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Original article

Intervention effect of total flavonoids of ilex pubesceus on tolerant rat models under cerebral anoxia

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ABSTRACT

Objective: To observe the intervention effect of total flavonoid of ilex pubesceus on animal models of cerebral ischemic tolerance. **Methods:** A rat model of global-focal cerebral ischemic tolerance was established by blocking bilateral common carotid artery blood flow and occluding left middle cerebral artery using thread-occlusion method. After the first operation, the Ginaton group and large-dosage, medium-dosage and small-dosage groups of total flavonoid of ilex pubesceus were given intragastric administration of corresponding drugs. The sham-operated group, pretreatment model group and ischemia-reperfusion group were given intragastric administration of the same volume of normal saline, 1 time a day, and administrated for 4d. At 24 h after the second operation, the neurological deficit was assessed, the whole blood viscosity, plasma viscosity, iNOS activity as well as NO level, IL-1β content and TNF-α content in the brain tissue of the rats were determined, and the morphological changes of brain tissue of the rats were observed by HE staining. **Results:** All the rat models of cerebral ischemic tolerance were established successfully. The total flavonoid of ilex pubesceus can obviously or significantly reduce the neurological deficit score, whole blood viscosity and plasma viscosity, obviously or significantly increase the NO level in the brain tissue of the rats, and significantly reduce the pathological damage of brain tissue of the rats. But compared with the ischemia-reperfusion group, the total flavonoid of ilex pubesceus can significantly or obviously increase the iNOS activity, IL-1β content and TNF-α content in the brain tissue of the rats.

Cerebral ischemic tolerance (IT) means that one or more times of sublethal transient ischemic injuries (ischemic preconditioning, IP) in advance can improve brain tissue's resistance to more serious recurring ischemic injury and reduce brain injury (Dirnagl et al., 2003). The mechanism of cerebral ischemic tolerance is not clear until now. In recent years, many bioactive substances that show increased expression or activity after ischemia reperfusion and play a cytotoxic role in ischemia-reperfusion injury, such as inflammatory mediators NO and iNOS as well as inflammatory cytokines TNF-α and IL-1β, play an important role in the induction of BIT (Jing, 2017; Yiming et al., 2017; Huiqing et al., 2003).

Traditional Chinese medicine (TCM) has the characteristics of holistic concept, while traditional Chinese drug has the characteristics of having many ingredients and being applied to multiple targets. The intervention of cerebral ischemic injury may present multiple pathways, which can improve the tolerance to cerebral ischemia. The cerebral ischemia belongs to "Apoplexy" in traditional Chinese medicine. Traditional Chinese medicine holds that the occurrence of stroke is mainly related with the wind, fire, phlegm, blood stasis and deficiency, and is most closely related with blood stasis. The treatment principle of promoting blood circulation to remove blood stasis is usually used to treat stroke. It is the basic method to improve cerebral ischemic tolerance (Fan et al., 2012), which provides a basis for the intervention effect of traditional Chinese drug on cerebral ischemia. Modern medicine thinks that the etiology and pathogenesis of cerebral ischemia tend to micro thrombosis and hemodynamics, blood rheology disorders (Jing et al., 2015), which is consistent with the concept of "blood stasis"in traditional Chinese medicine that is the pathological basis of cerebral ischemia (Can et al., 2017). Traditional Chinese medicine has achieved good results in treating cerebral ischemia with

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the method of promoting blood circulation to remove blood stasis, which has been widely affirmed.

The total flavonoid of ilex pubesceus in this paper is one of the effective components extracted from the dried root of ilex pubesceus (Xiao et al., 2012). Ilex pubesceus has the effect of promoting blood circulation to open vessels, dispersing swelling and relieving pain, as well as clearing heat and detoxicating (Xiaoyan et al., 2016). Recent studies show that ilex pubesceus has a protective effect on cerebral vessels, but the studies on the effect of total flavonoid of ilex pubesceus on improving cerebral ischemic tolerance are insufficient. The another purpose of this experiment was to establish rat models of cerebral ischemic tolerance, to observe the changes of iNOS activity and NO level as well as IL-1β content and TNF-α content in the rat models of cerebral ischemic tolerance, and to explore the intervention effect of total flavonoid of ilex pubesceus on the rat models of cerebral ischemic tolerance.

1. Experimental materials

1.1. Experimental drugs

Total flavonoid of ilex pubesceus (Analytical Chemistry Laboratories, Henan University of Chinese Medicine), content: 60%;

Ginaton (Ginkgo Biloba leaves extracts) (Dr.Willmar Schwabe), Batch No.: 9900908.

1.2. Reagents

NO, NOS and Coomassie Brilliant Blue Protein Assay Kits (NanJing Jiancheng Bioengineering Institute), Batch No.: 20160810, 20160810 respectively;

IL-1β and TNF-α ELISA Kits (Shanghai Senxiong Technology Industry Co., Ltd.), Batch No.: 1610056, 1610058 respectively;

1.3. Instruments

KDC-160HR High Speed Refrigerated Centrifuge (Keda Chuangxin Co., Ltd., Zhongjia Branch);

Automatic Blood Rheology Detector (Chongqing Maike Instrument and Meter Co., Ltd.), Model: XLB201;

TGL-16G Desk Centrifuge (Shanghai Anting Scientific Instrument Factory);

UV-2000 UV–Vis Spectrophotometer (Yonica (Shanghai) Instruments Co., Ltd.).

1.4. Experimental animals

SD rats, male, cleaning degree, weight 280–300 g; provided by Hebei Laboratory Animal Center, Certificate No.: 907048.

2. Experimental methods

2.1. Animal grouping and administration

98 male SD rats with a weight of 280–300 g were randomly divided into seven groups: sham-operated group, pretreatment model group, ischemia-reperfusion group, Ginaton group (0.02 g/kg), large-dosage group of total flavonoid of ilex pubesceus, medium-dosage group of total flavonoid of ilex pubesceus, and small-dosage group of total flavonoid of ilex pubesceus (0.2 g/kg, 0.1 g/kg, 0.05 g/kg, respectively). 14 male SD rats in each group. The drug administration groups were given intragastric administration of corresponding drugs from 1d after pretreatment; the sham-operated group, pretreatment model group and ischemia-reperfusion group were given intragastric administration of the same volume of normal saline. The volume of drug for intragastric administration in all groups was 1 ml/100 g, 1 time a day, and administrated for 4d.

2.2. Method of making models

First, a global cerebral ischemia model was established: The cerebral ischemia preconditioning was performed for the rats using the improved method by Simon et al. (Simon et al., 1993). All the rats were fasted for 12 h before the operation, and then anaesthetized by intraperitoneal injection with 0.3 ml/100 g of 10% chloral hydrate. Lie them on their back to immobilize them and wipe the neck clean with alcohol, and then incise the middle neck to separate layer by layer and expose the bilateral common carotid artery (CCA). The bilateral CCA blood flow was occluded with an arteriole clip for 10 min, then the mydriasis of the rats was observed. The righting reflex of the rats disappeared, namely a global cerebral ischemia was produced. (For the rats in the sham-operated group, only the bilateral CCA was exposed without other treatment).

Second, a focal cerebral ischemia model was established: A middle cerebral artery occlusion (MCAO) model was established using the improved method by Koizumi et al. (Koizumi et al., 1986) and Nagasawa et al. (Nagasawa and Kogure, 1989). All the rats were fasted for 12 h after the last time administration for 1 h, and then anaesthetized by intraperitoneal injection with 10% chloral hydrate again. Lie them on their back to immobilize them to separate layer by layer and expose the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA), which adopted thread-ligating therapy to serve as standbys. The ECA and CCA were ligated. After the distal end of ICA was occluded with an artery clip, an incision was made at the bifurcation of ECA and ICA rapidly. A smooth nylon coated with silicone adhesive 1 mm from the head end (0.25–0.26 mm in diameter, marked 2 cm from the head end) was inserted into the incision, and inserted upward about 20 mm above the bifurcation until a resistance was felt, namely a focal cerebral ischemia caused by the middle cerebral artery occlusion (MCAO) was produced. At the entrance of ligation, about 1 cm long nylon was reserved outside to suture the skin. After 2 h, the nylon thread was gently pulled until a slight resistance was felt, namely a middle cerebral artery reperfusion was produced. For the rats in the sham-operated group, only the CCA and ECA were exposed without other treatment, and the left middle cerebral artery of the rats in other groups was occluded. During the cerebral ischemia and ischemia reperfusion, the room temperature was maintained at 23–25 °C. The success of the rat models was marked by paralysis of the left limb, unsteadiness on feet and unilateral rotation when being lifted at the tail after sobering by anesthesia. Finally, rat models of global-focal cerebral ischemic tolerance were established. At the beginning of the experiment there were 98 male SD rats, and 29 rats died after two operations, so 69 rats were used in the result analysis.

3. Testing indexes

3.1. Neurologic deficit scoring

According to Zea Longa 5 grade scoring method (Zea longa et al., 1989), at 24 h after the operation (i.e. 22 h after ischemia reperfusion) neurologic deficit was assessed, and 0 score and unconscious rats were excluded. 0 score: no neurological deficit; 1 score: slight neurological deficit, the rats can not fully extend their right forepaw; 2 scores: moderate focal neurological deficit, the rats rotated to the right when walking; 3 scores: severe focal neurological