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Effects of androgen on the expression of brain aromatase cytopigment and nerve growth factor in neonatal rats with hypoxic-ischemic brain damage

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Abstract: **Objective** To study the effects of androgen on the expression of aromatase cytopigment P450 (AROM) and nerve growth factor (NGF) in the brain and brain ultrastructure in neonatal rats with hypoxic-ischemic brain damage (HIBD) in order to investigate the mechanism underlying the protective effect of androgen against HIBD. **Methods** Ninty-six seven-day-old Sprague-Dawley rats were randomly divided into three groups: sham-operation, HIBD and androgen treatment (n = 32 each). HIBD was induced by the ligation of left common carotid artery and hypoxia exposure. The rats in the androgen treatment and the HIBD groups were injected intraperitoneally with testosterone propionate (25 mg/kg) and arachis oil respectively immeadiatedly after hypoxia-ischemia (HI). After 24 and 72 hrs and 7 and 10 days of HI, AROM and NGF expression in the cortex and the hippocampus was detected with the immunohistochemical method. The ultrastructural changes of neurons in the cortex and the hippocampus were observed under a transmission electron microscope. **Results** Nerve cells of the HIBD group showed obvious injuries including cell organ decreasing, cellularoedema, nuclear swelling, chromatic agglutination, mitochondria decreasing and swelling, as well as an increase in apoptotic cells. Compared with the HIBD group, the nerve cells in the androgen treatment group had integrated nuclear membrane, well-distributed chromatin and abundant cell organs, and less cell apoptosis and increased axon regeneration. There was a positive expression of NGF and AROM in the brain cortex and the hippocampus in the HIBD group 24 hrs after HI. The expression of NGF and AROM increased significantly 72 hrs after HI, peaked 7 days after HI and then began to decrease but remained at a higher level than that in the sham-operation group 10 days after HI. The NGF and AROM expression in the cortex and the hippocampus in the androgen treatment group was significantly higher than that in the sham-operation and the HIBD groups 72 hrs, and 7 and 10 days after HI. **Conclusions** Androgen treatment can promote axon regeneration and morphous recovery of nerons and decrease neural apoptosis in neonatal rats with HIBD. The neuroprotection of androgen is produced possibly through an increase in the expression of NGF and AROM in the brain.

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Key words: Brain hypoxia/ischemia; Androgen; Aromatase; Nerve growth factor; Neonatal rats

雄激素对缺氧缺血性脑损伤新生大鼠脑组织芳香化酶和神经生长因子表达的影响

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[**摘　要**] **目的**　观察雄激素对缺氧缺血性脑损伤(HIBD)新生大鼠皮质及海马区芳香化酶细胞色素 P450(AROM)与神经生长因子(NGF)表达及脑组织超微结构的影响,探讨雄激素的神经保护作用机制。**方法**　96 只 7 日龄 Sprague-Dawley 大鼠随机分为假手术组、HIBD 组和雄激素组,每组 32 只。雄激素组和 HIBD 组制作 HIBD 模型。缺氧缺血(HI)后两组动物分别给予腹腔注射丙酸睾丸酮(25 mg/kg)和等量的花生油。于 HI 后 24 h、72 h、7 d、10 d 观察各组海马和皮层神经元超微结构变化及 AROM 和 NGF 表达的变化。**结果**　电镜下可见 HIBD 组神经细胞水肿,细胞器减少,核肿胀,染色质凝集成块状,线粒体减少、肿胀,可见大量凋亡细胞。与 HIBD 组相比,雄激素干预组神经细胞排列较整齐,神经元核膜完整,染色质均匀,细胞凋亡少见,神经元轴突再生明显。HIBD 组 HI 后 24 h NGF 与 AROM 表达出现阳性反应,HI 后 72 h 明显增多,7 d 达高峰,10 d 后开始下降,但仍高于假手术组。

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雄激素组皮层和海马 NGF 与 AROM 的表达在 HI 后 72 h、7 d 和 10 d 明显高于 HIBD 组和假手术组,均有统计学意义($P<0.01$)。**结论** 雄激素干预可增加脑组织 NGF 和 AROM 的表达,促进神经细胞形态的恢复及神经元轴突再生,减少细胞凋亡,具有明显的神经保护作用。

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[关 键 词] 脑缺氧/缺血;雄激素;芳香化酶;神经生长因子; 新生大鼠

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Some research has shown that androgen has neuroprotective effects against hypoxic-ischemic brain damage (HIBD) [1]. However, the relevant mechanisms have not been fully identified. A HIBD model of seven-day-old Sprague-Dawley (SD) rat was prepared in this study. The protection of androgen against HIBD and its possible mechanisms were studied by examining the changes of aromatase cytopigment P450 (AROM), nerve growth factor (NGF) and ultrastructure in the cortex and the hippocampus of the brain in HIBD rats after androgen administration.

Materials and methods

Materials

Ninety-six seven-day-old first-class SD rats of both sexes, weighing 12-18 g, were provided by the Center of Experimental Animals at the Fourth Military Medical University. Testosterone propionate injection (25 mg in 1 mL) was provided by Tianjin Jinyao Amino Acids Co. Ltd.. NGF and AROM rabbit anti-rat antibody, IgGII goat anti-rabbit antibody, SABC reagent kit and DAB chromogenic reagent kit were provided by Wuhan Boshide Bioengineering Co. Ltd..

Methods

Preparation of HIBD model of neonatal rats

The rats were anesthetized with ether, placed on their backs and their limbs were fixed to the operation board. The rats were cut right in the middle of the neck and the left common carotid artery was freed. Then the cut was ligated and sealed with 6-0 surgical silk. After 2-3 hours recovery, the rats were placed in the self-made hypoxic room. Mixed air with 8% oxygen and 92% nitrogen was introduced into the room at a speed of 1-2 L/min and the concentration of oxygen was monitored by an oxygen detector. Two and half an hours later the rats were taken out from the hypoxic room.

The rats which were only cut right in the middle of the neck and had their left common carotid artery freed but not ligated, had a sham operation.

Grouping of the animals

The 96 neonatal rats were randomly assigned to three groups: sham-operation, HIBD and androgen-treated HIBD ($n=32$ each). The rats in the androgen-treated HIBD and the HIBD groups were injected intraperitoneally with testosterone propionate (25 mg/kg) and arachis oil respectively immeadiatedly after hypoxia-ischemia (HI).

Preparation of brain tissue slices and immunohistochemical staining

At each time interval of 24 and 72 hrs and 7 and 10 days after HI, six rats in the three groups were randomly chosen and sacrificed for the preparation of brain tissue samples. Each rat was anaesthetized and the chest was cut to expose the heart. A tube was intubated from the left ventricle to the aorta and the heart was perfused and fixed with 4% parafofmal-dehyde in 0.55 mol/L phosphate buffer. In reference to the "Rat brain Stereotaxic Atlas", the skull was cut with a coronal cut from the mammillary body at the level of optic chiasma in order to obtain the brain tissue. The brain tissues were fixed in the 4% paraformal-dehyde for a night and were embedded in paraffin. The slice was 5 μm thick.

The SABC method was used for immunohistochemical stainings of AROM and NGF. Observing under a light microscope, the cells that had yellow particle sediment in the cytoplasm and the nucleus were positive cells. LEICA QWIN image signal collected and analytic system was used for measuring the gray scale of immunohistomhemical positive cells. Under the 40 × field lens, 6 fields of nonoverlapping cerebral cortex or hippocampuses in each slice of the left brain were chosen and the AROM and NGF immunohistochemical positive cells were measured. The gray scale was classified into 256 grades (0-255). Grade 0 was the darkest and Grade 255 was the brigtest. The average gray value was responsible for the degree of immunhistochemical stainings. The lower the gray value was, the higher the NGF or AROM expression was.

Preparation of transmission electron microscope specimen

At each time interval of 24 and 72 hrs and 7 and 10 days after HI, two rats in the three groups were sacrificed for the preparation of brain tissue samples. Each rat was anaesthetized and the chest was cut to expose the heart. A tube was intubated from the left ventricle to the aorta and the heart was perfused and fixed with

glutaraldehyde in 2.5% mol/L phosphate buffer. The brain tissues in the same position in the three groups were obtained to make electron microscope specimens.

Statistical analysis

The measured data were inputted into Excel 7.0 software and analyzed with SPSS10.0 software. Data were shown as $\bar{x} \pm s$ for ANOVA analysis. Student's t test was used for the mean comparision of two samples. $P < 0.05$ was believed to be statistically significant.

Results

The changes of ultrastructure of nerve cells

The sham-operation group showed normal ultrastructure of nerve cells. Nerve cells of the ischemic side (left side) hemisphere in the HIBD group presented obvious injuries including cell organ decreasing, cellularoedema, nuclear swelling, chromatic agglutination, mitochondria decreasing and swelling, endocytoplasmic reticulum distension, and ribosome sheding. Massive apoptotic cells and apoptotic body were observed. The reactive hyperplasia of astrocytes in the cortex and the hippocampus in the left hemisphere of the rats began to increase 24 hours, further increased 48 hours after HI, and reached a peak 7 days after HI. In the androgen treatment group, the nerve cells had integrated nuclear membrane, well-distributed chromatin and abundant cell organs. Compared with the HIBD group, the number of glial cells did not change obviously, but axon regeneration increased and cell apoptosis decreased in the cortex and the hippocampus of the left hemisphere in the androgen treatment group. See Figures 1-2.

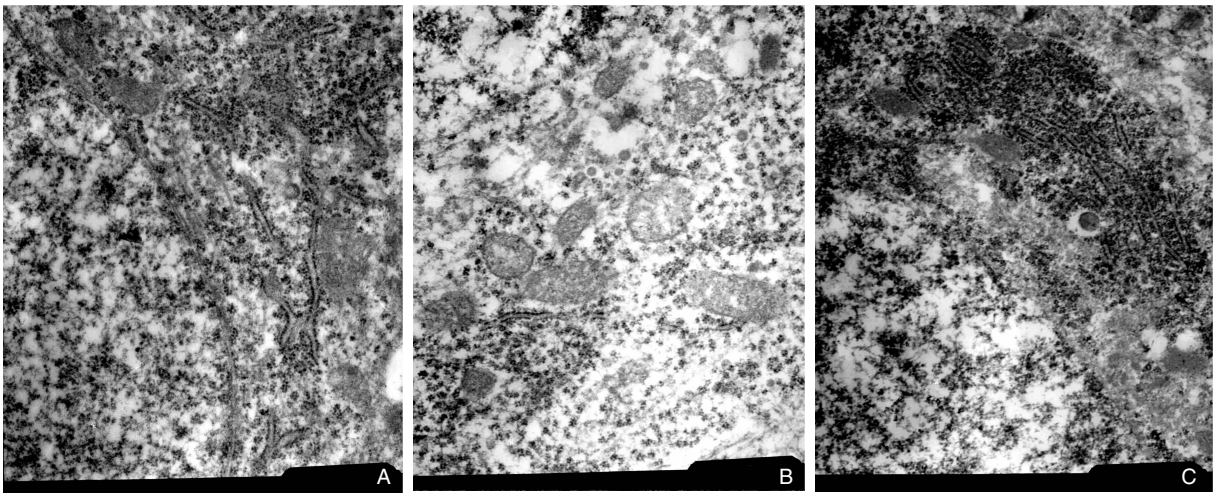

Figure 1 Ultrastructure of neurocytes 7 days after HI (×20 000) A: The sham-operation group showed normal ultrastructure of neurocytes. B: The neurocytes of the HIBD group presented obvious injuries including cell organ decreasing, cellularoedema, nuclear swelling, chromatic agglutination, and mitochondria decreasing and swelling. C: In the androgen treatment group, the neurocytes had integrated nuclear membrane, well-distributed chromatin and abundant cell organs.

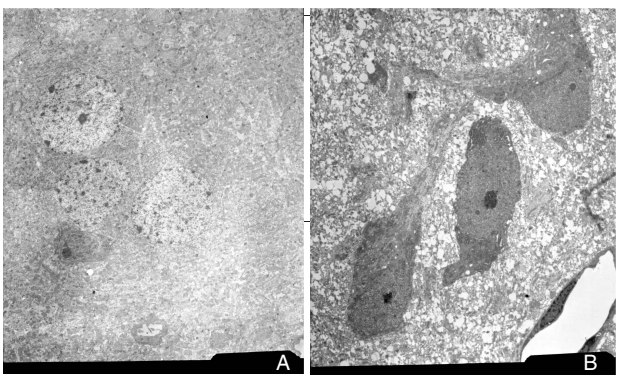

Figure 2 Axon regeneration in the cortex 7 days after HI (×2 500) A: There was little axon regeneration in the HIBD group. B: Axon regeneration increased in the androgen treatment group compared with the HIBD group.

The expression of NGF in neurocytes in the brain tissue

In the sham-operation group, there were few NGF immune positive cells in the cortex and the hippocampus of the left hemisphere. There was a positive expression of NGF in the brain cortex and the hippocampus in the HIBD group 24 hrs after HI. The expression of NGF in the HIBD group increased significantly 72 hours after HI, reached a peak 7 days after HI and then began to decrease but remained higher than the sham-operation group through 10 days aftert HI. NGF was presented as particle or streak in the cytoplasm and it had the same distribution as the cell organs. The nu-

cleus membrane was sometimes stained. The changed course of NGF expression in the cortex and the hippocampus in the androgen treatment group was similar to the HIBD group. However, the number of NGF immune positive cells in the androgen treatment group was significantly greater than that in the HIBD group 72 hours and 7 and 10 days after HI. See Table 1 and Figure 3.

The expression of AROM in the brain tissue

There were very few AROM immune positive cells in the cortex and the hippocampus of the left hemisphere in the sham-operation group. The expression of AROM immune positive cells in the cortex and the hippocampus of the left hemisphere in the HIBD group showed positive 24 hours after HI, increased significantly 72 hours after HI and reached a peak 7 days after HI. Then the AROM expression began to decrease but was higher than the sham-operation group 10 day after HI. The AROM expression in the cortex and the hippocampus of the left hemisphere in the androgen treatment group was significantly higher than that in the sham-operation and the HIBD groups 72 hours, 7 days and 10 days after HI. See Table 2 and Figure 4.

Table 1 NGF expression after HI (gray value) ($n=72, \bar{x} \pm s$)

Group	24 hrs		72 hrs		7 days		10 days	
	hippocampus	cortex	hippocampus	cortex	hippocampus	cortex	hippocampus	cortex
Sham-operation	168.08 ± 4.37	180.88 ± 2.02	167.16 ± 3.56	181.75 ± 2.15	165.93 ± 3.05	179.82 ± 3.05	166.58 ± 4.31	180.72 ± 4.21
HIBD	163.42 ± 3.27	178.22 ± 4.081	161.25 ± 5.31[a]	175.39 ± 2.38[a]	147.75 ± 4.12[a]	160.75 ± 3.23[a]	150.33 ± 2.87[a]	165.42 ± 5.10[a]
Androgen	159.25 ± 5.12[a]	178.39 ± 3.15	142.48 ± 4.76[a,b]	157.24 ± 2.11[a,b]	125.72 ± 5.56[a,b]	138.42 ± 4.30[a,b]	131.20 ± 3.63[a,b]	142.35 ± 3.42[a,b]

a: vs sham-operation group, $P<0.01$; b: vs HIBD group, $P<0.01$

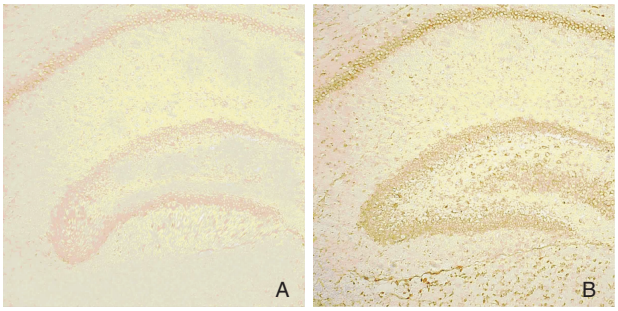

Figure 3 NGF expression in the hippocampus 7 days after HI (×100) NGF expression in the hippocampus 7 days after HI in the androgen treatment group (B) was significantly higher than that in the HIBD group (A).

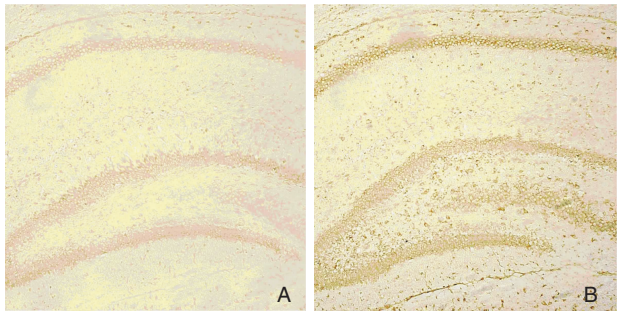

Figure 4 AROM expression in the hippocampus 7 days after HI (×100) AROM expression in the hippocampus 7 days after HI in the androgen treatment group (B) was significantly higher than that in the HIBD group (A).

Table 2 AROM expression after HI (gray value) ($n=72, \bar{x} \pm s$)

Group	24 hrs		72 hrs		7 days		10 days	
	hippocampus	cortex	hippocampus	cortex	hippocampus	cortex	hippocampus	cortex
Sham-operation	178.35 ± 2.21	195.78 ± 2.15	178.65 ± 4.15	197.42 ± 5.07	179.38 ± 2.51	197.32 ± 3.15	179.46 ± 3.12	196.85 ± 4.02
HIBD	176.95 ± 2.80	192.67 ± 3.18	170.08 ± 2.51[a]	189.92 ± 4.12[a]	150.23 ± 2.53[a]	180.11 ± 3.39[a]	165.83 ± 1.82[a]	185.78 ± 3.52[a]
Androgen	175.83 ± 1.82	193.22 ± 3.03	153.78 ± 3.10[a,b]	169.06 ± 5.79[a,b]	132.78 ± 3.27[a,b]	150.94 ± 3.62[a,b]	137.95 ± 2.75[a,b]	154.65 ± 4.31[a,b]

a: vs sham-operation group, $P<0.01$; b: vs HIBD group, $P<0.01$

Discussion

Research has shown that androgen can not only enhance antioxygenal ability and reduce injury caused by oxidative stress, but also can inhibit apoptosis of neurocytes after HI, thus providing a protective effect on brain tissues[1].

This study showed that nerve cells of the ischemic side hemisphere in the androgen treatment group had less severe injuries and fewer apoptosis than the HIBD group. Compared with the HIBD group, the number of

glial cells did not change obviously, but axon regeneration increased in the cortex and the hippocampus of the left hemisphere in the androgen ttreatment group. Astrocytes not only participate in the development of blood brain barrier, protect and nourish neurons, but also strengthen tolerance of nerve cells against low carbohydrates and hypoxia by gathering around ischemic brain tissues in the early stage. Furthermore, they have evident protections on nerve cells following ischemia by expressing neurotrophic factors, cytokines and their receptors. In addition, reactive astrocytes up-regulate cell surface molecules, including cell adhesion molecules and extracellular matrix, and provide groundplasm of axon regeneration. Cheng [2] reported that the reactive hyperplasia of astrocytes in the early stage of cerebral ischemia was a response to neural injury and it was related to neural survival. However, over-hyperplasia of glia cells may result in glial scar formation. The research indicated that androgen administration resulted in the changes in morphous of glial cells but did not change obviously the number of glia cells. Many experimental studies have shown that androgen can cause functional changes of glia cells through regulating their morphous and inhibits the reactiveness to injury of glia cells, thus reduces the formation of glical scar. This is hepful to functional reconstruction of regenerative neuraxons [3].

Many studies discovered that the expression of NGF increased 24 hours after HI and reached a peak 1-2 weeks after HI. The time course of NGF expression is associated with hypoxia time and the severity of HI. The more prolonged time of NGF expression is related to a more favorable protection[4]. This study observed that the expression of NGF in the cortex and the hippocampus of the left hemisphere began to increase 24 hrs after HI, increased significantly 72 hours after HI, reached a peak 7 days after HI and remained higher than that in the sham-operation group 10 days after HI. This was a response of the cortex and the hippocampus to HI insult, increasing NGF expression and thus providing neuronprotection. However the period and the level of NGF expression caused by HI were limited. This study showed that the expression of NGF in the androgen treatment group was significantly higher than that of the HIBD group 72 hours, 7 days and 10 days after HI. This suggested that androgen administration can increase significantly NGF expression in the brain and thus inhibits neural apoptosis in neonatal rats with HIBD. The result was consistant with that repoted by Bimonte-Nelson et al[5]. It was reported that androgen use following HI could regulate morphous and function of glia cells, including synthesising and expressing neurotrophic factors, which promote damaged nerves to regenerate [5]. So it is spectulated that androgen increases the expression of NGF by regulating the morphous, construction and function of glia cells.

AROM which catalysises androgen to estrogen, not only resides in placenta, sex gland and fatty tissue, but also in central nervous system. Nowadays it is considered that the expression of AROM decides estrogenic effective concentration in the brain, which influences cerebullar development, learning, memory, congnition and repairation.

Some research has shown that the AROM expression reached a peak at the late embroyo stage, decreased to low levels in the neonatal period and early stage of lives and further decreased after sexual maturation[6]. AROM is mainly expressed in special neurons and is not expressed in astrocytes. However in some special conditions, AROM can be induced to express in astrocytes [7]. In this study the expression of AROM in the cerebral cortex and the hippocamp began to increase 24 hours after HI, increased significantly after HI, reached a peak at 7 days, and then gradually decreased but remained higher than the sham-operation group 10 days after HI. The results showed that HI stimulation resulted in an increase of AROM expression in the brain tissue of damage area. In this study, androgen administration increased significantly AROM expression in the brain compared with the untreated HIBD group. Trainor et al [8] found that the activity of AROM is related to the level of estrogen in the brain which has obvious neurotrophy and neuroprotective effects. It is spectuated that the increased AROM expression promotes the conversion from androgen to destradiol after HI, increasing endogenous destradiol level in the brain. It is well known that reactively proliferated astrocytes following HIBD can excrete many kinds of bioactive compound, including growth factors and their receptors, cell kinase, antigen presentation mole-

cule and adhesion molecule, and up-regulate the activity of some enzymes. These responses are favorable to repairation of brain damage. With the increased expression and activity of AROM in the astrocytes in the damage area, estrogen synthesis increases, which promotes nerve regeneration[9].

In conclusion, androgen has protective effects against HIBD in neonatal rats, possibly through regulating the morphous, structure and function of astrocytes and increasing the expression of NGF and AROM in the brain.

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