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Neuroprotective effect of pAkt and HIF-1 α on ischemia rats

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ABSTRACT

Objective: To explore the neuroprotective effect of pAkt and HIF-1 α on ischemia rats. **Methods:** The rat model of cerebral ischemia which induced by permanent middle cerebral artery occlusion was established, Silybin were given respectively. The behavior was measured by modified Longa method, brain water content were measured by the dry-wet method. Infarct volume was measured by image analysis method, Akt, HIF-1 α , Bcl-2, Bax, NF- κ B protein expressions were detected by Western blotting. The Akt, HIF-1 α , Bcl-2, Bax, NF- κ B mRNA expression were detected by RT-PCR. **Results:** The control group, low-dose silibinin group and high-dose silibinin group showed paralytic of the left body of rats in various degrees, the brain water content increased and different infarction size. There was no abnormal of the neurobehavioral assessment and no cerebral infarction in the blank group. Compared with the control group, there was no significant improvement of neurological function ($t=1.341$, $P=0.188$) or significant changes of the infarct volume ($t=1.737$, $P=0.091$) in the low-dose silibinin group, while there was significantly improvement of the neurological function in the high dose silibinin group ($t=12.979$, $P<0.001$), and the infarct volume was significantly reduced ($t=23.503$, $P<0.001$), the difference had statistically significant. The brain water content of lesion side of the control group increased ($t=43.536$, $P<0.001$), while the brain water content of lesion side of the low-dose silibinin group and the high-dose silybin group were significantly reduced ($t=25.571$, $P<0.001$; $t=42.426$, $P<0.001$). The differences were statistical significance. The p-Akt 473, p-Akt 308, HIF-1 α , Bax, NF- κ B protein and the Akt, Bax, NF- κ B mRNA expression were increased of the control group, while the Bcl-2 protein and mRNA expression were decreased, the differences were statistically significant ($P<0.05$), there was no significant change of the Akt protein expression and HIF-1 α mRNA in the control group ($P>0.05$). In the high dose silybin group, the p-Akt 473, p-Akt 308, HIF-1 α , Bcl-2 protein and Akt, Bcl-2 mRNA expression were increased, while the Bax, NF- κ B protein and Bax, NF- κ B mRNA expression were decreased, the differences were statistically significant ($P<0.05$), there was no significant change of the Akt protein expression and HIF-1 α mRNA in the high dose silybin group ($P>0.05$). **Conclusions:** pAkt, HIF-1 α have neuroprotective effect on ischemia rats.

1. Introduction

In recent years, the incidence of ischemic cerebrovascular disease has increased year by year and become one important disease which is a threat to human health now. The local cerebral blood supply reduced or completely interrupted and can not be restored in time, the loss of

blood supply leads to the short supply of oxygen and glucose and cause the death of that area of brain tissue then results in cerebral infarction[1]. Cerebral ischemia damage process including inflammation, apoptosis and a series of complex pathological reactions, and Akt/HIF-1 α signal transduction pathways plays an important role in cell proliferation, growth, differentiation and apoptosis[2]. Research shows that in addition to anti-inflammatory, anti-apoptotic, anti-oxidation effects, silybin can also play a role of cerebral protection in ischemic brain injury[3]. In this study, we established the rat model of cerebral ischemia which induced by permanent middle cerebral artery

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occlusion and observed the Akt, HIF-1 α , Bcl-2, Bax, NF- κ B expression after administration of silybin, explore the neuroprotective effect of pAkt and HIF-1 α on ischemia rats and further study the possible mechanism.

2. Materials and methods

2.1. Materials

Silybin (Nanjing Zelang Medical Technology Co., Ltd.) as a neuroprotective drugs. 2,3,5-Triphenyltetrazolium chloride (Sigma, USA) as a coloring agent, Western blot reagent (Sigma, USA), RT-PCR Reagents (U.S. Promega Corporation).

2.2. Method

2.2.1. Experimental animals and grouping

Totally 80 male healthy adult Sprague-Dawley mice of clean grade were selected, average weighted (265.3 \pm 15.4) g. They were provided by the Experimental Animal Center of our Medical University, and were fed with standard diet and purified water. Rearing temperature was at 20–25 $^{\circ}$ C. The rats were randomly divided into four groups ($n=200$): blank group, control group, low-dose silybin group and high-dose silybin group. The behavioral assessments were performed 72 h after surgery for rats in each group. The animals were decapitated and the brain water content (BWC) were measured by the dry-wet method, stained with TTC then measured the infarct volume. Finally, the Akt, HIF-1 α , Bcl-2, Bax, NF- κ B expression were detected by Western blotting and RT-PCR.

2.2.2. Establish animal models

Middle cerebral artery occlusion were established with modified Longa method in the right side of rats[4].

2.2.3. Methods of administration

All drugs were administered by intragastric administration. Low-dose silybin group: 75 mg/kg silybin solution per rat daily, high-dose silybin group: 150 mg/kg silybin solution per rat daily, blank group and the control group received equivalent normal saline.

2.3. Evaluation

The praxiology evaluation was measured by modified Longa method[5]. 5 points: fall down when walking to the opposite side; 4 points: severe circling to the contralateral; 3 points: mild circling to contralateral; 2 points: contralateral forelimb flexion; 1 point: contralateral forelimb can not fully extend; 0: no neurological damage.

BWC were measured by the dry-wet method. The size of brain tissue infarct of every piece, the size on infarct side

and contralateral were obtained by Image-Pro Plus5.1.

Akt, p-Akt 473, p-Akt 308, HIF-1 α , Bcl-2, Bax, NF- κ B protein expression were detected by Western blotting. Akt, HIF-1 α , Bcl-2, Bax, NF- κ B mRNA expression were detected by RT-PCR.

2.4. Statistical analysis

All data involved in this study were analyzed with SPSS software. Neurobehavioral assessment, brain tissue water content, infarct volume, Western blotting, RT-PCR results were analyzed by ANOVA and SNK- q test, $P<0.05$ was regarded as statistically significant.

3. Results

3.1. Neurobehavioral assessment, BWC and infarct volume.

The control group, low-dose silibinin group and high-dose silibinin group showed paralytic of the left body of rats in various degrees and different infarction size. There was no abnormal of the neurobehavioral assessment and no cerebral infarction in the blank group. Compared with the blank group, the BWC of lesion side of the control group increased, the difference was statistically significant ($t=43.536$, $P<0.001$). Compared with the control group, there was no significant improvement of neurological function ($t=1.341$, $P=0.188$) or significant changes of the infarct volume ($t=1.737$, $P=0.091$) in the low-dose silibinin group, while the BWC of lesion side was significantly reduced, the difference had statistically significant ($t=25.571$, $P<0.001$). There was significantly improvement of the neurological function in the high dose silibinin group, BWC of lesion side was significantly reduced and the infarct volume was significantly reduced ($t=12.979$, $P<0.001$; $t=42.426$, $P<0.001$; $t=23.503$, $P<0.001$), the difference had statistically significant (Table 1).

3.2. Akt, p-Akt 473, p-Akt 308, HIF-1 α , Bcl-2, Bax, NF- κ B protein expression

Compared with the blank group, p-Akt 473, p-Akt 308, HIF-1 α , Bax, NF- κ B protein were increased of the control group after 72 hours of cerebral ischemia, while the Bcl-2 protein expression were decreased, the differences were statistically significant ($P<0.05$), there was no significant change of the Akt protein expression and HIF-1 α mRNA in the control group ($P>0.05$).

Compared with the control group, p-Akt 473, p-Akt 308, HIF-1 α , Bcl-2 protein and Akt, Bcl-2 mRNA expression of the high dose silybin group were increased, while the Bax, NF- κ B protein and Bax, NF- κ B mRNA expression were decreased, the differences were statistically significant

Table 1.

Neurobehavioral scores, BWC, infarct volume results of rats 72 hours postoperative(mean±SD).

Groups	n	Neurobehavioral score (points)	BWC (%)	Infarct volume (%)
Blank group	20	0.00±0.00	78.01±0.77	0.00±0.00
Control group	20	4.08±0.28	87.11±0.53*	47.50±2.45
Low dose silibinin group	20	3.96±0.29	82.27±0.66#	46.11±2.61
High dose silibinin group	20	2.78±0.35#	78.01±0.77#	30.23±2.19#

Note: Compared with the blank group, * $P<0.001$; compared with control group, # $P<0.001$.

($P<0.05$), there was no significant change of the Akt protein expression and HIF-1 α mRNA in the high dose silybin group ($P>0.05$) (Figure 1).

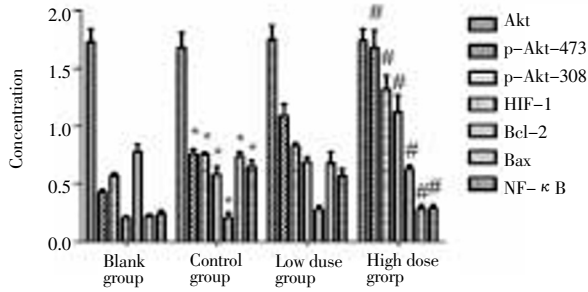


Figure 1. Protein expression results of the rats 72 hours postoperative. Compared with the blank group, * $P<0.05$; compared with control group, # $P<0.05$.

3.3. Akt, HIF-1 α , Bcl-2, Bax, NF- κ B mRNA expression

Compared with the blank group, Akt, Bax, NF- κ B mRNA expression were increased of the control group after 72 hours of cerebral ischemia, while the Bcl-2 mRNA expression were decreased, the differences were statistically significant ($P<0.05$), there was no significant change of the HIF-1 α mRNA expression in the control group ($P>0.05$).

Compared with the control group, Akt, Bcl-2 mRNA expression of the high dose silybin group were increased, while the Bax, NF- κ B mRNA expression were decreased, the differences were statistically significant ($P<0.05$), there was no significant change of the HIF-1 α mRNA expression in the high dose silybin group ($P>0.05$) (Figure 2, 3).

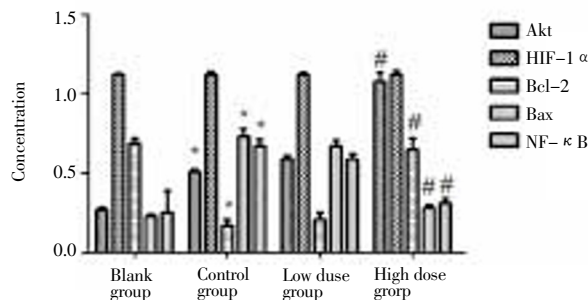


Figure 2. mRNA expression results of the rats 72 hours postoperative. Compared with the blank group, * $P<0.05$; compared with control group, # $P<0.05$.

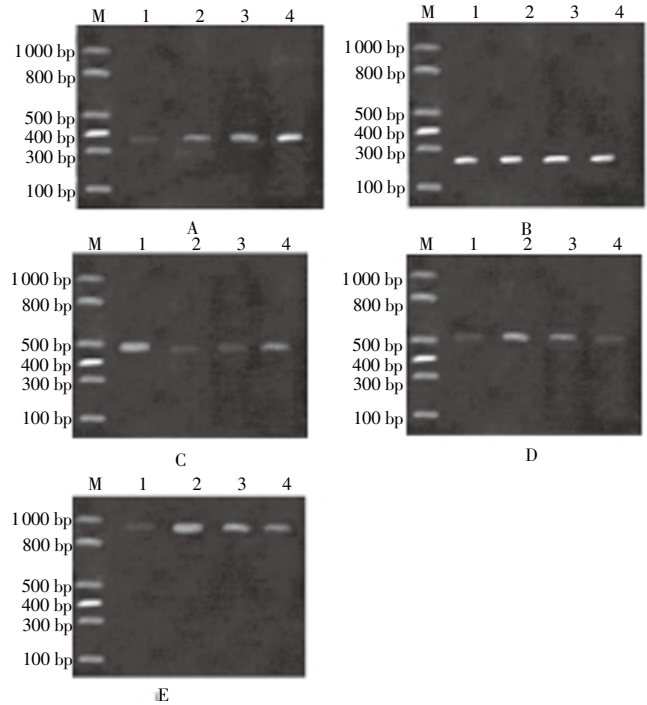


Figure 3. mRNA expression. A: Akt mRNA expression; B: HIF-1 α mRNA expression; C: Bcl-2 mRNA expression; D: Bax mRNA expression; E: NF- κ B mRNA expression.

4. Discussion

Ischemic cerebrovascular disease has a high disability rate and death rate, and has become one of the significant threats to human health and life. The disorder of circulatory system is an important reason for its formation. Establishment of the cerebral ischemia rat model is the key to study the neuroprotective effects of experimental rats. The middle cerebral artery occlusion model of rats has been universally accepted, which is a standard model to establish focal cerebral ischemia. The model can have cytotoxic effects in a few minutes, such as inflammation, apoptosis, nerve injury[7]. The experimental animal model was established strictly in accordance with the modified Longa, and to explore the the neuroprotective effect of pAkt and HIF-1 α on ischemia rats based on this model.

Silybin is a flavonolignan compound which can be

extracted from the medicinal plants of compositae. It has been proved that silibinin has anti-inflammatory, anti-apoptotic, anti-oxidation and other biological functions, Ryou *et al*[8] also found it has the anti-cancer and anti-cancer effect. In this study. The neurological function have been significantly improved of rats in the high dose group, and the BWC decreased to (78.32%±0.76%), infarct volume decreased by 36.4% compared with the control group. That suggested silibinin has a good neuroprotective effect on early ischemia, which may because silibinin can inhibit the NF- κ B and signal transduction, transcriptional activators and expression and activation[9].

Akt is a Ser / Thr protein kinase, the necessary prerequisite of Akt to play a catalytic role in cell survival is the phosphorylation of Ser473 sites and/or Thr308 sites, that is, the formation of p-Akt 473, p-Akt 308. Akt activation depends on phosphoinositide-dependent protein kinase synergies and the translocation from the cytoplasm to the plasma membrane[10]. In this experiment, compared with the blank group, after 72 hours of cerebral ischemia of rats in the control group, p-Akt 473, p-Akt 308 protein expression was significantly increased, in which p-Akt 473 increased by 76.7%, p-Akt 308 increase by 57.6%. Compared with the control group, the neurological function had a significant improvement of the silibinin high dose group, in which the p-Akt 473, p-Akt 308 protein expression increased more significantly, the expression is almost 2 times of the control group. This shows that the p-Akt 473, p-Akt 308 proteins play a protective role in the nervous system in experimental ischemia rat. Studies have shown that the activation of Akt is mainly through the promotion of the phosphorylation of downstream molecules such as the Bcl-2 apoptosis related family members, NF- κ B, mammalian target of rapamycin (mTOR), glycogen synthase kinase -3 to anti-apoptosis and promote cell survival[11].

mTOR is phosphoinositide kinase-related kinase family member, is also a Ser / Thr protein kinase, exist in the cytoplasm[12]. Study found that the mechanism of mTOR activity regulation and control is very complex, and is related with a variety of signaling proteins regulation. In which the most important two signal transduction pathway were PI3K/Akt/mTOR signaling pathway and LKB1/AMPK/mTOR signaling pathway[13]. Sato *et al*[14] reported that Akt/mTOR is closely related to HIF-1 α . Akt/mTOR's expression on HIF-1 α and activity regulation can be carried out in normoxic and hypoxic conditions. The detection of HIF-1 α in this experiment showed that compared with the blank group, the expression of HIF-1 α was significantly increased in the control group, especially in rats which nerve function

have improved significantly, and positively correlated with pAkt expression, which indicating that the Akt/mTOR/HIF-1 α neural pathway has the neuroprotective effect of pAkt and HIF-1 α on ischemia rats. Therefore, the role of Akt/mTOR/HIF-1 α neural pathway in ischemic brain disease has been very popular and become research hotspot.

HIF-1 α is a member of hypoxia inducible factor gene family. Its important feature is that can be only detected in the hypoxic environment. Its structure is divided into N-terminal domain and C-terminal domain[15]. Studies have shown that[16] C-TAD section has transcriptional gene activity. In ischemic hypoxia environment, HIF-1 α plays a key role in regulating acute and chronic physiological adaptation to brain tissue hypoxia. The mechanism may because HIF-1 α can regulate those genes which have the potential to protect and restore the role of neural genes, such as vasomotor control genes and erythropoietin genes[17], so that the body can adapt to hypoxia ischemia. Taking erythropoietin gene for example, the increasing of HIF-1 α expression will generate more hormone erythropoietin, which can promote a large number of red blood cell production into the blood, enhanced the blood vessel's ability to transport oxygen. At the same time the increase content of hemoglobin can enhance the oxygen transport capacity, thus can reduce the damage of brain tissue.

In this study, we also detected the Bcl-2, Bax, NF- κ B protein and mRNA expression of the experimental ischemia rats. Compared with the blank group, after 72 hours of the ischemic injury of rats in the control group, Bcl-2 protein and mRNA expression were significantly decreased, while the NF- κ B, Bax protein and mRNA expression were significantly increased, the differences were statistically significant ($P < 0.05$). NF- κ B inducing and activation in the case of cerebral ischemia, its signaling pathway in brain tissue cause neuronal death has become a research hotspot of ischemic animal experiments and clinical research at home and abroad[18,19]. Bcl-2, Bax are the most important genes in the regulation and control of ischemic neuronal apoptosis in rats, in which the Bcl-2 inhibits neuronal apoptosis while the Bcl-2 expression is positively correlated to the apoptosis of nerve sensitivity. Bax belongs to genes which can promote neuronal apoptosis and is a member of the Bcl-2 gene family[20]. Therefore, Bcl-2 expression increasing can reduce neuronal apoptosis, in contrast, Bax expression increasing can cause more neurons apoptosis. Consistent with this experimental results, p-Akt have the neuroprotective effect on ischemia rats by regulating Bcl-2, Bax.

Conflict of interest statement

We declare that we have no conflict of interest.

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