td>3 (13) 18 (18)</td>
<td>13 (52) 19 (19) 72 (12)</td>
<td>4</td>
<td>0.86</td>
</tr>
<tr>
<td>Sleep score</td>
<td>6.0±1.5 (6.8±1.2)</td>
<td>4.99±1.26 1.17±0.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Luciferase activity in the mutant sequence

Conclusion and prospects

In this study, we have offered novel insights into the mechanism of sleep disorders of child patients with HIE in different degrees. The results show that the circadian rhythm disorder has intimate correlation with the history of perinatal HIE; child patients with moderate HIE have a significantly increased risk of incidence of sleep relevant breath problems in the future, while those cases with mild HIE have a higher incidence of circadian rhythm disorders.

In regard to regulatory mechanisms, this study incites a series of programs to confirm that the target gene of miR-182 is CLOCK, and has explored an efficient and stable method to isolate pineal gland cells from SD's brain tissue for in vitro culture and purification and identification. The cell experiment proves the molecular mechanism that miR-182 affects post-HIE circadian rhythm disorder in neonatal SDs by CLOCK.

References


