Original article

Neuroprotective effect of aspirin combined with ginkgolide injection on cerebral ischemic stroke rats and its effect on ERK12 signal pathway

Yan Zhang, Qin Song, Yuanxin Wang, Bingshu Li, Jiming Pan, Haiyan Yang*

Department of Emergency, Yantaishan Hospital, Yantai 264000, China

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A B S T R A C T

The main aim of this study was to evaluate the neuroprotective effect of aspirin combined with ginkgolide injection on cerebral ischemic stroke model rats and its effect on extracellular regulated protein kinase 1/2 (REK1/2) signaling pathway, and to clarify the possible mechanism of aspirin combined with ginkgolide injection on neuroprotective mechanism. Experimental rats were randomly divided into sham group, model group, aspirin group, ginkgolide group and combination group (aspirin + ginkgolide injection) (n = 20). The results revealed scores of neurological dysfunction and infarct volume in aspirin group, ginkgolide group and combination group rats were lower than those in model group (P < 0.05). Score of neurological dysfunction and the volume of cerebral infarction in combination group rats were lower than those in aspirin group and ginkgolide group (P < 0.05). Combination of aspirin and ginkgolide injection could better reduce brain water content, reduce apoptosis rate of cortical cells (P < 0.05), reduce expression levels of caspase-3, Bax and p-REK1/2 proteins in ischemic brain tissue (P < 0.05), and increase expression level of Bcl-2 protein than aspirin and ginkgolide injection alone (P < 0.05). In conclusion, the synergistic neuroprotective effect of aspirin and ginkgolide injection on cerebral ischemic stroke rats is better than that of aspirin and ginkgolide injection alone. The mechanism of action may be that the two compounds can play a synergistic role and inhibit the activation of REK1/2 signaling pathway, thus inhibiting apoptosis of nerve cells and exerting neuroprotective effect.

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1. Introduction

Cerebral ischemic stroke is a common cerebrovascular disease. After cerebral ischemia occurs, brain tissue is in a decompensate state of ischemia, hypoxia and even necrosis due to reduction or interruption of blood perfusion (Johnston et al., 2018; Zhao et al., 2019). The pathogenesis of stroke is relatively complex, and nerve cell apoptosis is one of them. Ischemic brain injury can cause ischemia and hypoxia in brain tissue, thus causing apoptosis and necrosis of nerve cells and neurological dysfunction (Zhou et al., 2014; Oechmichen and Meissner, 2006). Reducing apoptosis of nerve cells has neuroprotective effect on ischemic brain injury (Gencpinar et al., 2011; Wang et al., 2015). The mechanism of apoptosis is very complex and is regulated by some specific apoptosis regulatory genes in cells. Changes in expression of Bcl-2 and Bax gene are considered to be main molecular mechanism of apoptosis after cerebral ischemia (Li et al., 2015; Theron et al., 2013). Caspase-3 is the final executor of apoptosis, and occupies the core position in the cascade reaction of apoptosis (Wang et al., 2005). REK1/2 signaling pathway is important in brain tissue injury (Huang et al., 2017). Cerebral ischemia injury can activate REK1/2 signaling pathway and phosphorylate REK1/2. p-REK1/2 can mediate inflammatory reaction and apoptosis of nerve cells. Medicine can inhibit activation of REK1/2 signaling pathway, thus inhibiting apoptosis of nerve cells and exerting neuroprotective effect (Wang et al., 2013; Steinmetz et al., 2004).

Aspirin is a non-steroidal anti-inflammatory medicine with broad spectrum pharmacological effects and multiple sites of action, which has been widely used to treat diseases (Larsson et al., 2006). Ginkgolide injection is a traditional Chinese medicine composed of effective components of Ginkgo biloba, which has the effects of promoting blood circulation, removing blood stasis, dredging channels and activating collaterals (Marcilhac et al., 1998; Moon et al., 2011; Nabavi et al., 2015;
At present, the synergistic neuroprotective effect of aspirin and ginkgolide injection on cerebral ischemic stroke model in rats is still unclear. The purpose of this study was to evaluate the neuroprotective effect of aspirin combined with ginkgolide injection on cerebral ischemic stroke in rat model and its effect on extracellular regulated protein kinase 1/2 (REK1/2) signaling pathway, and to analyze the possible neuroprotective mechanism of aspirin combined with ginkgolide injection.

2. Materials and methods

2.1. Experimental animals

One hundred healthy, clean, 8–9 week old, male, body mass about 300 g SD rats were selected and maintained in animal cages. Healthy food and water were provided and maintained in well established conditions.

2.2. Rat grouping and treatment

One hundred rats were divided into sham group, model group, aspirin group, ginkgolide group and combination group (aspirin + ginkgolide injection) with 20 rats in each group. Aspirin group, ginkgolide group and combination group rats were used to establish cerebral ischemic stroke rat models. Aspirin group rats were given aspirin (40 mg/kg) by intraperitoneal injection 30 min before modeling. Ginkgolide group rats were given intraperitoneal injection of ginkgolide injection (2.5 mg/kg) 30 min before modeling. Combination group were injected intraperitoneally with aspirin (40 mg/kg) and ginkgolide (2.5 mg/kg) 30 min before modeling.

2.3. Establishment of cerebral ischemic stroke rat model

According to Laing et al. (1993), the middle cerebral artery embolization in rats was used to establish a focal cerebral ischemic rat model. This work has been approved by the Institutional Ethical Committee.

2.4. Rat neurological dysfunction score

Twenty-four hours after modeling, rats in each group were scored with a 5-point scoring standard (Wang et al., 2015) for neurological dysfunction, with 0 points for no neurological dysfunction symptoms. When lifting the tail, the flexion of the left forelimb cannot be extended to 1 point. When walking, the symptoms of rear-end collision or circling to the left are 2 points. It is difficult to walk, and the body topples to the left for 3 points, 4 points for being unable to walk or in a coma.

2.5. Measurement of cerebral infarction volume in rats

After 24 h of modeling, 5 rats in each group were anesthetized deeply with chloral hydrate. The brain tissue was exposed and completely removed. The brain tissue was continuously sectioned from the forehead along the coronal face to the back, with a thickness of about 2 mm. The sections were immediately applied into TTC staining solution and incubated for 30 min. The staining conditions of the brain tissue of each group of rats were observed. Bright red was considered as normal brain tissue and pale was considered for analysis. Water content of brain tissue = (brain tissue mass-dry mass of brain tissue)/wet mass of brain tissue × 100%.

2.6. Determination of water content in brain tissue

After 24 h of modeling, 5 rats in each group were anesthetized after perfusion of cold PBS solution through the left ventricle. Brain tissue was taken out, the wet mass of brain tissue was weighed, the brain tissue was baked in an oven for 24 h, and the dry mass of brain tissue was taken out and weighed. The mean value was considered for analysis. Water content of brain tissue = (brain tissue mass-dry mass of brain tissue)/wet mass of brain tissue × 100%.

2.7. Determination of the apoptosis rate of cortical cells

After 24 h of modeling, 5 rats in each group were anesthetized with chloral hydrate, and the intact brain tissue was taken out and fixed in paraformaldehyde for 24 h. Brain tissue was dehydrated, paraffin-encapsulated and sectioned. The paraffin sections were dewaxed, added citrate buffer for antigen repair, sealed with sheep serum for 2 h. Then primary antibody TUNEL (dilution ratio 1: 600) was added and incubated overnight, then secondary antibody (dilution ratio 1: 1000) was added and incubated for 2 h. Then DAPI dye solution was added and incubated for 8 min. After fluorescence was observed under fluorescence microscope, 5 ischemic brain tissues were photographed under 200-fold visual field, and the apoptosis rate of cortical cells was calculated by ImageJ software. Apoptosis rate was calculated using the following formula:

Apoptosis rate (%) = number of apoptotic cells/total number of cells × 100

2.8. Determination of the expression level of target protein

After 24 h of modeling, 5 rats in each group were anesthetized deeply with chloral hydrate. The cerebellum and occipital lobe were removed, and the cortical part of the cerebral tissue around ischemia was taken and added into a homogenizer containing protein lysate for homogenization. Then the homogenate was transferred to the EP tube and centrifuge for 15 min at 10,000 rpm. Then the clear supernatant was taken and total protein concentration in brain tissue was detected by BCA method, and polyacrylamide gel was used for the separation of proteins. After loading, electrophoresis, membrane transfer and blocking were performed. Primary antibodies (caspase-3, Bax, Bcl-2, REK1/2 and p-REK1/2) were added for overnight incubation, and β-actin was used as internal reference, it was reheated for 1 h, and secondary antibodies were added and for 1 h. Finally ECL luminescent solution was added to emit light. The gray value of protein bands in each group was measured by Quantity One software to calculate expression level of target protein. Target protein expression level = target protein gray value/β – actin band gray value.

2.9. Statistical analysis

SPSS 25.0 was used for statistical analysis. Measurement data were expressed by means of single factor analysis of variance and LSD-t test. P < 0.05 was considered as statistically significant.

3. Results

3.1. Comparison of neurological dysfunction score and cerebral infarction volume in each group

Sham group rats had no neurological dysfunction and cerebral infarction. Model group rats showed obvious neurological dysfunc-
tion and cerebral infarction, indicating that the cerebral ischemic stroke rat model was successfully established.

The scores of neurological dysfunction and infarct volume in aspirin group, ginkgolide group and combination group rats were lower than those in model group \((P < 0.05)\) (Fig. 1). The score of neurological dysfunction and the volume of cerebral infarction in combination group rats were lower than those in aspirin group and ginkgolide group \((P < 0.05)\) (Fig. 2).

### 3.2. Comparison of water content in brain tissue of rats in each group

Compared with sham group rats, water content of brain tissue in model group rats increased \((P < 0.05)\); Compared with model group, the water content of brain tissue of aspirin group, ginkgolide group and combination group rats decreased \((P < 0.05)\). Water content of brain tissue in synergistic group rats was lower than that in aspirin group and ginkgolide group, respectively \((P < 0.05)\) (Fig. 3).

### 3.3. Apoptosis rate of cortical cells in ischemic lateral brain tissue of rats in each group

Compared with sham group, apoptosis rate of cortical cells in ischemic brain tissue of model group rats increased \((P < 0.05)\); compared with the model group, apoptosis rate of cortical cells in ischemic brain tissue of aspirin group, ginkgolide group and combination group rats \((P < 0.05)\). Apoptosis rate of cortical cells in ischemic brain tissue of combination group rats was lower than that of aspirin group and ginkgolide group \((P < 0.05)\) (Fig. 4).

### 3.4. Expression levels of caspase-3, Bax and Bcl-2 proteins in ischemic lateral brain tissue of rats in each group

Compared with Sham group, expression level of caspase-3 and Bax protein increased \((P < 0.05)\) and expression level of Bcl-2 protein decreased \((P < 0.05)\) in ischemic brain tissue of Model group rats.

Compared with model group, expression of caspase-3 and Bax protein decreased \((P < 0.05)\) and expression of Bcl-2 protein increased \((P < 0.05)\) in ischemic brain tissue of aspirin group, ginkgolide group and combination group rats. The expression of caspase-3 and Bax protein in ischemic brain tissue of synergistic group rats was significantly lower than that of aspirin group and ginkgolide group \((P < 0.05)\), and the expression of Bcl-2 protein was significantly higher than that of aspirin group and ginkgolide group \((P < 0.05)\) (Fig. 5).

### 3.5. Expression levels of REK1/2 and p-REK1/2 proteins in ischemic lateral brain tissues of rats in each group

There was no difference in expression of REK1/2 protein in ischemic brain tissue of sham group, model group, aspirin group, ginkgolide group and combination group rats \((P > 0.05)\). Compared with Sham group, the expression level of p-REK1/2 protein in ischemic brain tissue of model group rats increased \((P < 0.05)\). Compared with model group, the expression of p-REK1/2 protein in ischemic brain tissue of aspirin group, ginkgolide group and combination group rats decreased \((P < 0.05)\). Expression level of p-REK1/2 protein in ischemic brain tissue of combination group
rats was lower than that of aspirin group and ginkgolide group (P < 0.05) (Fig. 6).

4. Discussion

Inhibition of apoptosis of ischemic peripheral neurons can effectively improve brain injury. Caspases is the most important protease in apoptosis and the most critical apoptosis executing protease, also known as death protease, which can coordinate many protein factors to further regulate apoptosis, change its structure and promote apoptosis. Caspases is the key executor and main effectors of apoptosis. Its activation represents that apoptosis enters the irreversible damage stage (Thornberry and Lazebnik, 1998). Caspase-3-mediated signaling pathway is the only way to trigger apoptosis (Li et al., 2009). Bax, as an inactive monomer form, exists in cytoplasm. After receiving the stimulation of apoptosis signals, Bax can undergo molecular conformation changes, shift and insert into mitochondrial outer membrane, destroy mitochondrial membrane, and oppose Bcl-2, an inhibitor of apoptosis protein, etc. to promote the occurrence of apoptosis by affecting mitochondrial signaling pathways. Bcl-2 family proteins are a class of apoptosis proteins. Anti-apoptosis factor Bcl-2 has the effect of inhibiting cell apoptosis, and can inhibit cell apoptosis by controlling membrane potential such as inhibiting glutathione leakage (Carbott et al., 2002).

This study showed that ischemic stroke model rats have obvious neurological dysfunction and cerebral infarction foci. Water content of brain tissue increased, and apoptosis rate of cerebral cortex cells on ischemic side increased. Expression level of caspase-3 and Bax protein increased and expression level of Bcl-2 protein decreased in ischemic brain tissue, indicating that cerebral ischemia can cause brain tissue edema. Expression level of Bax and Caspase-3 increased while the level of Bcl-2 decreased, which resulted in neuronal apoptosis and neurological dysfunction. The expression level of Bax and Caspase-3 increased, and the expression level of Bcl-2 decreased, which resulted in neuronal apoptosis and neurological dysfunction.
ERK is an important signal transduction system that mediates cell response in the body. It is commonly found in a variety of mammals. The signal transduction pathway can gradually expand cell signals into cells, connect extracellular stimuli with effectors in cytoplasm and nucleus in cells, and promote cell growth and differentiation (Park et al., 1999; Wei et al., 2007). At present, ERK12 is the most widely studied ERK signaling pathway in clinical research. Results of this study showed that the expression level of p-REK1/2 protein in brain tissue of cerebral ischemic stroke model rats increased, indicating that cerebral ischemia can activate REK1/2, and the activated REK1/2 mediates neuronal apoptosis and leads to neurological dysfunction.

Aspirin and ginkgolide injection have protective effects on ischemic brain injury. It is not very clear how effective aspirin and ginkgolide injection are combined and signal pathway. This study showed that synergistic effect of aspirin and ginkgolide and these could reduce water content in brain tissue, reduce apoptosis rate of cortical cells in ischemic brain tissue, reduce expression levels of caspase-3, Bax and p-REK1/2 proteins in ischemic brain tissue, and increase the expression level of Bcl-2 protein than aspirin and ginkgolide injection alone. This shows that aspirin combined with ginkgolide injection can exert synergistic effect, jointly inhibit the activation of REK1/2 signaling pathway, inhibit nerve cell apoptosis, reduce cerebral edema, and reduce the apoptosis rate of cerebral cortical cells, thus reducing brain injury, neurological dysfunction and exerting neuroprotective effect.

5. Conclusions

The neuroprotective effect of aspirin combined with ginkgolide injection on cerebral ischemic stroke rats is better than that of aspirin and ginkgolide injection alone. The mechanism of action may be that the two can play a synergistic role and jointly inhibit the activation of REK1/2 signaling pathway through inhibition, thus inhibiting apoptosis of nerve cells and exerting neuroprotective effect.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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