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Full Length Research Paper

Neuroprotective effect of progesterone in newborn rats with hypoxic-ischemic encephalopathy

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Progesterone (PROG) is a type of neurosteroid, and it has physiological functions in the central nervous system. To date, most researches into progesterone have focused on its application in treatment of traumatic brain injury, stroke and neurodegenerative disorders, but its effect on hypoxic-ischemic encephalopathy (HIE) remains unclear. In the current study, we investigated the effect of exogenous progesterone in HIE newborn rats. Newborn Wistar rats were divided into the sham-operated group, hypoxic-ischemic (HI) group and the pretreated group with PROG to observe the effects of progesterone on the components in cortex and hippocampus. Our results showed that the levels of water, sodium, calcium, NO (nitric oxide) and glycogen synthatase kinase-3β (GSK-3β) in brain tissues were increased significantly in HI group compared to those in the sham-operated group except for significantly lower concentration of potassium while those in PROG pretreated group were lower significantly than those in HI group. The cell apoptosis rate and the concentration of malondialdehyde (MDA) in HI group were higher significantly than those in the sham-operated group or the pretreated group while superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities in HI group were lower significantly than those in any other two groups. Exogenous PROG can obviously reduce neuron apoptosis, oxygen free radical production, down-regulate the expression of glial fibrillary acidic protein (GFAP) and up-regulate the expression of PROG receptor (PR) after brain injury.

Key words: Progesterone, hypoxic-ischemic encephalopathy, newborn rats, neuroprotection.

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) or hypoxic ischemic brain damage (HIBD) can be caused by systemic asphyxia occurring at birth. HIE could not only seriously threaten the lives of newborns, but also it could lead to post-neonatal neurological sequelae such as cerebral palsy, mental retardation, learning disability and epilepsy (Karasev et al., 2010; Boggio et al., 2009; Kaur et al., 2008; Northington et al., 2011). As the consequences of HIE are devastating and permanent, it is urgently needed to identify and develop effective therapeutic strategies to lessen brain injury for newborns with HIE. Progesterone (PROG), as estrogen, affects many functions of the central nervous system (Chen et al., 2008; Stein et al., 2008). PROG plays an important role in promoting and enhancing repair after traumatic brain injury (TBI) and stroke, and there has been growing evidence that PROG treatment may be safe and effective for traumatic brain injury and other neural disorders in humans. Several reports have demonstrated that PROG can take the neuroprotective role by preventing neuronal loss and improving functional outcomes, which could improve intracranial pressure, cerebral perfusion pressure and neurological scores after TBI in ovariectomized rats (Wright et al., 2007). Acute PROG treatment post-TBI could provide an effective intervention to prevent or attenuate systemic, post-injury cortical and sub-cortical edema in young and aged females (Shahrokhi et al., 2010; Kasturi et al., 2009). Its neuro-protective

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Abbreviations: NO, nitric oxide; **SOD,** superoxide dismutase; **MDA,** malondialdehyde; **GPX,** glutathione peroxidase; **HIE,** hypoxic-ischemic encephalopathy; **GSK-3β,** glycogen synthatase kinase-3β; **GFAP,** glial fibrillary acidic protein; **PROG,** progesterone; **PR,** progesterone receptor.

mechanism may be involved in two independent processes: an acute protection by antagonizing sigma (1) receptor to inhibit NMDAr-Ca^{2+} influx and a delayed one by activating P4R-mediated Src-ERK signaling pathway (Cai et al., 2008).

The afore-mentioned data indicate that PROG can play a vital role in neuroprotection following brain injury. However, until now its effect on HIBD has still been unknown. Based on these facts, this study was intended to take the effect of PROG on HIBD as its target with the expectation of providing a promising and effective therapeutic intervention to maintain, and then, to improve the functions of the brain suffering from HIBD.

MATERIALS AND METHODS

Animals and drugs

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Xinxiang Medical University, China. Wistar rats aged one week (weighing 12~18 g) were maintained at the same center and housed individually in cages with free access to water and laboratory chow. PROG was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and dissolved in the sesame oil at the final concentration of 0.5 mg/ml for experimental use. All kits were purchased from Nanjing Jiancheng Bioengineering Institute, China.

HIE model

The model for perinatal hypoxic-ischemic brain injury was prepared according to the method described previously (Kumral et al., 2003; Taniguchi et al., 2008). Newborn Wistar rats were anaesthetized using isoflurane (5%) and underwent permanent unilateral carotid ligation 4 min later. The midline of the neck was incised at the longitudinal plane under halothane anesthesia. The left carotid artery was permanently tied with 5/0 surgical silk. Total time of surgery never exceeded 5 min. Animals were excluded from the study if the animals did not appear left rotation movement after 6 h. Following recovery and feeding for 2 h, the animals were exposed to hypoxia (92% N_2, 8% O_2) for 2.5 h, and then partially submerged in a 37°C water bath in airtight containers with a constant thermal environment.

Groups and drug administration

Rats were randomized divided into three groups: pretreated group (receiving an intraperitoneal injection of PROG (4, 8 and 16 mg/kg, respectively) just 30 min before the surgical procedures), hypoxic-ischemic group (undergoing the same surgical procedures and receiving an intraperitoneal injection of the same dose of sesame oil), and sham-operated group (undergoing the same surgical procedures but without ligation of the carotid artery or hypoxia and receiving an intraperitoneal injection of the same dose of sesame oil 30 min before the surgical procedures). Firstly, 100 rats were divided into the aforementioned three groups, and then returned to their dams for 24 h until sacrifice. Each sample was subjected to the detection of water content, sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), nitric oxide (NO) and glycogen synthatase kinase-3β (GSK-3β) in cortex and hippocampus. Secondly, 90 rats were divided into the sham-operated group, hypoxic-ischemic group and pretreated group injected with 8 mg/kg PROG, and sacrificed at 6, 24, 48, 72h and 7 d, respectively. After hypoxia they were subjected to the detection of brain neuron apoptosis, oxygen free radicals including enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX), and the content of malondialdehyde (MDA). Finally, 48 rats were divided into the sham-operated group, hypoxic-ischemic group and pretreated group injected with 8 mg/kg PROG. 8 rats were sacrificed in each group at 24 h after hypoxia.

Brain tissues were removed immediately and fixed in 10% formalin overnight, and then subjected to the detection of protein expressions of PROG receptor (PR) and glial fibrillary acidic protein (GFAP). And the remnant rats were sacrificed and subjected to the total RNA extraction from the brain tissues for the detection of PR and GFAP mRNA expressions.

Assay of water content, Na^+, K^+, Ca^{2+}, NO and GSK-3β in cortex and hippocampus

Eppendorf tubes were weighed before use (tube weight, W1). After the harvest of the cortex and hippocampus tissues, one portion of them was collected into the separate pre-weighed tubes. The tube was weighed once again (pre-weight, W2) with the lid left open, and kept in a vacuum oven maintained at 60° for 48 h. The tube was removed from the oven and allowed to cool at room temperature for 30 min in an enclosed space to prevent any accidental gross contamination that might affect the weight. The lid was closed and the tube was weighed again (post-weight, W3). Then, W1 was respectively subtracted from W2 and W3 to get the wet weight (WW) and dry weight (DW). The percent of water content (WC) (mg) was calculated as follows:

$$WC = [(WW - DW)/WW] \times 100\%.$$

Meanwhile, the other portion of harvested cortex and hippocampus tissues was stored in liquid nitrogen immediately for enzyme-linked immunosorbent assay (ELISA) and other assays. The frozen cortex and hippocampus tissues were homogenized with a glass homogenizer in 1 ml of buffer containing 1 mmol/L fluoride, 1 mg/L pepstatin A, 1 mg/L aprotinin and 1 mg/L leupeptin in phosphate-buffered saline solution (pH 7.2). The concentrations of Ca^{2+}, Na^+, and K^+ were analyzed using the atomic absorption spectrophotometer and the flame spectrophotometer, respectively. The content of NO was detected through nitrate reductase method by specific NO kit according to the manufacturer's instructions. Specifically, the cortex and hippocampus tissues were collected into a separate pre-weighed tube. 100 mg of tissues was homogenized with a glass homogenizer in 1 ml cold phosphate-buffered saline solution (pH 7.2) and centrifuged at 12,000 g for 10 min at 4°C. And 0.1 ml of supernatants was picked out and measured using the spectrophotometer. Nitrate/nitrite was assayed to represent nitric oxide content in brain tissues.

The content of GSK-3β in cortex and hippocampus tissues was quantified using specific GSK-3β ELISA kit for rats according to the manufacturer's instructions. The brain homogenates prepared from the same samples were centrifuged at 12,000 g for 10 min at 4°C. The supernatants were collected and used for the ELISA.

Assay of brain neuron apoptosis, content of MDA and SOD, and GPX activity

To analyze neuron apoptosis in brain tissues, the rats were sacrificed and the brain tissues were harvested on ice immediately to make cell suspension. The cells (5×10^5~1×10^6/ml) were washed with cold PBS twice, then resuspended in 300 µl of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated with 3 µl of FITC-conjugated annexin V (Invitrogen, USA)

according to the manufacturer's instructions. Afterward, cells were gently centrifuged and incubated for 15 min at room temperature in darkness. Propidium iodide (PI) (20 µg/ml) was added, and flow cytometry was carried out. For SOD activity, GPX activity and MDA concentration assays, the brain tissues were harvested on ice immediately to prepare homogenates, centrifuged at 10,000 g for 10 min at 4°C. The supernatants were stored at -70°C before use. The content of MDA was determined by the modified thiobarbituric acid method. SOD activity was measured by the method of xanthine oxidase. GPX activity was measured by the method of disulfide II-nitrobenzoic acid.

Assay of PR and GFAP by immunohistochemistry

Rats were deeply anesthetized, sacrificed 24 h later after hypoxia. The harvested brain tissues were 10% formalin-fixed, paraffin-embedded and fresh-frozen sections (5 µm) were taken. Nonspecific binding was blocked for 30 min with 4% horse or goat serum (depending on the species used to raise the secondary antibody) in PBS. Immunohistochemical study was conducted. The rabbit anti-rat monoclonal antibody of PROG receptor and GFAP (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Immunohistochemical assay was performed as recommended by the manufacturer (Beijing Zhongshan Biotechnology, Inc, China). Evaluation of sections was undertaken by assessing the intensity of staining. The positive cells of PR and GFAP were indicated by cytoplasm and nucleus with brown staining.

Assay of PR and GFAP mRNA by RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen, USA) from brain tissues. All RNA samples were synthesized into the first strand cDNA with reverse transcriptase and random primers (TaKaRa). The cDNA was stored at -20° until use. RT-PCR primer sequences for PR amplification were: forward, 5'-AACTGGTTCCGCCACTCAT-3', reverse, 5'-AACTGGTTCCGCCACTCAT-3' (product length, 460 bp). Primer sequences for GFAP were: forward, 5'-CGTTGACATCCGTAAAGACC -3', reverse, 5'-GCTAGGAGCCAGGGCAGTA -3' (product length, 488 bp). The housekeeping gene β-actin was used as an internal control to confirm the PCR product. Primer sequences for β-actin were: forward, 5'-CGTTGACATCCGTAAAGACC-3', reverse, 5'-GCTAGGAGCCAGGGCAGTA-3' (product length, 260 bp). The products of PCR were separated by 2% agarose gel and analyzed by Bander leader 3.0 software.

Statistical analysis

All values were expressed as the mean± standard deviation (SD). Statistical comparisons among pretreated, sham, and HI groups were performed by the one-way ANOVA t-test, and least-significant differences (LSD) were adopted to determine significant differences. All statistical analyses were computed by using SPSS ver. 13 software. *P* value less than 0.05 represented the statistical significance.

RESULTS

Effect of PROG on water content, Na^+, K^+, Ca^{2+}, NO and GSK-3β in cortical and hippocampus

As shown in Figure 1, the water content, concentrations of Na^+, K^+, Ca^{2+}, NO and GSK-3β in cortex and hippocampus of HI group were significantly higher than those of the sham-operated group ($P < 0.01$). The water content of the pretreated group with PROG (4, 8, 16 mg/kg, respectively) was significantly lower than that of HI group ($P < 0.05$) with the subgroup pretreated with 8 mg/kg PROG lowest. The concentration of K^+ of the pretreated group was significantly higher than that of HI group ($P < 0.05$).

Effect of PROG on neuron apoptosis, content of MDA and SOD, and GPX activity in brain tissue

Neuron apoptosis in brain tissues of HI group at 6 h after hypoxia was significantly higher than that of sham-operated group ($P < 0.01$), and it reached the peak at 24 h, and afterward decreased gradually. There was no significant difference at 7 d between them ($P > 0.05$). Neuron apoptosis of the pretreated group with 8 mg/kg PROG at 6 h after hypoxia was significantly lower than that of HI group ($P < 0.05$), and afterward descended gradually. No statistically significant differences were detected at 7 d between them ($P > 0.05$, Figure 2). The content of MDA in HI group at 6 h was significantly higher than that in sham-operated group ($P < 0.05$), and it reached the highest at 24 h, afterward decreased gradually. There was no significant difference at 7 d between them ($P > 0.05$). The content of MDA in the pretreated group with 8 mg/kg PROG at 6 h was significantly lower than that in HI group ($P < 0.05$), afterward descended gradually. There was no significant difference at 7 d between them ($P > 0.05$), as shown in Figure 2. The enzyme activities of SOD and GPX in the brain tissues of the sham-operated group were highest while lowest in those of HI group, and the differences between them at 6, 24, 48 and 72 h after hypoxia ($P < 0.01$) were significant. Significant differences were also detected between the pretreated group and HI group at 6, 24, 48, 72 h after hypoxia ($P < 0.05$), as shown in Figure 2.

Effect of PROG on expression of PR and GFAP in brain tissue

The immunohistochemical assay showed PR immunorectivity was highest in the brain tissues of the pretreated group with 8 mg/kg PROG. The number of PR positive cells with brown-stained cytoplasm and nucleus was 53.46 ± 5.37 in the brain tissues of the pretreated group with 8 mg/kg PROG, 21.57 ± 1.88 of the HI group, and 30.22 ± 2.16 of the sham-operated group. All the differences among them were statistically significant ($P < 0.05$, Figure 3A1, B1 and C1). In the sham-operated group, the GFAP positive astrocytes were thinner, scattered with fine and short projections and the number of them was scarce. In the HI group and the pretreated

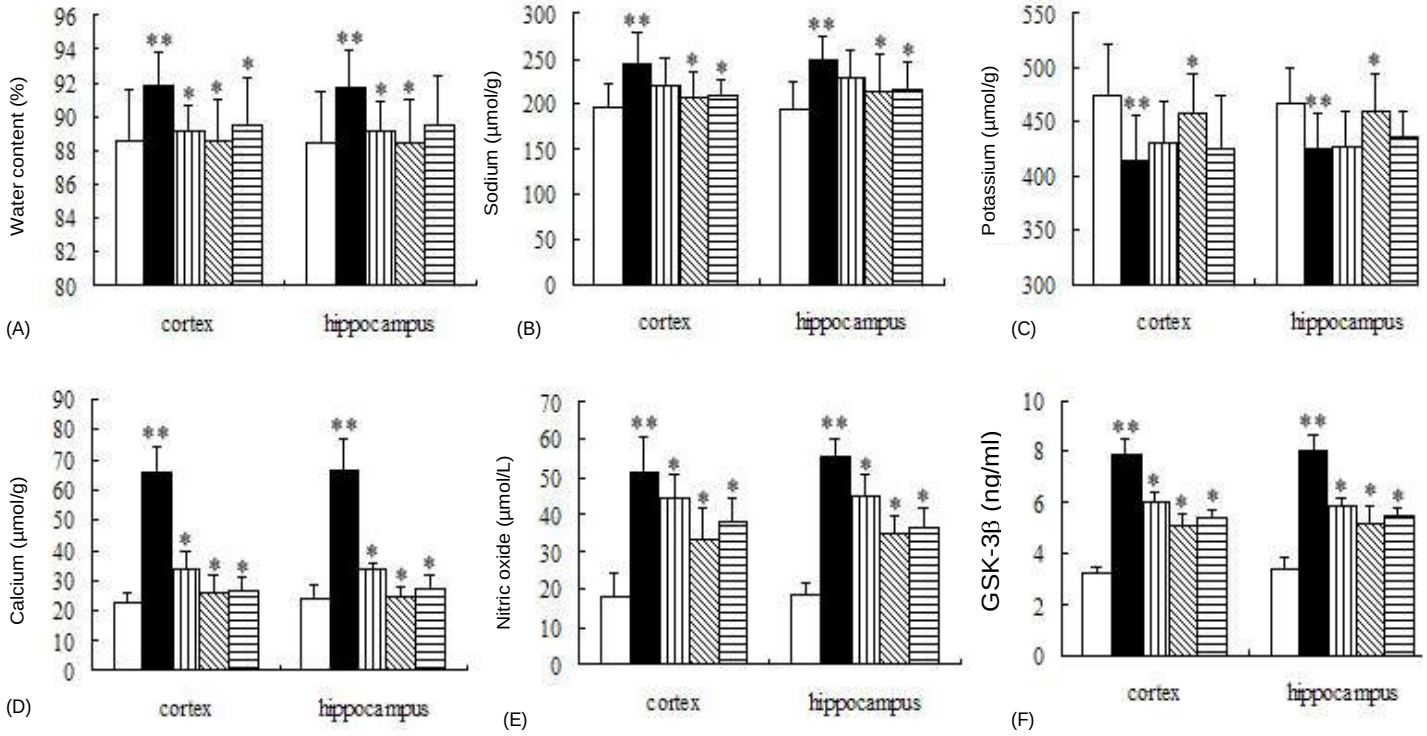

Figure 1. Effects of PROG on water content (A), Na^+ (B), K^+ (C), Ca^{2+} (D), NO (E), and GSK-3β (F) in cortex and hippocampus of newborn rats. Comparisons were made among sham-operated group (white bar), hypoxic-ischemic group (black bar), pretreated group with PROG (4 mg/kg- vertical bar, 8 mg/kg-oblique bar, 16 mg/kg- horizontal bar, respectively).**P<0.05 compared with sham-operated group; * P<0.05 compared with hypoxic-ischemic group.

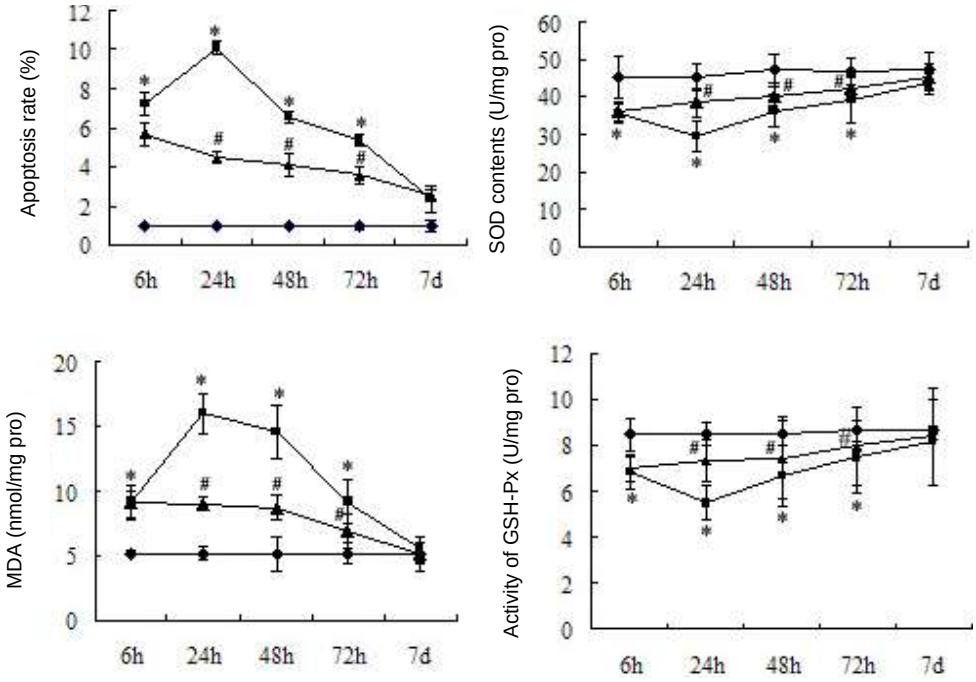

Figure 2. Effects of PROG on neuron apoptosis, content of MDA and SOD, GPX activity in brain tissues of newborn rats. Comparisons were made among sham-operated group (rhombus line), hypoxic-ischemic group (rectangular line), pretreatment group with PROG (triangle line).*P<0.05 compared with sham-operated group; # P<0.05 compared with hypoxic-ischemic group.

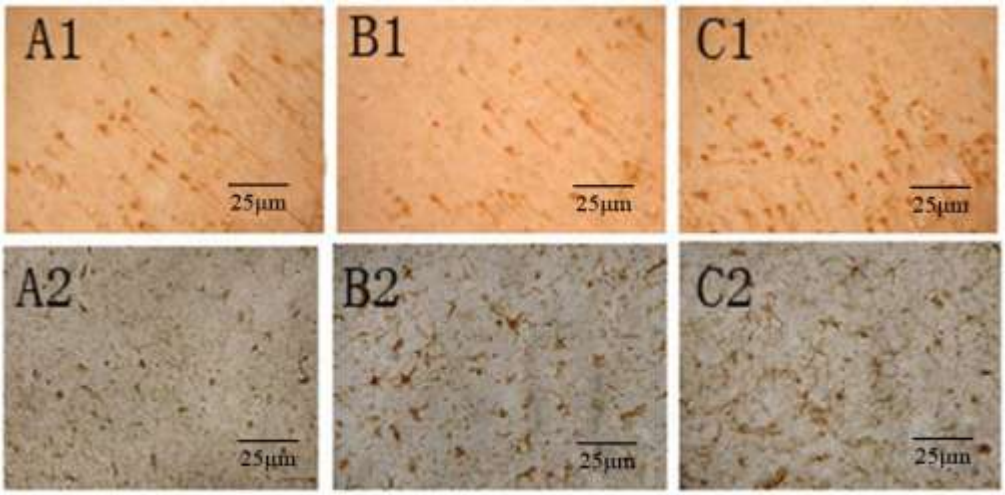

Figure 3. Expressions of progesterone receptor (A1B1C1) and GFAP (A2B2C2) by immunohistochemistry. A) sham-operated group; B) hypoxic-ischemic group; and C) pretreated group with PROG.

group, the astrocytes cell body was bigger with more projections and the numbers were larger (Figure 3A2, B2 and C2). Furthermore, the number of GFAP positive cells was significantly increased in the HI group (25.52 ± 2.6) compared with that in the sham-operated group (3.23 ± 1.46) ($P < 0.05$) while that in the pretreated group was significantly decreased (15.49 ± 3.72) compared within the HI group ($P < 0.05$).

Effect of PROG on mRNA expression of PR and GFAP in brain tissue of newborn rats

RT-PCR assay indicated that mRNA expression of the PR in newborn brain rats exhibited statistically significant differences among groups (Figure 4). It was significantly decreased in the HI group (0.47 ± 0.26) compared to that in the sham-operated group (0.93 ± 0.31) ($P < 0.05$), or to that in the pretreated group (0.78 ± 0.27) ($P < 0.05$). The mRNA expression of GFAP in newborn brain rats also displayed the significant differences among groups (Figure 4). It was significantly increased in the HI group (0.89 ± 0.34) compared to that in the sham-operated group (0.20 ± 0.13) ($P < 0.05$), or to that in the pretreated group (0.52 ± 0.19) ($P < 0.05$).

DISCUSSION

The occurrence of hypoxic-ischaemic encephalopathy (HIE) during the perinatal period could cause the neurodevelopmental disabilities in the infants (Aly et al., 2010; Doyle et al., 2010) and it is a leading cause of morbidity and mortality in the perinatal period with an incidence of 1/4000 newborns. Biochemical events such as brain edema, the increase in intracellular Ca^{2+}, production of oxygen-free radicals, lipid peroxidation, and inadequate blood flow are triggered by HIE may lead to brain dysfunction and neuronal death (Calvert et al., 2005; Sheldon et al.,1996; Nakata et al., 2002). NO plays an important role in the regulation of cerebral blood flow in the perinatal period (Liu et al., 2011). GSK-3β is a potent regulator of cell apoptosis *in vivo*, and involved in the regulation of cell growth and development. Traumatic brain injury resulted in the increased phosphorylation of inhibitory site serine (9) of GSK-3β, which was consistent with previous reports (Shapira et al., 2007; Kajta et al., 2007). Our study demonstrated that water content, the concentrations of sodium, calcium, NO and GSK-3β in cortex and hippocampus were increased significantly in HI group compared with those in the sham group with the exception of a significantly lower concentration of potassium. In contrast, water content, the concentrations of sodium, calcium, NO and GSK-3β in the pretreated group with PROG were lower significantly than those in HI control group with the subgroup with 8 mg/kg PROG the most significant. These findings indicated that PROG could recover the normal Na^+ and K^+ exchange to keep ion balance in brain tissues and reduce the hydrocephalus to lessen cerebral injury. After perinatal hypoxia-ischaemia, brain neurons undergo apoptosis (Edwards et al., 1996; Choi et al., 1996). Primary antioxidant enzymes such as SOD (scavenging superoxide anions), GPX (removing hydrogen peroxide and lipid peroxides) perform significant activities in HIE. In the present study, the activities of the antioxidant enzymes and the brain neuron apoptosis were also estimated.

Results exhibited that the brain neuron apoptosis rate and level of MDA in HI group were significantly higher

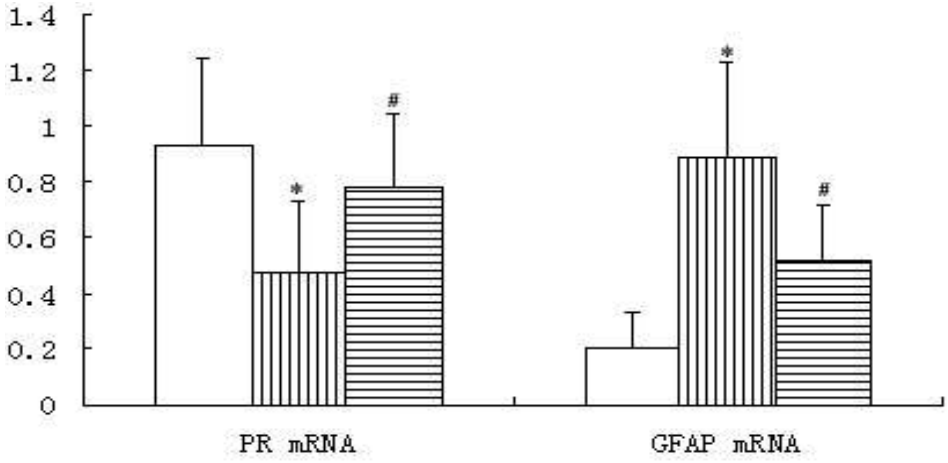

Figure 4. mRNA expressions of progesterone receptor and GFAP detected by RT-PCR. Comparisons were made among sham-operated group (white bar), hypoxic-ischemic group (vertical bar), pretreated group (horizontal bar). **P*<0.05 compared with sham-operated group; # *P*<0.05 compared with hypoxic-ischemic group.

than those in sham group or the pretreated group at 6 h after HI, and they rose to the highest level at 24 h, and then gradually declined. The levels of SOD and GPX in HI group were lower significantly than those in sham group or the pretreated group at 6 h after HI, and they decreased to the lowest level at 24 h, and then gradually rose till 7 d, but the difference was not significant. These data suggested that exogenous PROG can play a neuroprotective function by reducing neuron apoptosis and oxygen free radical production after brain injury. In our study, effects of PROG on the expressions of the PR and GFAP in the brain tissues of newborn rats with HIE were measured by immunohistochemistry staining and RT-PCR. The results showed that expression of PR was down-regulated in HI group, but increased in pretreated group with PROG, indicating that PROG might play a vital role in up-regulating the expression of PR, which in turn, may play a critical and specific role in the normal development of the reproduced brain areas. The expression of hormone receptors is intimately involved in the regulation of gonadotropin secretion and female sexual cycle (Ezquer et al., 2008; Quadros et al., 2008). Astrocytes play multiple roles in maintaining an optimally suitable milieu for neuronal functions. Reactive astrocytes could secrete neurotrophic factor, nerve growth factor and extracellular matrix, which induce axonal outgrowth and regeneration of the neural network after brain injury. GFAP has been recognized as an astrocyte marker. The GFAP-positive reactive astroglia increased around the damaged area (Callahan et al., 1990; Van et al., 1984; Itoh et al., 2007). The present results indicated that there were fewer small GFAP-positive astroglia with finer and shorter projections in the sham group while many larger GFAP-positive cells with long elongated projections in the brain tissues at 7-days after HI. Up-regulation of GFAP has a key role in the development of some of the long-term inhibitory features of astrogliosis. In fact, the attenuation of reactive gliosis observed in mutant mice deficient for GFAP correlates with improved integration of neural grafts and enhanced posttraumatic regeneration (Li et al., 2008; Buffo et al., 2010).

Our data demonstrated that PROG could protect the impaired brain tissue by inhibiting the activation of astrocytes and down-regulating GFAP expression of reactive astrocytes. PROG belongs to neurosteroid and has a wide range of physiological functions in the central nervous system and can significantly improve neurologic recovery (Romeo et al., 1998; Dubrovsky et al., 2000; Thomas et al., 1999; Baulieu et al., 2000). Several studies have demonstrated that PROG appears to exert its protective effect by protecting or rebuilding the blood-brain barrier, decreasing the development of cerebral edema, down-regulating the inflammatory cascade, and limiting cellular necrosis and apoptosis (Coughlan et al., 2009; Hu et al., 2009; Nicola et al., 2009; Junpeng et al., 2011; Wali et al., 2011). In summary, to our knowledge, this is the first study to demonstrate the effects of progesterone on the pathophysiological changes in newborn rats with HIE. Through the establishment of HIE experimental animal models, to observe the effect of PROG on newborn rats by using the technology of atomic absorption spectrophotometer, flame spectrophotometer, ELISA, immunohistochemistry and RT-PCR. We found that PROG administration could reduce brain tissue edema, intracellular calcium overload, the generation of NO, neuron apoptosis and oxygen free radical production after HI injury in newborn rats. The present results suggested that the therapeutic benefit of progesterone treatment might be by virtue of its salutary effects in modulating PR and GFAP expressions to exert its

neuroprotective function and apoptosis, as is true of exogenous PROG in our study.

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