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Original Article

The expression of PI3K/Akt signaling pathway and PTEN in hippocampus of the brain and the correlation with cognitive impairment after neonatal hypoxic ischemic brain damage in rats

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Abstract: Objective: Since the PI3K/Akt signaling pathway and PTEN gene have been known to be closely related to hypoxia-ischemia brain injury, we hypothesized that they are involved in neonatal hypoxic ischemic brain damage (HIBD) and as well as cognitive impairment. Methods: To test this hypothesis, 7 day old newborn rats were subjected to HIBD by ligating the common carotid artery followed by hypoxia. Rat brains were collected to detect the expression of PI3K/Akt and PTEN using Western blot analysis. The Morris Water Maze Test in rats was used for evaluating learning and memory ability when the rats were 28 days old. Results: The expression of p-Akt and p-PTEN were found to be significantly up-regulated after hypoxia-ischemia (HI) compared with the sham operation group. Through the Morris Water Maze (MWM) test, showed that the rats' learning and memory cognitive abilities were impaired after HI, and the cognitive impairment were more severe after blocking the PI3K/Akt signaling pathway. Conclusions: Our findings suggest that the PI3K/Akt signaling pathway and PTEN gene play important roles to cognitive impairment in HIBD.

Keywords: Hypoxia-ischemic, Morris water maze, neonatal rat

Introduction

Neonatal hypoxia ischemia brain damage (HIBD) is a leading cause of birth death, as well as high incidences of neurological sequelae in survivors such as cerebral palsy, mental retardation, learning disabilities and epilepsy, etc. With the development of prenatal medicine, the mortality rates in children with HIBD decreased greatly however the incidence of different levels of HIBD increased [1]. Cognitive impairment is the most common sequela. Recently, therapeutic measures of HIBD are investigated in clinical as well as animal studies [2, 3]. However the relation between cognitive impairment in the developing brain and HI remains unclear. Therefore, intensive research of the mechanisms of cognitive processes in the developing brain after HIBD is becoming important for prevention and even of cure cognitive impairment in children [4].

The PI3K/Akt signaling pathway is a classic anti-apoptosis, urge-survival signal transduction pathway in the cell [5]. It has been reported to play an important role in protecting the brain against ischemic, in angiogenesis and anti-apoptosis, et al [6, 7]. PI3K can phosphorylate inositol lipids producing phosphatidylinositol-3,4,5-trisphosphate (PIP3). One of the downstream targets of PI3K is a serine-threonine kinase, AKT. It works closely with PI3K and plays an important role in the signal transduction of cell growth [8]. The PTEN gene (phosphatase and tensin homolog deleted on chromosome 10) is the first ever discovered highly conservative tumor suppressor gene with a dual-specificity phosphatase activity [9, 10]. Its encoding protein PTEN can specifically dephosphorylate phosphatidylinositol-3,4,5-triphosphate (PIP3), and antagonize the PI3K/Akt signaling pathway [11]. Thus it interferes with cell growth signals, induces cell death and cell

cycle arrest, and plays a negative feedback role in cell survival and growth [12].

Although the roles of the PI3K/Akt signaling pathway and PTEN in injury, protection and regeneration in the central nervous system have been previously reported, their relativity with cognitive impairment in the developing brain with HI still remain unclear.

Therefore, we hypothesized that the PI3K/Akt signaling pathway and PTEN gene are involved in neonatal HIBD and closely related to cognitive impairment. To test this hypothesis, we generated neonatal hypoxia-ischemia brain injury models using 7-day-old rats to explore the expression regularity of the PI3K/Akt signaling pathway and PTEN and also to investigate the relation between PI3K/Akt signaling pathway and cognitive impairment with or without PI3K/Akt inhibitor.

Methods

Source of experimental animals and grouping

The experiment was approved by the Zhejiang University Animal Ethics Committee according to the local government legislation. Female Sprague-Dawley (SD) rats with litters of mixed gender were acquired from Zhejiang University's animal center. The pregnant rats were given food and water and housed in a temperature and light controlled facility until the litter was 7 days old.

For the HIBD model, we used a method referred to the "Rice method" [13]. Each 7 day-old neonatal rat was anesthetized with ethylether. With the rat supine on the minor board, the left common carotid artery (CCA) was exposed and permanently ligated with a 7-0 silk suture through a midline cervical incision. Then the rats were returned to the dam for 1-2 h to recover from anesthesia. The rats were subsequently placed in a hypoxic chamber of 8% O_2+92% N_2 maintained at 37°C for 2 hour to create hypoxia-ischemia brain damage [14]. The sham operation group rats just received ethylether anesthesia and exposure of the left CCA without ligation or hypoxia. All pups were returned to their cages for breastfeeding after the surgery.

For the wortmannin-treated HIBD group, rats received hippocampus regions injection of wortmannin (16 μg/kg) with the help of stereotaxic apparatus 30 min before HIBD as described above; while the DMSO-treated HIBD group rats just received hippocampus regions injection of 2 μL DMSO with the same dilution instead of wortmannin.

From 1 h, 4 h, 12 h and 24 h of 4 time points in the HIBD model group and sham operation group after HI (each group n = 6), from 4 h in wortmannin-treated HIBD group and DMSO-treated HIBD group after HI, rats brain were collected for Western blot analysis (n = 8). Whole brains were removed for dissection. The hippocampus regions were separated from both ischemic and contralateral sides and immediately frozen in liquid nitrogen. The remaining rats in each group were used to take the Morris water maze test to assess learning and memory ability at the age of 28 days old.

Western blot analysis

The isolated hippocampus tissues at different time points as described above were homogenized in ice-cold lysis buffer containing: cytosol extraction buffer containing.

HEPES (pH 7.9) 10 mmol/L, KCL 10 mmol/L, EDTA (ethylene diamine tetraacetic acid) 0.1 mmol/L, EGTA (ethylene glycol tetraacetic acid) 0.1 mmol/L, DTT (dithiothreitol) 1 mmol/L, PMSF (phenylmethanesulfonyl Fluoride) 0.5 mmol/L, protease inhibitor aprotinin 5 μg/mL, leupeptine (5 μg/mL), and phosphokinase inhibitor 10 μg/mL. Lysates were centrifuged at 14,000 rpm for 30 min at 4°C. Cytosol proteins were purified as described previously [15]. Protein concentration was determined by a BCA protein assay kit (Pierce) using bovine serum albumin (BSA) as the standard. Protein samples (50 μg per lane) were separated on 10% SDS-polyacrylamide gels. Protein was then transferred to PVDF membranes. The membranes were blocked in 5% nonfat milk in TBS containing 0.02% Tween-20 for 1 h at room temperature with rotation. The membranes were incubated over night at 4°C with the following antibodies: rabbit anti-AKT polyclonal antibody (Cell Signaling, 1:1000), rabbit anti-phospho-Akt polyclonal antibody (Cell Signaling, 1:1000) and rabbit anti-phospho-PTEN polyclonal antibody (Cell Signaling, 1:2000). Rabbit anti-β-actin polyclonal antibody (Sigma 1:2000) was used as an internal loading control. Membranes were then incubated with peroxi-

dase-conjugated goat anti-rabbit IgG (Sigma, 1:2000) in blocking solution for 1 h. Signals of bound antibodies were developed by enhanced chemiluminescence (Pierce, Rockford, IL). NIH images were used to measure the densities of the protein signals on X-ray films after scanning. Protein levels were normalized to β-actin as a loading control. Relative optical density of protein bands was measured following extraction of the film background. All experiments were repeated at least three times to assure reproducibility of the results.

Learning and memory ability assessment

The Morris water maze (MWM) test employed in the present investigation is one of the most widely accepted models to assess learning and memory in rodents [16]. For assessment of behavioral function, the rats were tested in a MWM at the age of 28 days. The experimental apparatus consisted of a circular water pool (diameter, 120 cm; height, 60 cm) filled with carbon ink-clouded water and the temperature controlled at 22±0.5°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 × 10 cm) situated in the center of the target quadrant was submerged 1 cm below the surface of water (thereby made invisible) for testing of spatial learning. A camera was mounted above the center of the maze. The animal's motion could be recorded and sent to the computer. A tracking system was used to measure the escape latency (EL), traveled path and swimming speed [17, 18].

Acquisition trials

All the rats performed a block of four trials during four daily sessions. The hidden platform position remained stable during the four days of the assessment. Each rat was put in the water gently while facing the wall, at every quadrant each day, and rats were allowed a maximum of 120 seconds at each trial to find the hidden platform. The duration to find the platform was called the escape latency (EL). If the rat succeeded to escape, it was allowed to stay on the platform for 30 additional seconds 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 seconds, it was gently guided onto the platform by the experimenter with 120 seconds scored and allowed to remain there for 30 seconds. Then, it was taken from the platform and the next trial was started. After completion of the fourth trial, each rat was kept warm for an hour and returned to its home cage. The mean data of EL from four trials each day was taken as the daily average of the above mentioned parameters and used to form cognitive function-time curves. Throughout the tests, the experimenter always stood in the same position. Care was taken to maintain the location of the water maze relative consistent to other objects in the laboratory so that prominent visual clues would not be disturbed during the test. All the trials were completed between 10.00 am and 3.00 p.m.

Retrieval trial: On the fifth day, the hidden platform was removed and each rat was allowed to explore the pool for 120 seconds. 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 three days after the retrieval trial. Then, on the eighth day, the MWM assessment was repeated to obtain retention memory data. The method was similar with the acquisition trials, in order to assess its long-term memory retention.

Statistical analysis

Experimental data are presented as mean ± standard deviation (SD), using SPSS 17.0 statistical software. Statistical differences between multiple groups were compared using one way ANOVA with LSD post-hoc tests. Pairwise Comparisons used independent sample t test. Repeated measures analyses of variance (Repeated measures ANOVO) were used to compare the escape latency of the experiment. The process of multi-factor analysis of variance made comparisons between the groups at each time point. A value of $P < 0.05$ was considered statistically significant.

Results

The expression of PI3K/Akt pathway and PTEN in hippocampus region of brain after HI

To quantify AKT, p-Akt and p-PTEN expression after HI in this model, we measured AKT, p-Akt and p-PTEN protein expression in the hippo-

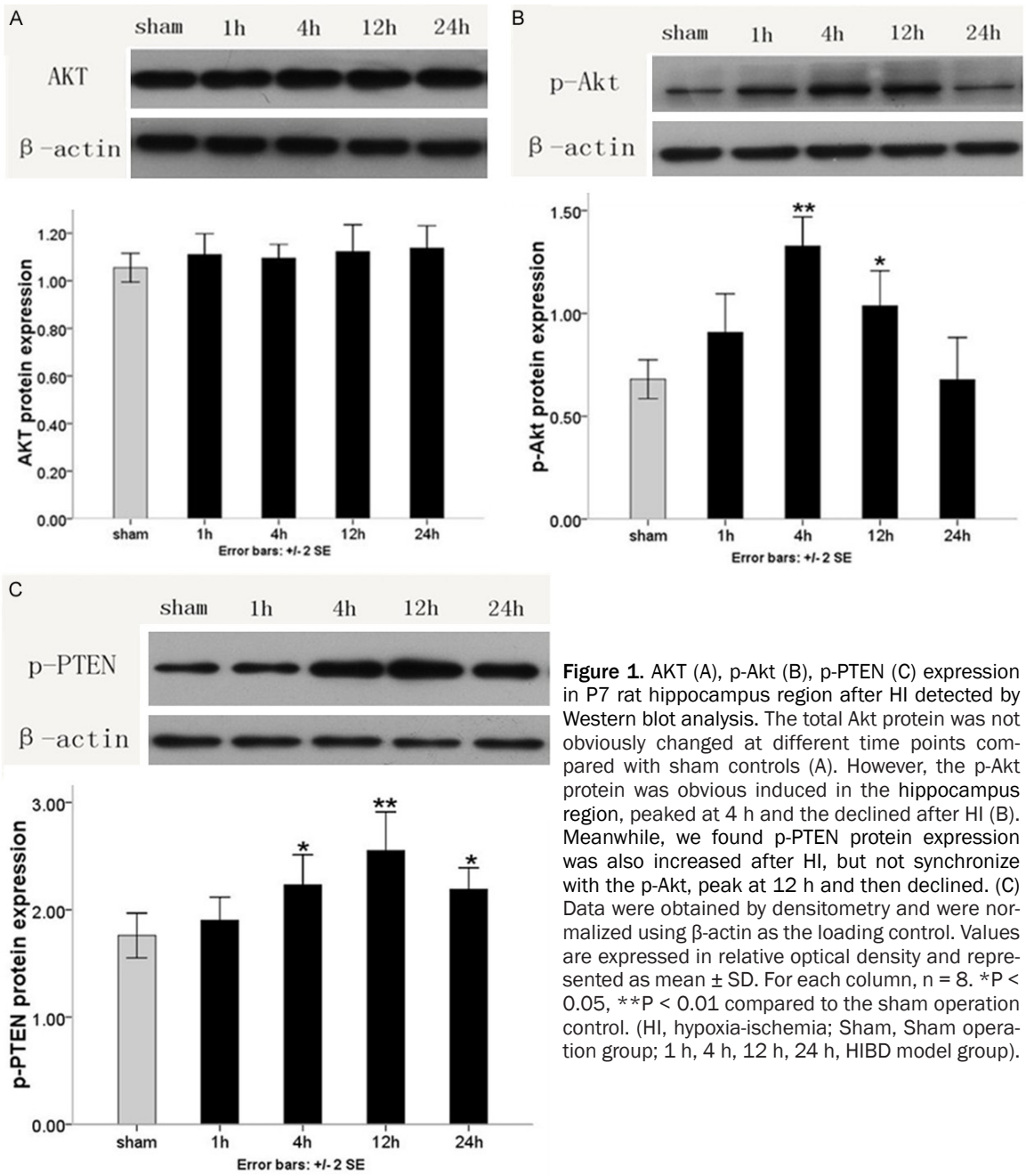

Figure 1. AKT (A), p-Akt (B), p-PTEN (C) expression in P7 rat hippocampus region after HI detected by Western blot analysis. The total Akt protein was not obviously changed at different time points compared with sham controls (A). However, the p-Akt protein was obvious induced in the hippocampus region, peaked at 4 h and the declined after HI (B). Meanwhile, we found p-PTEN protein expression was also increased after HI, but not synchronize with the p-Akt, peak at 12 h and then declined. (C) Data were obtained by densitometry and were normalized using β-actin as the loading control. Values are expressed in relative optical density and represented as mean ± SD. For each column, n = 8. *P < 0.05, **P < 0.01 compared to the sham operation control. (HI, hypoxia-ischemia; Sham, Sham operation group; 1 h, 4 h, 12 h, 24 h, HIBD model group).

campus region using Western blot analysis. Comparison within all the time points in the sham operation group showed that each protein showed no significant difference (P > 0.05). Therefore, we took 4 h to unify when compared to the HIBD model group.

To detect whether PI3K/Akt is involved in this model, the expression of AKT and its phosphorylated form p-Akt was detected. In HIBD model group, the expression of AKT protein in 1 h, 4 h, 12 h, and 24 h was 1.1100±0.0879, 1.0950±0.0290, 1.1225±0.0580 and 1.1375±0.0935 respectively, which showed no significant variation between different time points (P > 0.05). Meanwhile, the difference had no statistical significance either when compared to the sham operation group (P > 0.05). The sham operation group had a small amount of AKT protein expression 1.0550±0.0603 (**Figure 1A**).

However, we found that p-Akt protein was obviously induced after HI, peaked at 4 h and then declined, returning to the control level at 24 h.

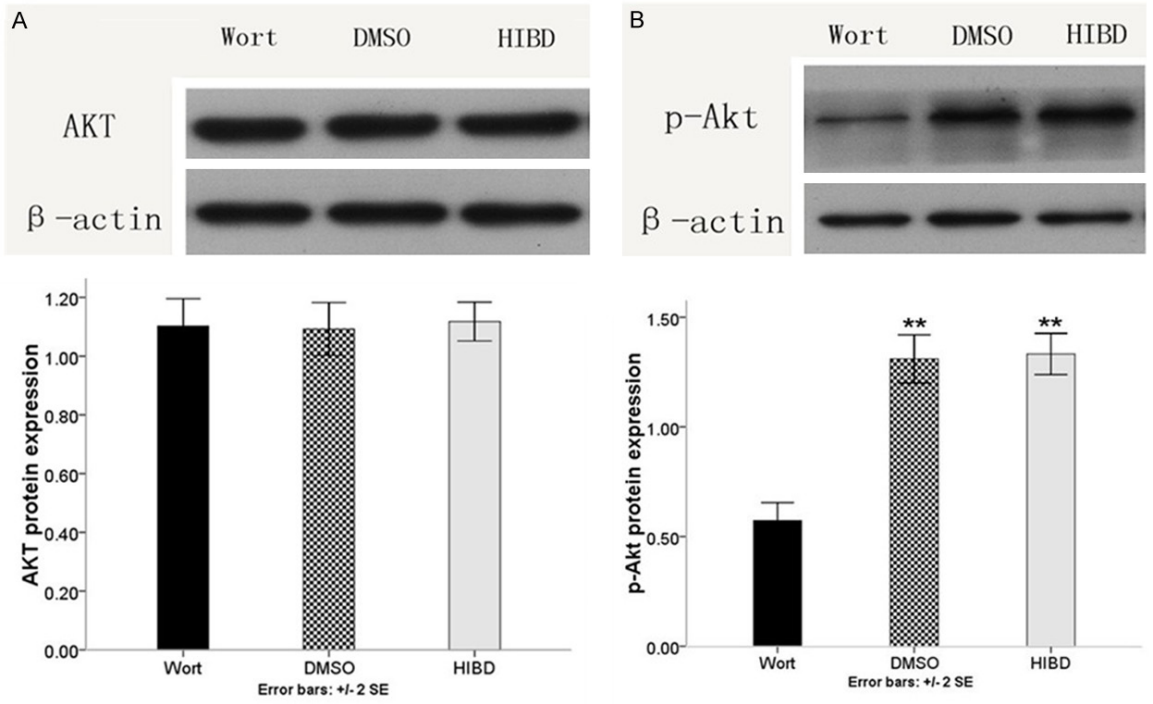

Figure 2. The expression of AKT (A) and p-Akt (B) protein after Wortmannin intervention in P7 rat hippocampus region detected by Western blot analysis. We found the total AKT protein expression was not obviously changed, no significant difference with Wortmarmin-treated HIBD group, DMSO-treated HIBD group and HIBD model group (A). While the p-Akt protein expression was significantly inhibited in Wortmannin-treated HIBD group rats compared with that in DMSO-treated HIBD group and HIBD model group rats (B). Data were obtained by densitometry and were normalized using β-actin as the loading control. Values are expressed in relative optical density and represented as mean ± SD. For each column, n = 8. **P < 0.01 compared to the sham operation group. (Wort, Wortmannin-treated HIBD group; DMSO, DMSO-treated HIBD group; HIBD, HIBD model group).

The expression in 1 h, 4 h, 12 h, and 24 h was 0.9075±0.1882, 1.3275±0.1413, 1.0375±0.1702 and 0.6775±0.2053 respectively. The p-Akt protein expression in 4 h and 12 h showed significant difference compared to the sham operation group (P < 0.001; P = 0.008), but at 24 h, no significant difference showed. The sham operation group had a small amount of p-Akt protein expression 0.6801±0.0942 (**Figure 1B**).

Meanwhile, we found that p-PTEN protein expression was also increased after HI, but not synchronize with p-Akt, peaked at 12 h and then declined. The expression in 1 h, 4 h, 12 h, and 24 h was 1.9725±0.0996, 2.2525±0.2707, 2.6125±0.3038 and 2.1950±0.2098 respectively. The expression in 4 h, 12 h and 24 h showed significant difference compared to sham operation group (P = 0.006; P < 0.001; P = 0.013). The sham operation group had a small amount of p-Akt protein expression 1.765±0.1015.

The expression of AKT and p-Akt protein after Wortmannin intervention

To investigate whether PI3K/Akt signaling pathway was involved in cognitive impairment after HIBD, a PI3K/Akt signaling pathway specific inhibitor, Wortmannin, was used in this study. Quantitative analysis showed that the p-Akt protein expression peaked at 4 h after HI, so we determined the expression of AKT and p-Ak at 4 h after HI within Wortmannin-treated HIBD group, DMSO-treated HIBD group and the HIBD model group (each group n = 8). With the isolated hippocampus protein, the AKT and p-Akt protein expression was detected using Western blot analysis.

Total AKT protein expression was not substantially changed. There was no significant difference within Wortmarmin-treated HIBD group, DMSO-treated HIBD group and HIBD model group (P > 0.05) (**Figure 2A**).

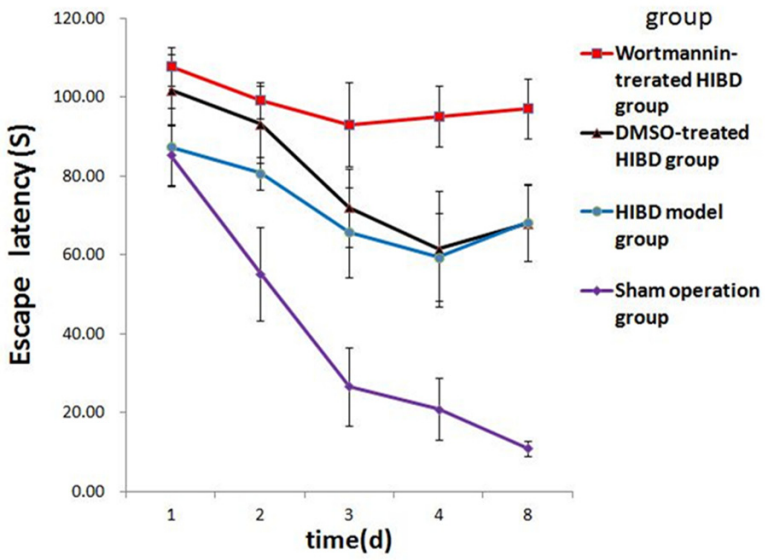

Figure 3. The trend chart about the mean EL data change of each group rats in the first four days and on day 8 from repeated measure indicators. We observed that the EL curves showed a tendency to decrease in each group animals, but the EL values of the sham operation group dropped the most obvious. When compared to the sham operation group, the EL values of other three groups were longer ($P < 0.05$).

In contrast, p-Akt protein expression was significantly inhibited in Wortmannin-treated HIBD group rats compared with that in DMSO-treated HIBD group and HIBD model group rats ($P < 0.001$) (**Figure 2B**). However, there was no significant difference between DMSO-treated HIBD group and HIBD model group ($P > 0.05$).

Learning and memory ability assessment

Acquisition trials: Repeated measures ANOVA results: The tests of Within-Subjects Effects showed that the time factor (day) had a very statistically significant ($F = 15.578$, $P < 0.001$). This denoted that the escape latency (EL) of rats in each group had a variation trend over time. Meanwhile, day and group interaction (day*group) also had statistical significance ($F = 2.067$, $P = 0.025$). This indicated that the role of the time factor varies within the groups. After 4 days of seeking the hidden platform training, the EL curves showed a tendency to decrease in each group of rats, but the sham operation group's EL values had a more evident drop. The EL values of the other three groups were higher when compared to the sham operation group ($P < 0.05$), which indicated that different degrees of damage from spatial learning ability were caused in rats after HI (**Figure 3**).

Pairwise comparison between the four groups at each time point: One the first day, the EL values of the four groups were narrow in range. While with the increasing number of swimming, the EL values had different levels of shortening among groups. From the second day, the EL value of the Wortmannin-treated HIBD group was higher than the control group ($t = 2.637$, $P = 0.023$), and the gap was enlarged as time passed by. On the fourth and eighth day, the EL values of the Wortmannin-treated HIBD group were obvious higher compared with other three groups ($P < 0.05$, $P < 0.001$). These indicated that the Wortmannin-treated HIBD group had the most serious spatial learning and memory ability impairment. The impact of long-term memory was also more significant after blocking the PI3K/Akt pathway. Representative tracking charts of Morris Water Maze have been showed in **Figure 4**.

Retrieval trial: On the fifth day, the control group rats mostly focused on the path to the former platform location, while the rats of the other three groups were mostly swimming along the wall. This denotes that rats of each group had different spatial searches, but the one that the control group chose was more reasonable. The number of times crossing the former platform location within 120 seconds after removing the platform was lower in DMSO-treated HIBD group and HIBD model group compared to the control group ($P < 0.05$). However the number of times crossing the former platform location in Wortmannin-treated HIBD group was lower compared with the DMSO-treated HIBD group and the HIBD model group ($P < 0.05$), also significantly less compared to the control group ($P < 0.001$) (**Figure 5**). This indicated that rats had varying degrees of damage after HI in spatial memory ability and positioning capabilities, which was much worse after blocking the PI3K/Akt signaling pathway.

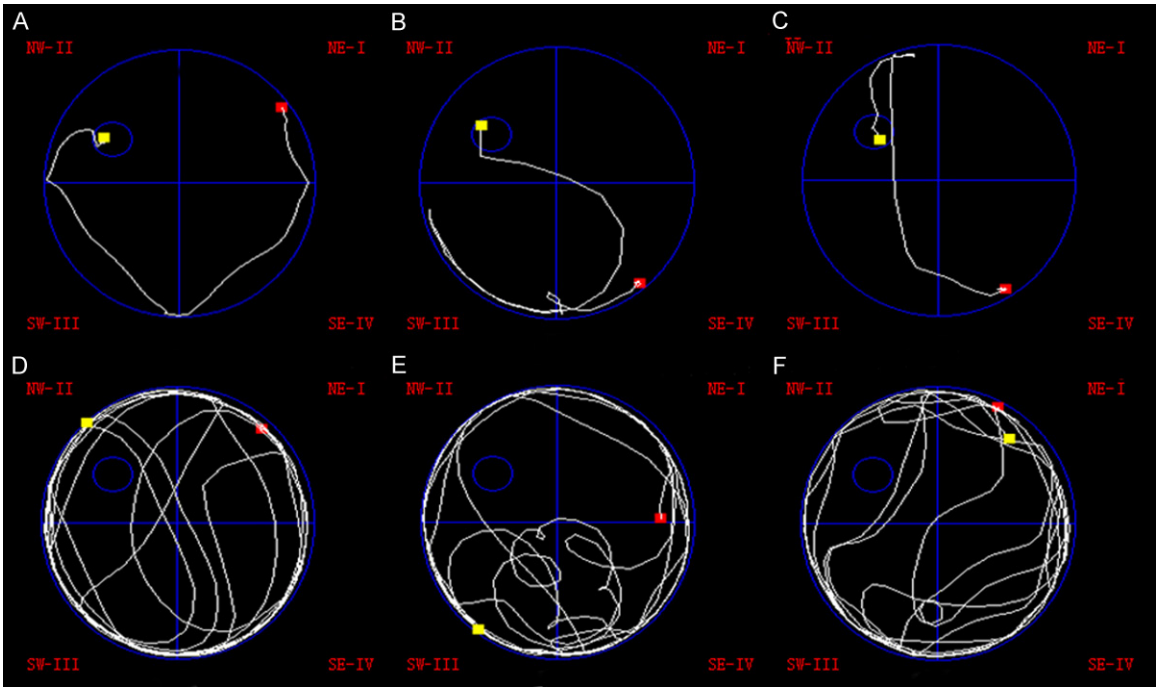

Figure 4. Swimming trajectories of different situation. A-C: Rates who can find the hidden platform rapidly; D-F: Rates who fail to find the hidden platform.

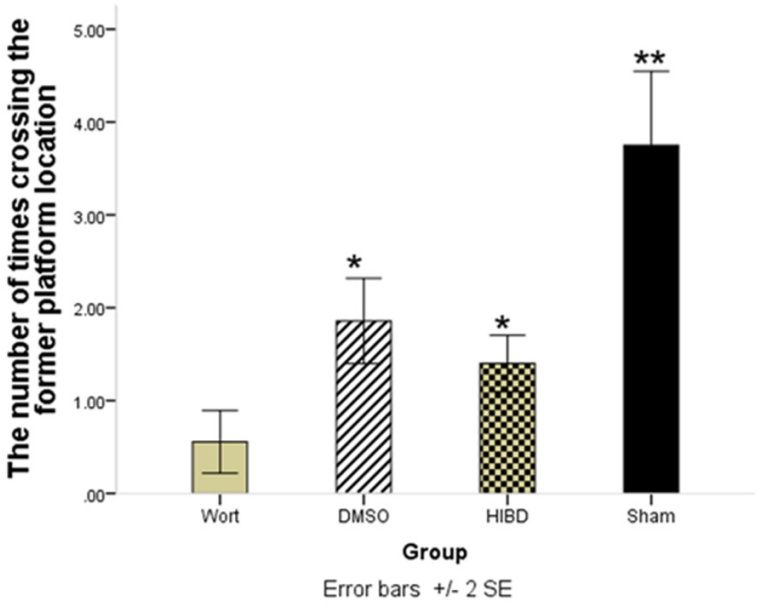

Figure 5. The comparison of the number of times crossing the former platform location within each group rats. *P < 0.05; **P < 0.001, compared to Wortmannin-treated HIBD group. (Wort, Wortmannin-treated HIBD group; DMSO, DMSO-treated HIBD group; HIBD, HIBD model group; Sham, Sham operation group).

Discussion

Both localized and diffused cerebral ischemia can cause neuronal apoptosis, which is an important way of cell death in brain after ischemic injury [19, 20]. However, the activation of signal transduction is a prerequisite to apoptosis and the early stages of apoptosis. Phosphoinositide3-kinase (PI3K) family is a kind of kinase which can specifically catalyze phosphatidylinositol lipid and is also involved in intracellular signal transduction. It can phosphorylate inositol (which is on the cell membrane after activated), and generates phosphatidylinositol-3-phosphate (PI3P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) and phosphatidylinositol -3,4,5-triphosphate (PI-3,4,5-P3). Functionally, PI3K is the primary regulator of AKT activation. AKT is an important signal protein kinase in the transduction pathway as a direct target gene of PI3K. In the heart of PI3K/Akt signaling pathway, it plays an important role in anti-apoptotic regulation and can induce a variety of biological effects [21].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was originally identified as a tumor suppressor because of its high frequency mutations in various types of tumors [9, 22]. It is a negative regulator of the PI3K/Akt signaling pathway that prevents the recruitment of AKT to the cellular membrane for phosphorylation [23-25]. Thus it interferes with cell growth signals and induces cell death and cell cycle arrest, showing its negative regulation of cell proliferation and survival [12].

Previous studies have shown that PI3K/Akt pathway and PTEN is related to hypoxic-ischemic brain injury [26-28]. In this study, we have found that the expression of p-Akt and p-PTEN is significantly up-regulated in the developing brain after HI compared to the sham operation group. However the expression of p-Akt peaks at 4 h, earlier than p-PTEN which is at 12 h. This showed that the PI3K/Akt signaling pathway is activated after HI, and AKT phosphorylation increased. Also the expression of p-Akt protein is regulated by time; it peaks at 4 h after reoxygenation and then starts to lower. However the expression trend of p-PTEN is related with p-Akt's, but the expression peak time is at 12 h later than p-Akt. This indicated that while PI3K/Akt signaling pathway works as an upstream regulator, the role of PTEN is to antagonize PI3K/Akt signaling pathway. So the increased expression of p-PTEN can reduce the phosphorylation levels of AKT after HI. Meanwhile, the expression of AKT does not change throughout the process, which indicated that p-Akt is the one involved in regulating the activities. The protein expression at different time points in the sham operation group showed no significant difference, indicating that anesthesia and exposure to carotid artery does not affect the protein expression.

This shows that PI3K/Akt signaling pathway can be activated and induced by AKT phosphorylation in the brain after HI. But also hypoxia-ischemia activates PTEN, and induces the expression of p-PTEN protein. p-PTEN manifests as the main negative regulatory protein to the PI3K/Akt signaling cascades. So it in turn inhibits the PI3K/Akt signaling pathway, antagonizes the AKT phosphorylation level, and leads to neuronal damage. From this it can be seen that protection signals and injury signals exist and interact simultaneously in the cell. However, injury signals are stronger after HI, which consequently lead to neuronal damage. PTEN inhibitor has been reported in the literature that it can significantly increase the expression of p-Akt [29]. This shows that the protection signals can be enhanced after inhibiting the injury signals, thereby protecting the ischemic neurons. It illustrates interactions between injury signals and protective signals from another point of view.

In recent years, AKT was used as the molecular target of treating cerebral ischemia via signaling pathway. It has taken a large number of drug intervention studies and its neuroprotective effect has been confirmed. However, the relation between PI3K/Akt signaling pathway and cognitive impairment after HI was not clear. Therefore, we applied the specificity and efficiency of the PI3K/Akt signaling pathway inhibitors, Wortmannin, intervened in neonatal rat HIBD model, and found that the expression of p-Akt is completely suppressed in Wortmannin-treated HIBD group, while in the DMSO-treated HIBD group and the HIBD model group the expression of p-Akt does not change. Meanwhile, AKT has no effect on the expression in the three groups. This shows that the use of an inhibitor is effective.

Furthermore, from MWM test we observed that Wortmannin can not only significantly reduce the expression of p-Akt in the developing rat brain, but also deteriorate the learning and memory ability of long-term cognition in the rats after HI. Thus, PI3K/Akt signaling pathway can affect the learning and memory ability in the developing brain after HI, and is closely related to the later recovery of brain function [30]. In summary, we have shown that PI3K/Akt signaling pathway and PTEN involved in neuronal apoptosis in the developing rat brain after HI are closely related to the later recovery of brain function. Agents targeting AKT/PTEN might help to study the protective mechanisms in neonatal after HI, and to discover a more effective way to treat newborn children with HIBD.

What is already known

Recently, therapeutic measures of HIBD are investigated in clinical as well as animal studies.

The roles of the PI3K/Akt signaling pathway and PTEN in injury, protection and regeneration

in the central nervous system have been previously reported.

What this study adds

The PI3K/Akt signaling pathway and PTEN gene are involved in neonatal HIBD and closely related to cognitive impairment.

The mechanisms of cognitive processes in the developing brain after HIBD is becoming important for prevention and even of cure cognitive impairment in children.

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Disclosure of conflict of interest

None.

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