

Effects of androgen on expression of OMgp of the HIBD neonatal rat brain

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Aim: To investigate the protective effects of testosterone propionate (TP) on hypoxic–ischemic brain damage (HIBD) neonatal rat brain by observing oligodendrocyte myelin glycoprotein (OMgp) expression in the cortex and hippocampus after HIBD.

Materials and methods: Rats were randomly divided into 3 groups: TP pretreatment, HIBD control, and a sham treatment group. Rats 7 days old from the TP group and HIBD control group were subjected to HIBD, and OMgp expression in the hippocampus and cerebral cortex of the different doses and groups was observed after hypoxic–ischemic induction at 24 h, 48 h, 72 h, 7 days, and 14 days.

Results: The OMgp expression in the brain tissue of the HIBD control group was significantly higher than that of the sham group at the same point in time ($P < 0.01$). After intervention with TP, OMgp expression in the hippocampus and cortex (30 mg/kg and 120 mg/kg) was significantly reduced compared with the HIBD control group ($P < 0.01$).

Conclusion: OMgp expression in neonatal rat brain tissues was increased after HIBD; OMgp overexpression was inhibited after intervention with TP. Therefore, androgen may play an important role in removing inhibition of OMgp on axon growth, and thus promote axonal regeneration, playing a protective role in the brain.

Key words: Oligodendrocyte cells, myelin glycoprotein, hypoxic–ischemic brain damage, testosterone propionate, cortex, hippocampus

Introduction

Hypoxic–ischemic brain damage (HIBD) is a common cause of damage to the neonatal central nervous system, and is caused by perinatal hypoxia and cerebral blood flow reduction or suspension (1,2). There is a high mortality rate in children with severe HIBD; most deaths happen in the neonatal period, and survivors are left with varying degrees of nervous system sequelae, such as cerebral palsy, mental retardation, learning disabilities, and epilepsy. Therefore, HIBD is a serious threat to children's health (3–5).

The oligodendrocyte myelin glycoprotein (OMgp) is an important component of axonal growth inhibition in central nervous system (CNS) myelin, which is a specific glycoprotein within the CNS white matter. It is now clear that OMgp inhibits axonal growth, which can lead to growth cone collapse. The human OMgp gene can inhibit cell proliferation, is expressed by mature oligodendrocytes in both the CNS and in the peripheral nervous system, and is mainly located in the membrane (6–9). So far, almost all research on OMgp expression in brain injury has concentrated on adult rat ischemic brain injury, and

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has rarely focused on OMgp expression changes in the brain tissues of neonatal rat brains after HIBD damage.

Androgen is a hormone with the main function to promote organ development of the reproductive system and maintenance of secondary sexual characteristics. Moreover, studies have reported that androgen plays an important role in the brain's normal development, including differentiation, maintenance of nerve function, and resistance to oxidative damage. In recent years, it has been suggested that androgen and its receptor play a protective role against hypoxic-ischemic nerve injury (10,11).

In recent years, the in-depth study of nerve regeneration has made axon regeneration inhibitors a research hotspot. While OMgp is 1 of 3 myelin inhibitors of nerve growth, research about the regulation of axon regeneration of HIBD, particularly in OMgp expression change, is limited at this time (12,13). Therefore, to further an in-depth understanding of the neuroprotective roles of androgen in the occurrence and development of HIBD, we investigated the effects of androgen on OMgp expression of excessive growth inhibitor of axons in the HIBD rat brain tissue, using an HIBD newborn rat model. This would provide a reliable experimental and theoretical basis for finding new therapeutic approaches to neonatal brain damage. The purpose of this study was to explore the effects of androgen pretreatment on OMgp expression of HIBD hippocampus and cortex, and the dose dependence of this relationship and its significance for axonal regeneration.

Materials and methods

Animals

A total of 200 Sprague Dawley rats (3 days old, 6–10 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University, Xi'an. They were housed at 21 ± 1 °C under a 12 h light/12 h dark cycle and had free access to a standard pellet diet (Purina chow) and tap water. Each animal was used only once in the experiment. The experimental protocols were approved by the Animal Care and Use Committee of our institute.

Drugs and reagents

The SABC immunohistochemistry kit and DAB chromogenic kit were both purchased from the Wuhan Boster Biological Engineering Co., Ltd. The injection of testosterone propionate was purchased from Shanghai GM Pharmaceutical Co., Ltd.

Experimental apparatus

The optical microscope was manufactured by Japanese Olympus, the measuring oxygen analyzer was produced by the Jiande Lida Instrument Factory, and the computer image analysis equipment was purchased from the German company Leica.

Animal groups and preparation of the animal model

Two hundred rats from the same growth conditions were randomly divided into 3 groups: the sham group ($n = 40$), the HIBD control group ($n = 80$), and the androgen group ($n = 80$). The androgen group and HIBD control group were divided into 2 doses, so that in each dose group there were 40 rats. At 3 days postpartum, each rat from each dose of the androgen group was dosed with testosterone propionate by intraperitoneal injection (30 mg/kg and 120 mg/kg). Each rat from each dose of the HIBD control group was separately given an intraperitoneal injection with the corresponding amount of peanut oil, once daily for 3 days.

Making the HIBD newborn rat model

The HIBD newborn rat model was established according to the method described by Rice with some modifications (14). The rats (7 days postpartum) were placed in supine position, and their limbs were fixed on the operating panel. After routine disinfection, the neck incision was taken under anesthesia to perform common carotid artery ligation on the left, and finally the incision was sutured. The entire surgical procedure took less than 10 min. Four hours after the surgery, the rats were placed in a 10,000 mL airtight container for 2.5 h, with an 8% O₂/92% N₂ gas mixture added at 2 L/min. For the sham group, rats underwent the same surgical procedures, but without the left common carotid artery ligation and there was no hypoxia treatment.

Behavioral observations to determine the success of the HIBD rat model

HIBD group: after hypoxia for about 10 min, all the animals showed irritability; 30 min after hypoxia,

animals showed cyanosis, shortness of breath, and they demonstrated weakness with the hind legs dragging while crawling; after hypoxia for 60 min, most of the rats showed drowsiness or irritation to phenomena, sometimes including twitching limbs and incontinence; 1 h after reoxygenation, only a small number of rats demonstrated much activity, and most of those who showed activity rotated to the left, suggesting that the model was successful.

Specimen preparation

Eight rats were taken from each group at 24 h, 48 h, 72 h, 7 days and 14 days after hypoxic-ischemic induction (HI), respectively. After a 10% chloral hydrate (40 mg/100 g) intraperitoneal injection of anesthesia, the heart was exposed by thoracotomy, and the right atrial appendage was cut. Intubation through the left ventricle to the aorta allowed for a rapid infusion of 20 mL of heparin saline, followed by 20 mL of 4% paraformaldehyde (4 °C), and then with 50 mL of 4% paraformaldehyde over a slow infusion of 2 h. Then the whole brain was decapitated, and the meninges removed. From this, 1/3 of the brain tissue was coronally cut to 4 mm thick, and fixed in 4% paraformaldehyde for 24 h, followed by a gradient of alcohol and xylene dehydration. Finally the sample was embedded in wax to prepare for sectioning into serial 5 μ m thick sagittal slices. Tissue sections were stained with H&E by the routine procedure, and the expression of OMgp in the brain tissues was assessed by an immunohistochemical SABC assay.

Histopathological examination and determination of OMgp

When the stained sections were observed with a light microscope, the cytoplasm from OMgp-positive cells showed brown particles. The LEICA QWIN image signal acquisition and analysis system was used for processing these results. Eight fields were randomly selected in the cortex and hippocampus of each slice, and then LEICA DC 300F software was applied to measure the average optical density; the average optical density reflects the immunohistochemical staining intensity, suggesting that the deeper the color, the stronger the expression. The average of 8 fields was used to calculate the average optical density, the average values of 2 slices from each sample were used to calculate the sample test result, and the average

values of the 8 animals from each sample group were used to calculate the group results.

Statistical analysis

All data were analyzed with GraphPad Prism 5 statistical software (GraphPad Software, Inc., San Diego, CA, USA). Measurement data are presented as mean \pm standard deviation (SD), and comparisons among groups used single factor analysis of variance (ANOVA), with significance levels of $P < 0.05$ and $P < 0.01$.

Results

The pathological changes in rat brain tissues

Under the microscope (Figure 1), rat brain tissues in the sham group showed significant levels of brain tissue structure, cell contours and a clear structure at all time points (Figure 1A–B). In the HIBD control group, 24 h after HI, the brain lesion was not obvious, the left side of the brain showed necrosis after 48 h, and after 72 h large areas of the left brain tissue also showed necrosis. As a result, the number of neurons decreased; nerve cells became disordered; cells degenerated, showed necrosis, and collapsed; and the cellular organizational structure was unclear. Cells swelled, the cell nuclear membrane and nucleolus were unclear, some cells were only a profile, as the cell contents were released, and there was infiltration of inflammatory cells. After 7 days, the proliferation of glial cells and apoptosis of nerve cells appeared around the lesions, apoptotic cells showed rounding and cell shrinkage, and were out of contact with neighboring cells, and the cell cytoplasm was darkly stained. After 14 days, there was formation of the glial scar around the cerebral cortex and hippocampus, while the nerve cells showed degeneration, necrosis, and inflammatory cell infiltration, and the proliferation of glial cells and apoptotic nerve cells appeared around the lesions (Figure 1C–D). The necrosis and degeneration of nerve cells from the androgen intervention group were less than that from the HIBD group, the nerve cells from the androgen intervention group were arranged in neat rows, and the structure of the nerve cells from the androgen intervention group were more complete than those in the HIBD group. There was no clear infarction in the androgen intervention group (Figure 1E–F).

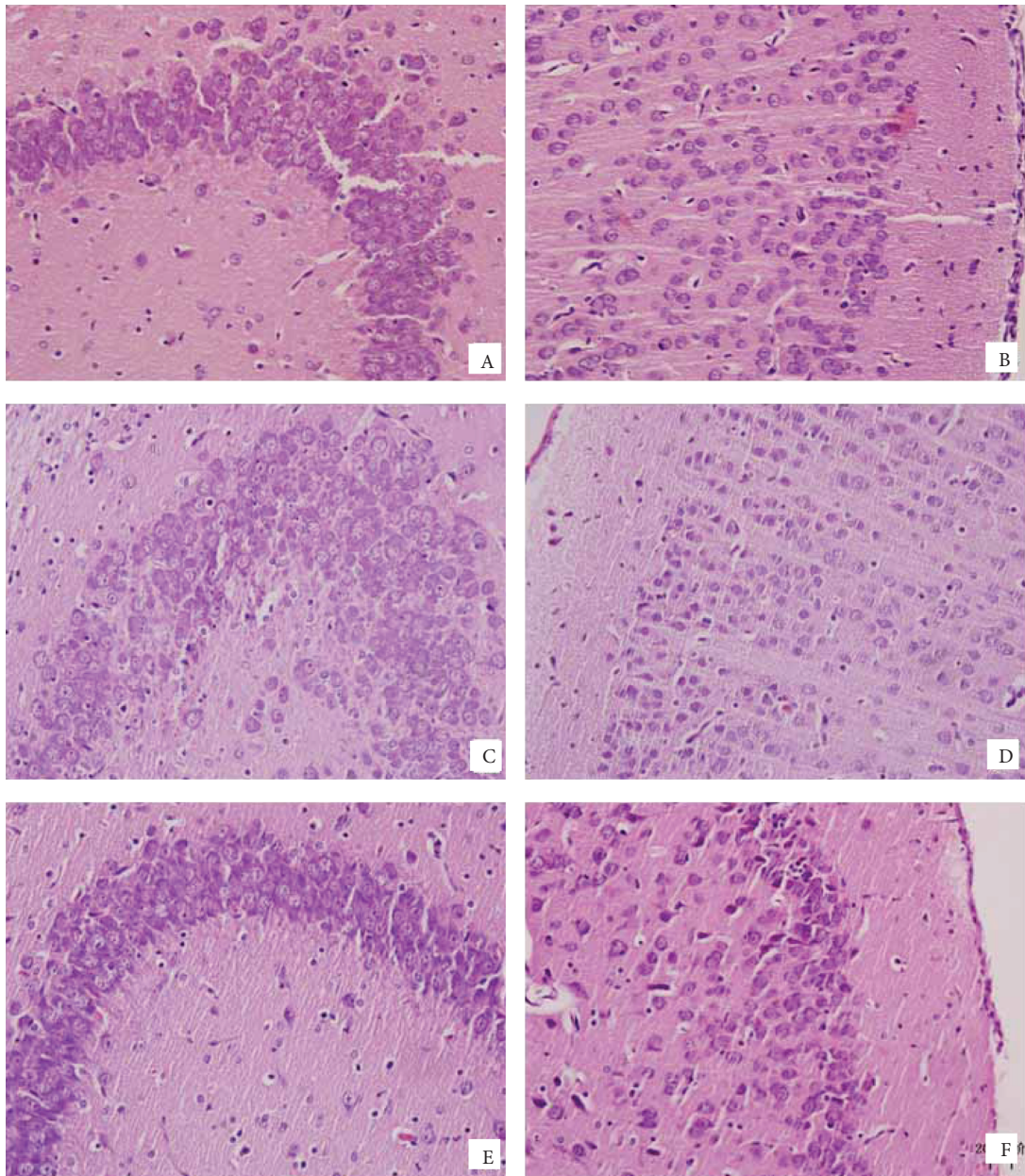


Figure 1. The pathological changes in rat brain tissues 7 days after HIBD.

HIBD = hypoxic-ischemic brain damage. Tissue sections of the tissues were H&E stained (magnification, $\times 400$). (A) The hippocampus of the sham group; (B) The cortex of the sham group; (C) The hippocampus of the control group (D) The cortex of the control group (E) The hippocampus of the TP treated group (F) The cortex of the TP treated group.

The expression of OMgp in rat brain tissues from different groups

OMgp expression was seen in the hippocampus and cortex in the sham group (Figure 2A–B) and showed changes in volatility over time, and the hippocampus and cortex had synchronized fluctuations. After 24

h, OMgp was expressed in the hippocampus and cortex, after 48 h the average optical density of OMgp increased, then decreased after 72 h, reached a peak after 7 days, and after 14 days the average optical density decreased. In the HIBD control group (Figure 2C–D), OMgp expression in the hippocampus and

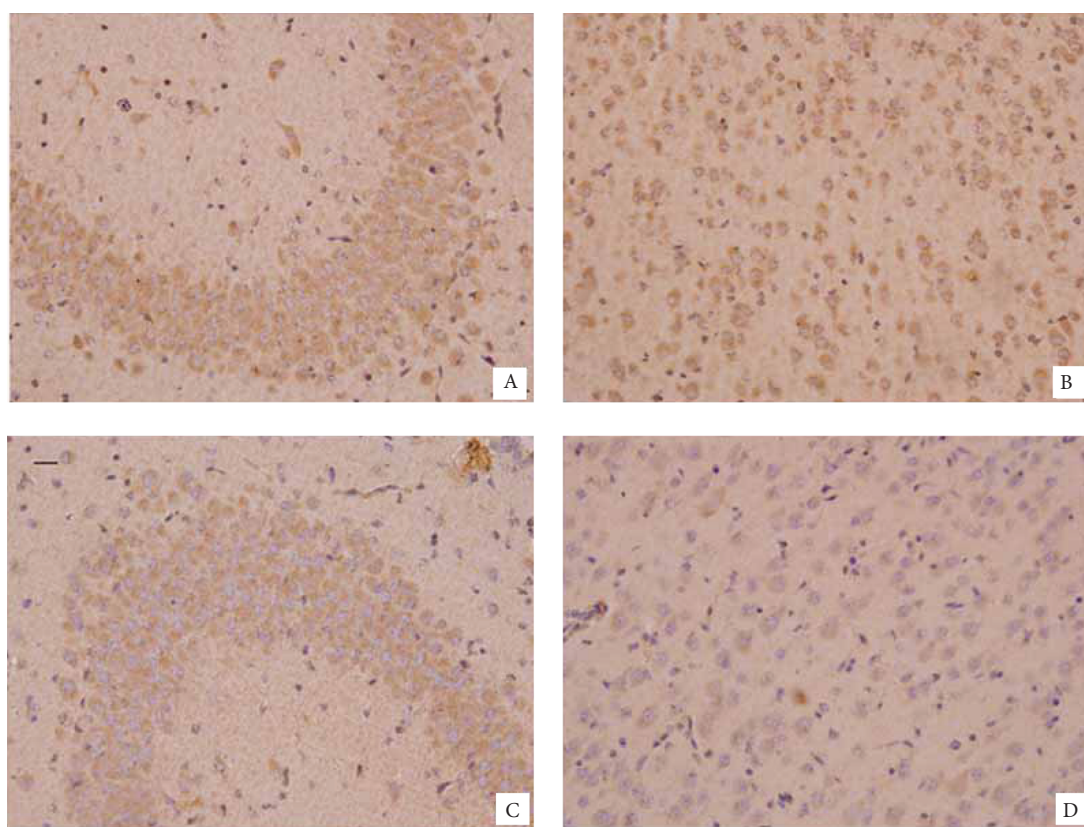


Figure 2. The expression of OMgp in rat brain tissues 7 days after HIBD.

HIBD = hypoxic–ischemic brain damage. Tissue sections were visualized by immunohistological staining (magnification, $\times 400$). (A) OMgp expression in the hippocampus of the sham group; (B) OMgp expression in the cortex of the sham group; (C) OMgp expression in the hippocampus of the control group (D) OMgp expression in the cortex of the control group.

cortex was higher than that of the sham group at each time point, and there was a statistically significant difference between the 2 groups ($P < 0.01$). Meanwhile, in the hippocampus and cortex from the 2 dose groups of the HIBD control group, OMgp expression fluctuated over time distinctly from the sham group, showing OMgp expression significantly increased 24 h after HI, which continued to rise at 48 h and 72 h, peaked at 7 days, and decreased at 14 days. These values were always higher than the average optical density values of the sham group at the same period. The average difference of OMgp expression between the 2 dose groups of the HIBD control groups, 30 mg and 120 mg, was not statistically significant ($P > 0.05$).

After androgen intervention, in both the hippocampus and cortex the average optical density

of OMgp expression in both dose groups, 30 mg and 120 mg, was significantly reduced at each time point compared to the HIBD group ($P < 0.01$). OMgp expression after treatment with either 30 mg or 120 mg of androgen was less than that of the sham group (Figure 3) ($P < 0.05$). Both androgen treated groups fluctuated in OMgp expression over time in the hippocampus and cortex, similar to the HIBD control group. At the same time, the difference in OMgp expression between the 2 androgen dose groups was not statistically significant ($P > 0.05$). The average optical density of OMgp expression during each period was slightly lower in the hippocampus than in the cortex, but the difference was not statistically significant ($P > 0.05$). OMgp expression from the left hippocampus and cortex of rats is shown in Tables 1 and 2.

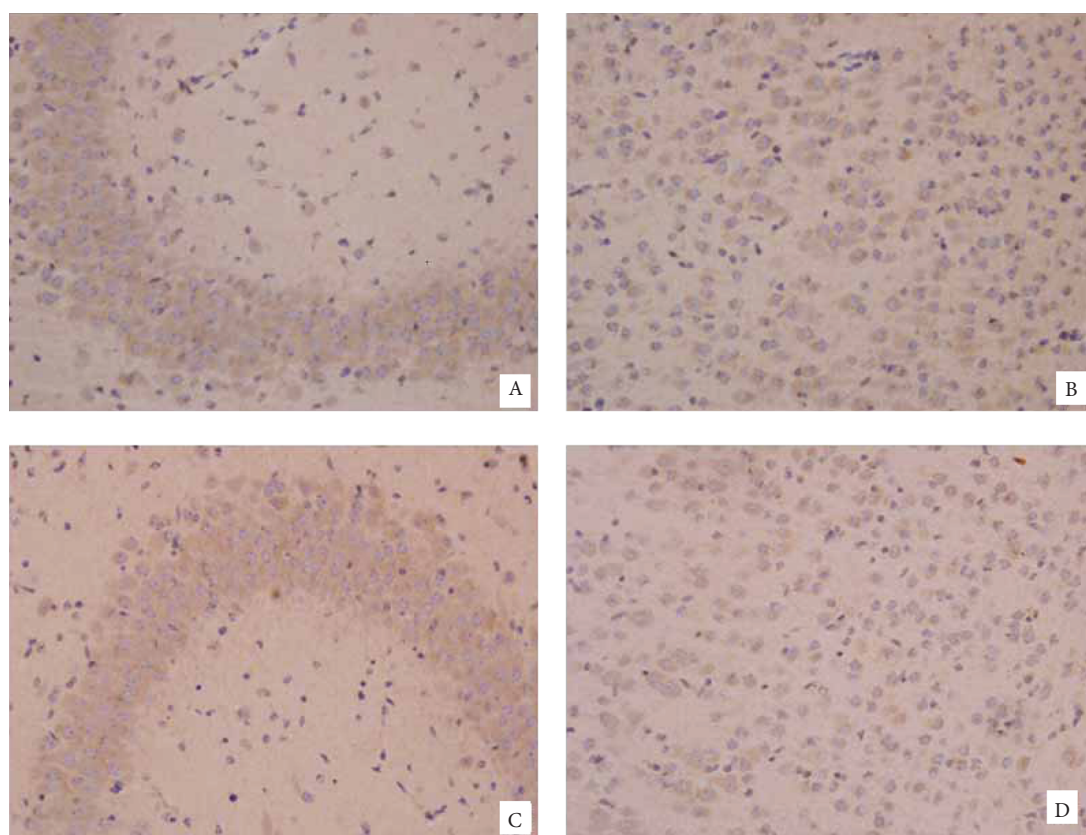


Figure 3. The expression of OMgp in the brain of rat treated with TP 7 days after HIBD.

HIBD = hypoxic–ischemic brain damage, TP = testosterone propionate. Tissue sections were visualized by immunohistological staining (magnification, ×400). (A) OMgp expression in the hippocampus after a dose of 30 mg/kg; (B) OMgp expression in the cortex after a dose of 30 mg/kg; (C) OMgp expression in the hippocampus after a dose of 120 mg/kg (D) OMgp expression in the cortex after a dose of 120 mg/kg.

Table 1. OMgp expression in the left hippocampus of rats after HI.

Group	The OMgp expression at different times after HI				
	24 h	48 h	72 h	7 days	14 days
Sham	0.177 ± 0.01	0.211 ± 0.01	0.202 ± 0.02	0.22 ± 0.02	0.186 ± 0.01
HIBD 30 mg	0.218 ± 0.02 ^a	0.232 ± 0.03 ^a	0.245 ± 0.02 ^a	0.250 ± 0.01 ^a	0.234 ± 0.01 ^a
HIBD 120 mg	0.212 ± 0.02 ^a	0.235 ± 0.02 ^a	0.244 ± 0.01 ^a	0.252 ± 0.01 ^a	0.229 ± 0.02 ^a
Androgen 30 mg	0.165 ± 0.02 ^b	0.153 ± 0.02 ^b	0.178 ± 0.01 ^b	0.180 ± 0.02 ^b	0.168 ± 0.01 ^b
Androgen 120 mg	0.164 ± 0.02 ^b	0.150 ± 0.03 ^b	0.174 ± 0.01 ^b	0.179 ± 0.01 ^b	0.167 ± 0.01 ^b

Values are presented as mean ± SD (n = 8). HI = hypoxic–ischemic; ^aP < 0.05, compared with the sham group; ^bP < 0.01, compared with the same dose group from HIBD.

Table 2. The OMgp expression in the left cerebral cortex of rats after HI.

Group	The OMgp expression at different time points after HI				
	24 h	48 h	72 h	7 days	14 days
Sham	0.188 ± 0.03	0.212 ± 0.01	0.203 ± 0.01	0.223 ± 0.02	0.189 ± 0.01
HIBD 30 mg	0.232 ± 0.02 ^a	0.234 ± 0.02 ^a	0.240 ± 0.02 ^a	0.251 ± 0.02 ^a	0.201 ± 0.02 ^a
HIBD 120 mg	0.229 ± 0.02 ^a	0.242 ± 0.05 ^a	0.243 ± 0.03 ^a	0.253 ± 0.01 ^a	0.205 ± 0.03 ^a
Androgen 30 mg	0.169 ± 0.04 ^b	0.151 ± 0.01 ^b	0.171 ± 0.02 ^b	0.178 ± 0.02 ^b	0.170 ± 0.02 ^b
Androgen 120 mg	0.165 ± 0.02 ^b	0.160 ± 0.02 ^b	0.169 ± 0.01 ^b	0.172 ± 0.02 ^b	0.168 ± 0.01 ^b

Values are presented as mean ± SD (n = 8). HI = hypoxic–ischemic; ^aP < 0.05, compared with the sham group; ^bP < 0.01, compared with the same dose group from HIBD.

Discussion

Currently, the cellular and molecular mechanism of HIBD is still unclear, and it remains difficult to find an effective method for the prevention and treatment of HIBD. The role of androgen and its receptor in hypoxic–ischemic brain injury have received increasing attention in recent years (15,16). Studies have shown that androgen in hypoxic–ischemic neurons can provide endogenous protection, and that adding exogenous androgen can enhance this protection (17–19). However, the neuroprotective role of androgen in the neonatal HIBD model has rarely been studied (20,21).

In the present study, using a neonatal rat HIBD model, we observed the expression of OMgp in newborn rat brain tissues at different time points after HI, and observed the expression of OMgp in newborn rat brain tissues after pretreating rats with different doses of testosterone propionate. The results showed that there was OMgp expression at all time points in the rat hippocampus and cerebral cortex in the sham group after surgery, the expression of OMgp over time showed changes in volatility, and fluctuations in OMgp levels in the hippocampus and cortex were synchronized.

OMgp expression levels in the HIBD control group were higher than that of the sham group at the same time points, indicating that the expression levels

of the neuroinhibitor OMgp respond quickly after HIBD. One week after the HI, expression of OMgp reached its peak, but was maintained at a high level afterwards. This suggests that the inhibiting protein OMgp maintains a high level of expression for a long time after HIBD, which may be a major cause for CNS regeneration problems after injury. However, our observations of OMgp expression in brain tissues showed a slight difference from previous experiments. Vourc'h used reverse transcriptase polymerase chain reaction to analyze OMgp expression in some regions of the postnatal rat CNS, and found that OMgp expression had been increasing until 6 weeks after birth. The proliferation of oligodendrocytes was also at a peak before and after birth, with its mature and immature myelin, and this synchronized with OMgp expression. OMgp expression levels were consistent with the myelination process, with expression peaking in the final stage of myelin formation (22). The main reasons for the inconsistency with our experiments may be that the time frame of observation in this experiment did not extend to 6 weeks postpartum. In addition, the observational methods used were different between the 2 studies, and these differences may reflect changes to developmental maturity after an injury to brain tissue. These results suggest that OMgp expression is slightly different between brain injuries that occur during development and brain damage that occurs after maturity. In order to use

OMgp expression to promote axonal regeneration of neurons in the central nervous system after injury, it is important to understand the differences in neuronal maturity and the type of brain damage.

To the best of our knowledge, there have been no previous studies of testosterone propionate dosage in neonatal rats. In preliminary experiments, the pre-test dose had been the single dose (25 mg/kg); later screening experiments dosed with 30 mg, 60 mg, 120 mg, 240 mg, or multiple increasing doses and it was determined that 24 h after HIBD the best dose was 30 mg/kg. In each dose group, there was only a significant difference between the 30 mg/kg and 120 mg/kg groups. Previous experiments did not show different time points after HI, and the time- and dose-dependence of the dynamic relationships between the androgen dose and brain protection was therefore not clear. Therefore, this experiment was based on our preliminary studies, expanding those studies by selecting 2 different doses (30 mg/kg, 120 mg/kg), and time points of 24 h, 48 h, 72 h, 7 days, and 14 days after HI were selected. In each dose group and at each time point there were 8 rats; otherwise treatment between the groups was kept constant to eliminate the extent of interfering factors and to make the results more comparable.

The experimental results showed that when pretreated with different doses of androgen, OMgp expression in the hippocampus and cortex declined at all the time points, which was statistically significant when compared to the control group HIBD at the same time point. These results also showed that, when comparing the 30 mg/kg and 120 mg/kg dose groups, OMgp expression decreased as the dose increased, although this trend was not statistically significant. OMgp expression was lower in the hippocampus than in the cortex, but the trend was the same as that of the cortex. Thus we had 2 conclusions: on the one hand, testosterone propionate can reduce the expression of OMgp of HIBD newborn rat brains, so as to reduce levels of inhibitory factors, and playing a role in brain protection; on the other hand, the protective effect of testosterone propionate at a dose of 30 mg/kg was best. The H&E staining showed that necrosis and degeneration of nerve cells

in the androgen intervention group was less than that in the HIBD group at each time point. Brain cells were arranged in neat rows, and the structure of the nerve cells was incomplete compared to the HIBD group, and there was no clear infarction. These results indicated that the hormone could reduce the expression of OMgp, preventing inhibition of axon regeneration after HIBD, promote the recovery of nerve cell morphology and axon regeneration, and thus played a neuroprotective role.

Recent studies have confirmed that after cerebral ischemia the glial cells are extremely active; there are changes in hypertrophy and hyperplasia, which may facilitate the growth and survival of neurons. Several experimental studies have reported that regulating glial cell morphology causes changes in its functions, which to some extent inhibits the response of the glial cells to injury (23,24). In this experiment, androgen intervention decreases the expression of OMgp in rat brain tissues at each time points after HI, and possibly by regulating the cell morphology, structure and function of the glial cells. Due to the regulatory role of glial cells, decreased expression of OMgp in glial cells at the site of brain injury results in decreased expression of inhibitory axonal growth factors and plays a neuroprotective role. This pathway may be one of the mechanisms for promoting the functional recovery of HIBD newborn rat brains. From the point of view of androgen dose after HI, androgen at 30 mg/kg dose had a significant protective effect on rat brain tissue. The average density measured solely from the hippocampus and cortex using androgen of 120 mg/kg dose was lower than that of the 30 mg/kg dose group, which appeared to inhibit the OMgp expression in those brain tissues, but these differences were not statistically significant.

In conclusions, After HIBD in neonatal rats, the expression of OMgp increased, reaching its peak 7 days after HI, and after 14 days the expression decreased to higher than normal levels. After androgen intervention, OMgp expression in brain tissues was reduced, which resulted in a reduction in the inhibition of axon growth, thereby promoting axonal regeneration and playing a neuroprotective role. Androgen treatment at 30 mg/

kg significantly suppresses the expression of OMgp in the brain. Androgen intervention can promote the recovery of nerve cell morphology after HIBD and improve the brain's structure, with a significant neuroprotective effect.

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