Saudi Journal of Biological Sciences 24 (2017) 1871–1878

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

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

Le Kang [a], Mingsan Miao [b,*]

[a] College of Pharmacy, Henan University of Chinese Medicine, Zhengzhou 450046, China
[b] Department of Science and Technology, Henan University of Chinese Medicine, Zhengzhou 450046, China

ARTICLE INFO

Article history:
Received 14 October 2017
Revised 9 November 2017
Accepted 9 November 2017
Available online 11 November 2017

Keywords:
Total flavonoid of ilex pubesceus
Ischemic tolerance
Animal model
Promoting blood circulation to remove blood stasis

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

* Corresponding author.
E-mail address: miaomingsan@163.com (M. Miao).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2017.11.030
1319-562X/

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

deficit, the rats dumped to the right when walking; 4 scores: the rats can not walk spontaneously, and their level of consciousness dropped.

3.2. Whole blood viscosity and plasma viscosity

At 24 h after the operation, all the rats were decapitated to obtain blood, which adopted heparin anticoagulation, then the whole blood viscosity and plasma viscosity were measured.

3.3. Observation of the morphological changes of brain tissue

After the neurologic deficit was assessed within 24 h after the operation, the rats were anaesthetized by intraperitoneal injection with 0.3 ml/100 g of 10% chloral hydrate, and then craniotomy was performed to take out the whole brain. At the coronal plane the tissue blocks 3–4 mm from the caudad of optic chiasm were taken, and fixed with 10% formaldehyde solution, and then made into sections and stained with HE to observe the morphological changes of brain tissue.

3.4. Preparation and detection of brain homogenate

At 24 h after the rat models were made, the rats were decapitated to obtain brain, after the cerebellum, olfactory bulb and lower brainstem were removed, the brain tissue from the anterior pole of the brain on the ischemic side to the midpoint of the optic chiasm line to the optic nerve root was taken, and weighed accurately. Then, the normal saline was added according to the ratio of mass and volume to prepare 10% brain homogenate. After being centrifuged at 2,300 r/min and 4 °C for 10 min, the supernatant was stored at -20 °C in a freezer for determination of iNOS activity, NO level, IL-1β content and TNF-α content. The protein content in the brain tissue was determined with a Coomassie Brilliant Blue Protein Assay Kit, and the iNOS activity was determined according to the operation instructions of NOS Assay Kit, unit: U/mgprot. The NO_2^-/NO_3 content in the brain homogenate was determined by colorimetric methed according to the operation instructions of NO Assay Kit (nitrate reductase method) to indicate the NO level, unit: μmol/g. The IL-1β content and TNF-α content in the brain homogenate were determined using ELISA method.

4. Statistical method

Data were analyzed using SPSS13.0 for windows. Measurement data among groups were compared using single factor variance analysis, and expressed by $\bar{x}\pm$ s. Ranked data were tested using Ridit test.

5. Results

5.1. Effect of total flavonoid of ilex pubesceus on neurological deficit score of the rat models of cerebral ischemic tolerance

Table 1 showed that: the rats in the sham-operated group showed no neurological deficit, and the rats in other groups showed different degrees of neurological deficit 24 h after ischemia. For example, the rats can not fully extend their right forepaw, rotated or dumped to the right when walking, and can't even walk. Compared with the sham-operated group, the pretreatment model group and ischemia-reperfusion group had statistical significance ($P < .01$), showing that the model was made successfully. Compared with the pretreatment model group, the neurological deficit score in the large-dosage group of total flavonoid of ilex pubesceus significantly decreased ($P < .05$); compared with the ischemia-reperfusion group, the large-dosage group of total flavonoids of ilex pubesceus and Ginaton group had statistical significance ($P < .01$), and there was a significant difference ($P < .05$) between the medium-dosage group of total flavonoid of ilex pubesceus and small-dosage group of total flavonoid of ilex pubesceus. But in the pretreatment model group, the neurological deficit score only showed a decreasing trend and was not significantly different ($P > .05$).

Table 1
Effect of total flavonoid of ilex pubesceus on neurological deficit score of the rat models of cerebral ischemic tolerance ($\bar{x}\pm$s).

Group	Dosage (g/kg)	Number of rats	Neurological deficit score
Sham-operated group	–	14	$0.00 \pm 0.00^{**\,\triangle\triangle}$
Pretreatment model group	–	9	2.00 ± 0.71
Ischemia-reperfusion group	–	8	2.75 ± 0.89
Ginaton group	0.02	9	$1.44 \pm 0.53^{\triangle\triangle}$
Large-dosage group of total flavonoid of ilex pubesceus	0.2	10	$1.30 \pm 0.48^{*\,\triangle\triangle}$
Medium-dosage group of total flavonoid of ilex pubesceus	0.1	9	$1.56 \pm 0.73^{\triangle}$
Small-dosage group of total flavonoid of ilex pubesceus	0.05	10	$1.60 \pm 0.70^{\triangle}$

Compared with the pretreatment model group: *P < .05, **P < .01; compared with the ischemia-reperfusion group: △P < .05, △△P < .01.

5.2. Effect of total flavonoids of ilex pubesceus on whole blood viscosity and plasma viscosity of the rat models of cerebral ischemic tolerance

Table 2 showed that: the whole blood viscosity and plasma viscosity of the rats in the sham-operated group significantly increased ($P < .01$), showing that the model was made successfully. Compared with the pretreatment model group, the whole blood viscosity and plasma viscosity in the large-dosage group of total flavonoids of ilex pubesceus and Ginaton group significantly decreased ($P < .01$); compared with the ischemia-reperfusion group, the whole blood viscosity and plasma viscosity in the large-dosage group of total flavonoids of ilex pubesceus and Ginaton group significantly decreased ($P < .01$), and the whole blood viscosity and plasma viscosity in the medium-dosage group of total flavonoids of ilex pubesceus significantly or obviously decreased ($P < .01$ or $P < .05$), and the whole blood viscosity in the small-dosage group of total flavonoids of ilex pubesceus significantly decreased ($P < .01$). There was no statistical difference in the pretreatment model group.

5.3. Synergetic protective effect of total flavonoids of ilex pubesceus on brain tissue of the rat models of cerebral ischemic tolerance

The pathological changes of brain nerve cells of the rats in all groups can be divided into four grades: "-" the brain nerve cells were large, the cytoplasm was rich, and the nuclei were normal; "+" most brain nerve cells, the cytoplasm and nuclei were normal, and individual brain nerve cells were atrophied; "++" few brain nerve cells were atrophied, and most brain nerve cells, cytoplasm and nuclei were normal; "+++" most brain nerve cells were obviously atrophied, the cytoplasm obviously decreased, and the nuclei and nucleoli blurred. The pathological changes of brain nerve cells of the rats in all groups were determined according to semi-quantitative standard.

Table 3 showed that: for the rats in the sham-operated group, the brain nerve cells, cytoplasm and nuclei were normal; the cytoplasm was rich and the nuclei were obvious; for the rats in the pretreatment model group, the volume of partial brain nerve cells decreased, the cytoplasm decreased, and the nuclei were

Table 2
Effect of total flavonoids of ilex pubesceus on whole blood viscosity and plasma viscosity of the rat models of cerebral ischemic tolerance ($\bar{x}\pm s$).

Group	Number of rats	Whole blood viscosity (mPa s)			Plasma viscosity (mPa.s)
		High cut	Middle cut	Low cut	
Sham-operated group	14	$5.73 \pm 0.30^{**\Delta\Delta}$	$6.83 \pm 0.41^{**\Delta\Delta}$	$14.30 \pm 0.91^{**\Delta\Delta}$	$1.61 \pm 0.05^{**\Delta\Delta}$
Pretreatment model group	9	7.08 ± 0.78	8.54 ± 0.88	17.75 ± 1.42	1.87 ± 0.06
Ischemia-reperfusion group	8	7.85 ± 0.71	9.67 ± 0.99	19.44 ± 1.41	1.88 ± 0.06
Ginaton group	9	$5.72 \pm 0.79^{**\Delta\Delta}$	$7.01 \pm 0.63^{**\Delta\Delta}$	$15.06 \pm 1.34^{**\Delta\Delta}$	$1.68 \pm 0.12^{**\Delta\Delta}$
Large-dosage group of total flavonoids of ilex pubesceus	10	$5.80 \pm 0.75^{**\Delta\Delta}$	$7.00 \pm 0.79^{**\Delta\Delta}$	$15.05 \pm 1.45^{**\Delta\Delta}$	$1.66 \pm 0.09^{**\Delta\Delta}$
Medium-dosage group of total flavonoids of ilex pubesceus	9	$6.01 \pm 0.73^{\Delta\Delta}$	$7.39 \pm 0.93^{\Delta\Delta}$	$15.76 \pm 1.51^{\Delta\Delta}$	$1.73 \pm 0.12^{\Delta}$
Small-dosage group of total flavonoids of ilex pubesceus	10	$6.54 \pm 0.93^{\Delta\Delta}$	$7.94 \pm 1.12^{\Delta\Delta}$	$16.70 \pm 1.59^{\Delta\Delta}$	1.76 ± 0.10

Compared with the pretreatment model group: *P < .05, **P < .01; compared with the ischemia-reperfusion group: $^{\Delta}P < .05$, $^{\Delta\Delta}P < .01$.

Table 3
Synergetic protective effect of total flavonoids of ilex pubesceus on brain tissue of the rat models of cerebral ischemic tolerance.

Group	Dosage (g/kg)	Number of rats	–	+	++	+++
Sham-operated group	–	14	12	2	0	0
Pretreatment model group	–	9	0	2	5	2
Ischemia-reperfusion group	–	8	0	0	4	4
Ginaton group	0.02	9	4	4	1	0
Large-dosage group of total flavonoids of ilex pubesceus	0.2	10	4	5	1	0
Medium-dosage group of total flavonoids of ilex pubesceus	0.1	9	3	5	1	0
Small-dosage group of total flavonoids of ilex pubesceus	0.05	10	1	3	4	2

understained or disappeared; for the rats in the ischemia-reperfusion group, the brain nerve cells were obviously atrophied, the cytoplasm obviously decreased, and the nuclei blurred or disappeared; for the rats in the large-dosage group of total flavonoid of ilex pubesceus and Ginaton group, the volume of partial brain nerve cells increased, partial brain nerve cells were atrophied, the cytoplasm decreased, and the nuclei were understained or disappeared; for the rats in the small-dosage group of total flavonoid of ilex pubesceus, the volume of brain nerve cells decreased, partial brain nerve cells were atrophied, the cytoplasm decreased, and partial nuclei were understained or disappeared. See Appendix for Pathology Photos: HE staining of brain tissue of the rat models of cerebral ischemic tolerance by total flavonoid of ilex pubesceus.

Ridit test showed that: Compared with the sham-operated group, the pretreatment model group and ischemia-reperfusion group were had significant statistical significance ($P < .01$), showing that the model was made successfully. Compared with the pretreatment model group, the pathological damage ($P < .01$) of ischemic side brain tissue of the rats in the large-dosage group of total flavonoids of ilex pubesceus and Ginaton group significantly decreased, and the medium-dosage group of total flavonoid of ilex pubesceus had obvious statistical significance ($P < .05$); compared with the ischemia-reperfusion group, there was a significant difference ($P < .01$) between the large-dosage and medium-dosage groups of total flavonoid of ilex pubesceus and Ginaton group; the pretreatment model group had no statistical significance ($P > .05$).

5.4. Effect of total flavonoid of ilex pubesceus on iNOS activity and NO level in the brain homogenate of the rat models of cerebral ischemic tolerance

Table 4 showed that: Compared with the sham-operated group, the iNOS activity and NO level in the brain homogenate of the rats in the pretreatment model group and ischemia–reperfusion group significantly increased ($P < .01$). Compared with the pretreatment

Table 4
Effect of total flavonoid of ilex pubesceus on iNOS activity and NO level in the brain homogenate of the rat models of cerebral ischemic tolerance ($\bar{x}\pm s$).

Group	Dosage (g/kg)	Number of rats	iNOS(U/mgprot)	NO(μmol/gprot)
Sham-operated group	–	14	$0.15 \pm 0.08^{**\Delta\Delta}$	$6.14 \pm 1.13^{**\Delta\Delta}$
Pretreatment model group	–	9	$0.43 \pm 0.08^{\Delta\Delta}$	$16.31 \pm 2.18^{\Delta\Delta}$
Ischemia-reperfusion group	–	8	$0.29 \pm 0.07^{**}$	$12.25 \pm 1.00^{**}$
Ginaton group	0.02	9	$0.48 \pm 0.09^{\Delta\Delta}$	$21.75 \pm 1.81^{**\Delta\Delta}$
Large-dosage group of total flavonoids of ilex pubesceus	0.2	10	$0.49 \pm 0.09^{\Delta\Delta}$	$21.33 \pm 2.09^{**\Delta\Delta}$
Medium-dosage group of total flavonoids of ilex pubesceus	0.1	9	$0.47 \pm 0.08^{\Delta\Delta}$	$19.61 \pm 2.84^{\Delta\Delta}$
Small-dosage group of total flavonoids of ilex pubesceus	0.05	10	$0.45 \pm 0.05^{\Delta\Delta}$	$17.81 \pm 2.16^{\Delta\Delta}$

Compared with the pretreatment model group: *P < .05, **P < .01; compared with the ischemia-reperfusion group: $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$.

Table 5
Effect of total flavonoid of ilex pubesceus on IL-1β content and TNF-α content in the brain homogenate of the rat models of cerebral ischemic tolerance ($\bar{x}\pm s$).

Group	Dosage (g/kg)	Number of rats	IL-1β (×103 pg/gprot)	TNF-α (×103 pg/gprot)
Sham-operated group	–	14	$1.24 \pm 0.53^{**\Delta}$	$0.68 \pm 0.30^{**\Delta}$
Pretreatment model group	–	9	$4.46 \pm 1.60^{\Delta}$	$2.44 \pm 0.88^{\Delta}$
Ischemia-reperfusion group	–	8	$2.75 \pm 1.43^{*}$	$1.51 \pm 0.78^{*}$
Ginaton group	0.02	9	$4.77 \pm 1.25^{\Delta\Delta}$	$2.68 \pm 0.78^{\Delta\Delta}$
Large-dosage group of total flavonoids of ilex pubesceus	0.2	10	$4.92 \pm 1.19^{\Delta\Delta}$	$3.00 \pm 1.10^{\Delta\Delta}$
Medium-dosage group of total flavonoids of ilex pubesceus	0.1	9	$4.59 \pm 1.71^{\Delta\Delta}$	$2.51 \pm 0.94^{\Delta}$
Small-dosage group of total flavonoids of ilex pubesceus	0.05	10	$4.57 \pm 1.74^{\Delta\Delta}$	$2.50 \pm 0.95^{\Delta}$

Compared with the pretreatment model group: *P < .05, **P < .01; compared with the ischemia-reperfusion group: $^{\Delta}P < .05$, $^{\Delta\Delta}P < .01$.

model group, there was no significant difference of iNOS activity between the 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; compared with the ischemia-reperfusion group, the NO level ($P < .01$) in the brain homogenate of the rats in the pretreatment model group, large-dosage, medium-dosage and small-dosage groups of total flavonoid of ilex pubesceus and Ginaton group significantly increased; compared with the pretreatment model group, the NO level ($P < .01$) in the brain homogenate of the rats in the large-dosage group of total flavonoid of ilex pubesceus and Ginaton group significantly increased; compared with the ischemia-reperfusion group, the NO level ($P < .01$) in the brain homogenate of the rats in the large-dosage, medium-dosage and small-dosage groups of total flavonoid of ilex pubesceus and Ginaton group significantly increased.

5.5. Effect of total flavonoid of ilex pubesceus on IL-1βcontent and TNF-α content in the brain homogenate of the rat models of cerebral ischemic tolerance

Table 5 showed that: Compared with the sham-operated group, the IL-1β content and TNF-α content in the brain homogenate of

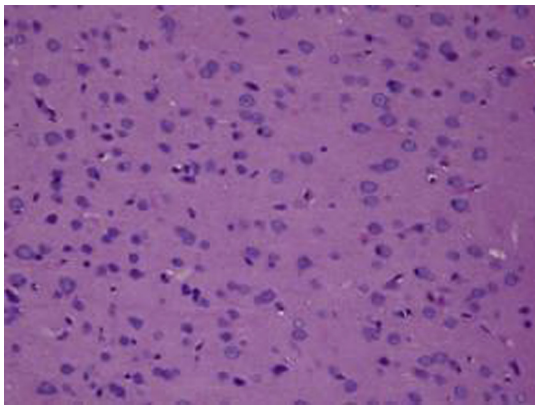

Photo 1. Cortex in the sham-operated group HE × 400.

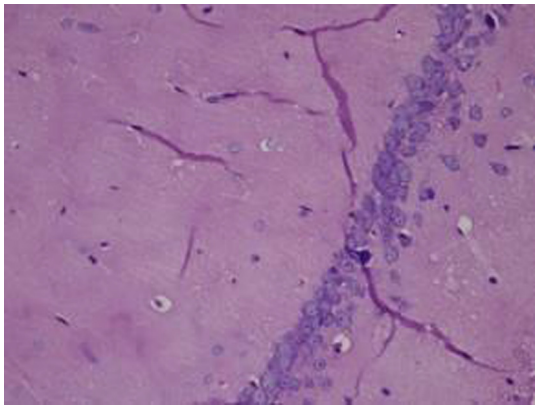

Photo 2. Hippocampus in the sham-operated group CA1 HE × 400.

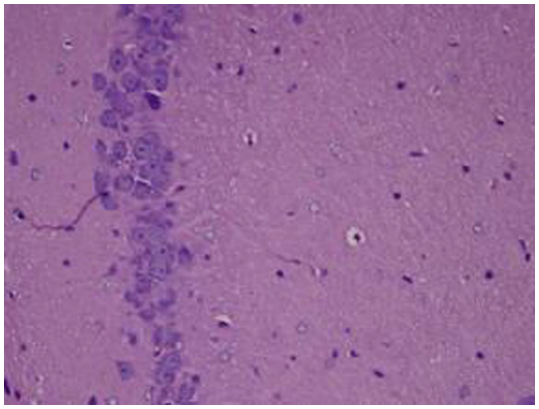

Photo 3. Hippocampus in the sham-operated group CA3 HE × 400.

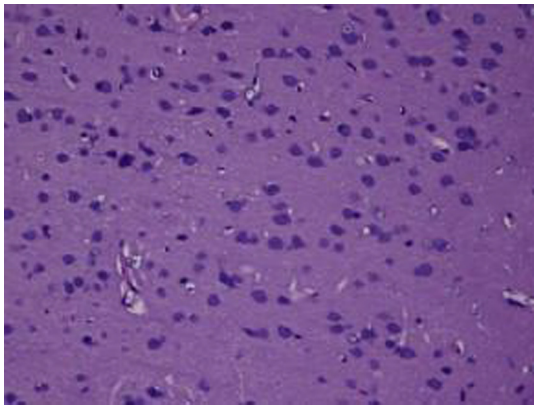

Photo 4. Cortex in the pretreatment model group HE × 400.

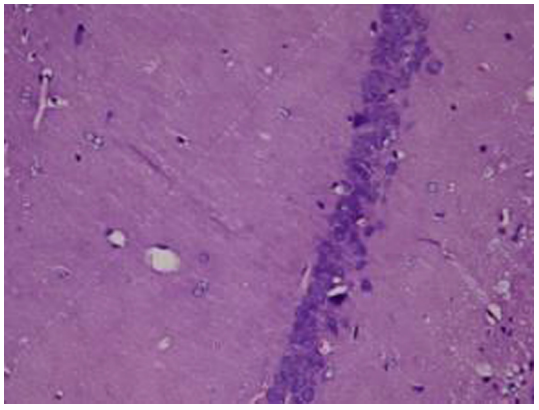

Photo 5. Hippocampus in the pretreatment model group CA1 HE × 400.

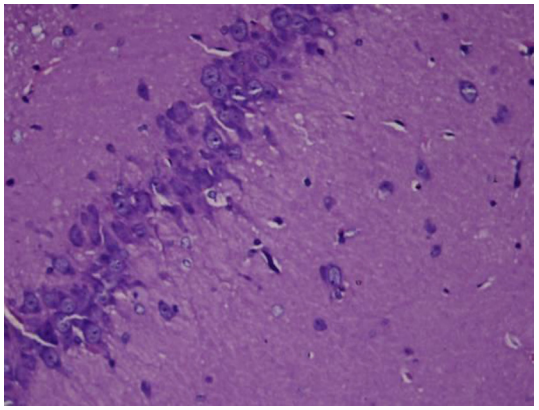

Photo 6. Hippocampus in the pretreatment model group CA3 HE × 400.

the rats in the pretreatment model group and ischemia-reperfusion group significantly or obviously increased ($P < .01$ or $P < .05$). Compared with the pretreatment model group, the IL-1β content and TNF-α content in the brain homogenate of the rats in the large-dosage, medium-dosage and small-dosage groups of total flavonoid of ilex pubesceus and Ginaton group showed an increasing trend, but had no statistical difference ($P > .05$); compared with the ischemia-reperfusion group, the IL-1β content and TNF-α content ($P < .01$) in the brain homogenate of the rats in the large-dosage group of total flavonoid of ilex pubesceus and Ginaton group significantly increased, the IL-1β content and TNF-α content ($P < .01$) in the brain homogenate of the rats in the medium-dosage and small-dosage groups of total flavonoids of ilex pubesceus significantly or obviously increased, and the IL-1β content and TNF-α content ($P < .05$) in the brain homogenate of the rats in the pretreatment model group obviously increased.

6. Discussion

The experimental results showed that the ischemic preconditioning reduced severe ischemia response, and produced significant tolerance to longer period of ischemic injury by inducing

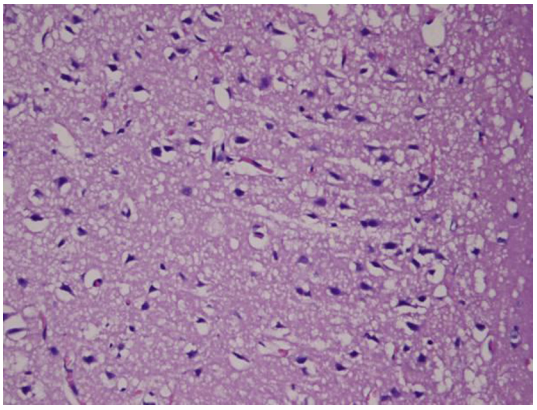

Photo 7. Hippocampus in the ischemia–reperfusion group HE × 400.

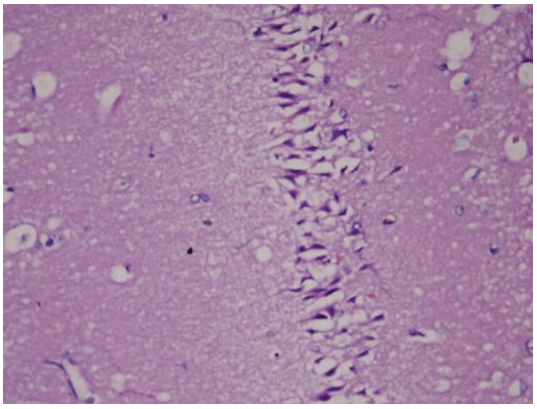

Photo 8. Hippocampus in the ischemia–reperfusion group CA1 HE × 400.

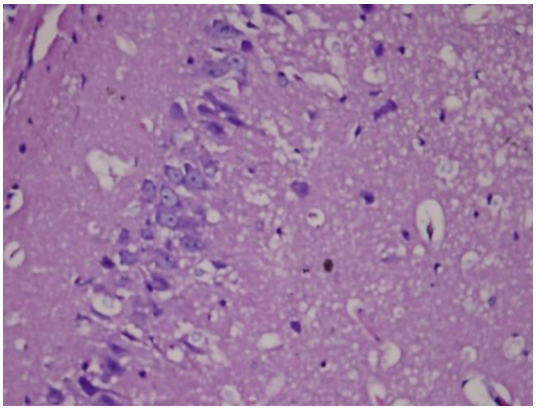

Photo 9. Hippocampus in the ischemia–reperfusion group CA3 HE × 400.

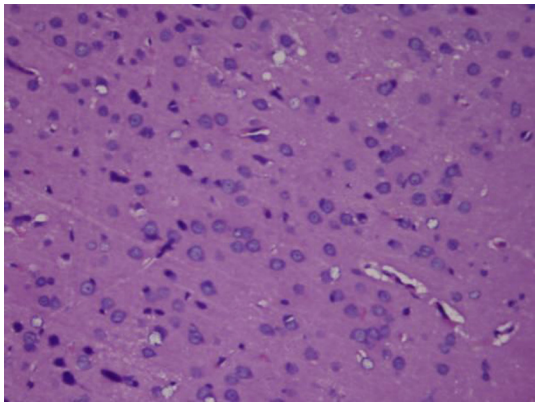

Photo 10. Cortex in the Ginaton group HE × 400.

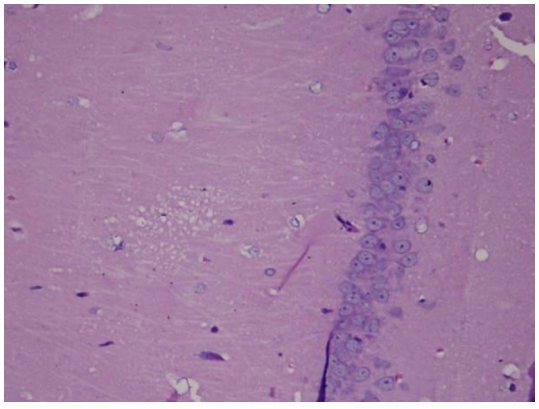

Photo 11. Hippocampus in the Ginaton group CA1 HE × 400.

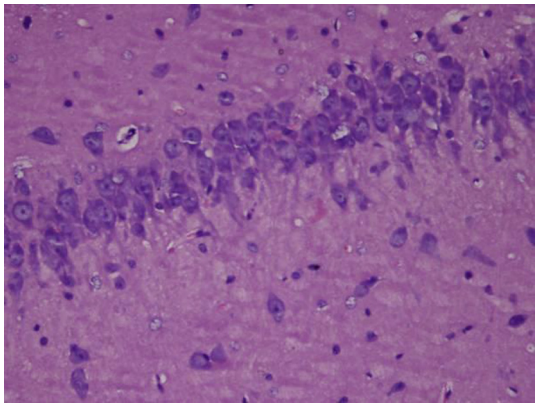

Photo 12. Hippocampus in the Ginaton group CA3 HE × 400.

the brain tissue to produce endogenous protective mechanism. The iNOS and NO as well as IL-1β and TNF-α participated in the production of cerebral ischemic tolerance, which was speculated to be the mechanism inducing cerebral ischemic tolerance.

The total flavonoid of ilex pubesceus can significantly improve the neurological deficit score and significantly reduce the ischemic injury, which showed that it had benign intervention effect on cerebral ischemic tolerance and can enhance the protective effect of brain tissue.

The total flavonoids of ilex pubesceus can significantly reduce the whole blood viscosity and plasma viscosity, which showed that the effect of improving cerebral ischemic tolerance by total flavonoid of ilex pubesceus may be achieved by increasing the whole blood viscosity and plasma viscosity.

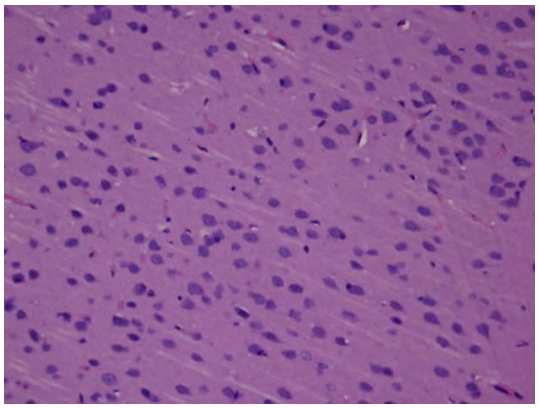

Photo 13. Cortex in the large-dosage group of total flavonoid of Ilex pubesceus HE ×400.

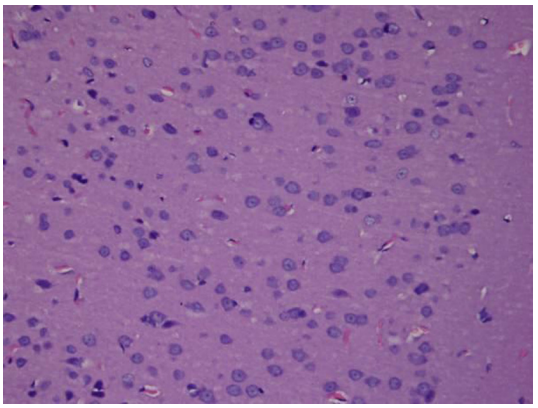

Photo 16. Cortex in the medium-dosage group of total flavonoid of Ilex pubesceus HE ×400.

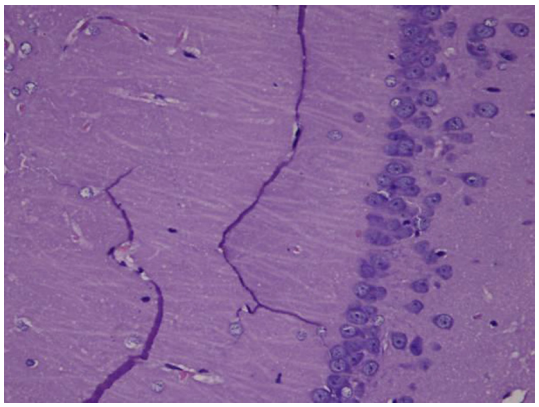

Photo 14. Hippocampus in the large-dosage group of total flavonoid of Ilex pubesceus CA1 HE ×400.

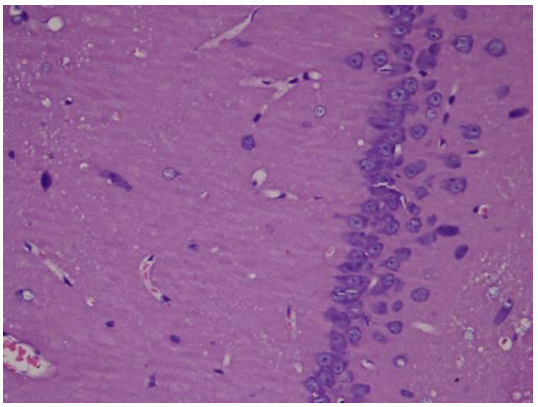

Photo 17. Hippocampus in the medium-dosage group of total flavonoid of Ilex pubesceus CA1 HE ×400.

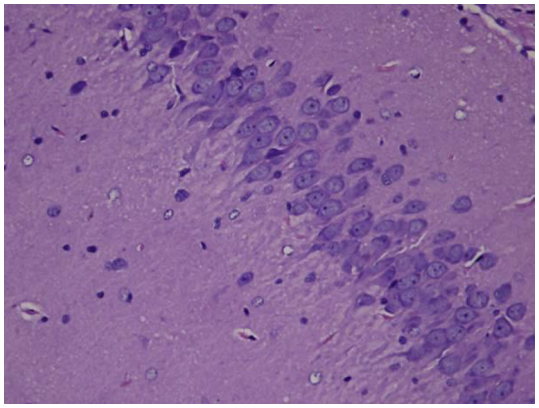

Photo 15. Hippocampus in the large-dosage group of total flavonoid of Ilex pubesceus CA3 HE ×400.

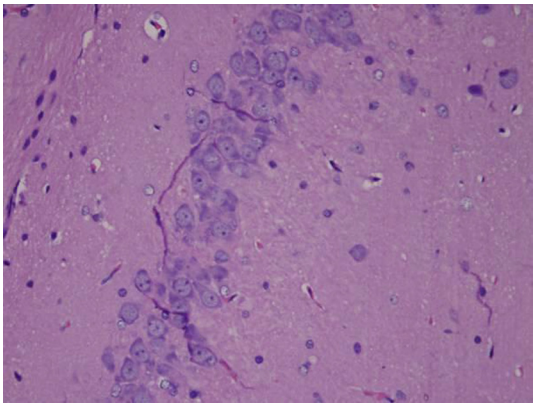

Photo 18. Hippocampus in the medium-dosage group of total flavonoid of Ilex pubesceus CA3 HE ×400.

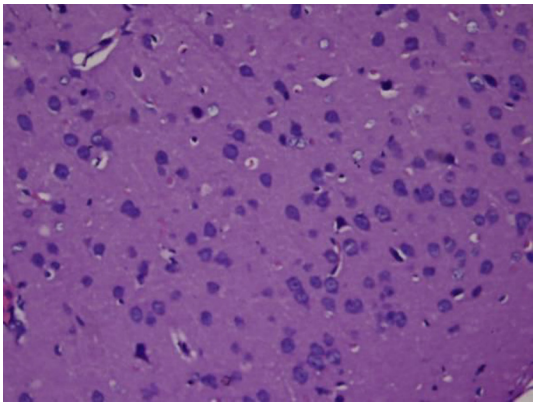

Photo 19. Cortex in the small-dosage group of total flavonoid of Ilex pubesceus HE ×400.

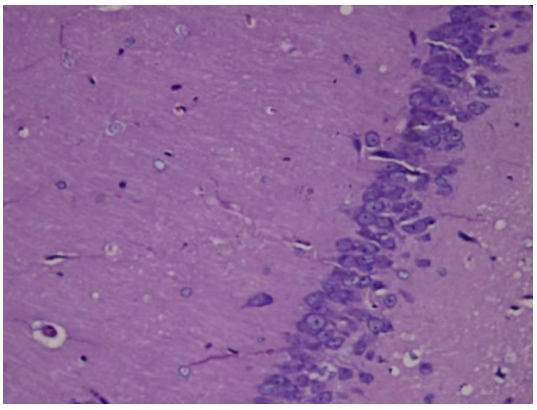

Photo 20. . Hippocampus in the small-dosage group of total flavonoid of Ilex pubesceus CA1 HE ×400.

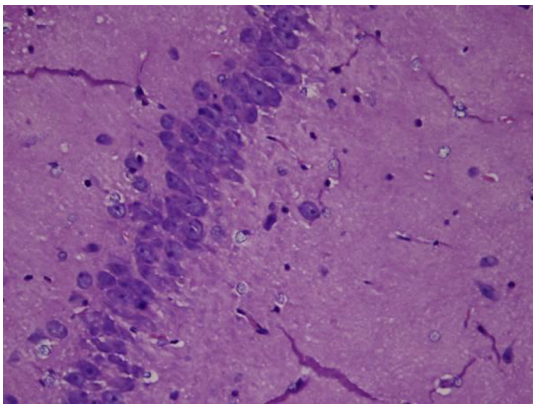

Photo 21. Hippocampus in the small-dosage group of total flavonoid of Ilex pubesceus CA3 HE ×400.

The total flavonoid of ilex pubesceus can obviously increase the iNOS activity and NO level as well as IL-1β content and TNF-α content, which showed that the effect of improving cerebral ischemic tolerance by total flavonoid of ilex pubesceus may be achieved by improving the iNOS activity and NO level as well as IL-1β content and TNF-α content.

The analysis of the experimental results showed that the total flavonoid of ilex pubesceus had a synergistic effect on the ischemic preconditioning as it can inhibit platelet aggregation by increasing the whole blood viscosity and plasma viscosity; it can moderately increase the iNOS activity and NO level as well as IL-1β content and TNF-α content; it can reduce brain damage after ischemia and promote the production of cerebral ischemic tolerance.

Acknowledgements

The work was financially supported by Major Scientific and Technological Special Projects of "Major New Drug Discovery" of China (2009ZX09103-324), National Natural Science Foundation of China (U1204827), Excellent Technology Innovation Team (TCJ2014-391).

A Pathology Photos

HE staining of brain tissue of the rat models of cerebral ischemic tolerance by total flavonoid of ilex pubesceus

See (Photos 1–21).

References

Can, Wang, Fang Xiaoyan, Xu., Kun, Xiang Liling, Mingsan, Miao, 2017. Effect of total flavonoid of ilex pubesceus on focal cerebral ischemia reperfusion injury in rats. Pharmacol. Clinics Chinese Materia Medica 33 (02), 70–74.

Dirnagl, U., Simon, R.P., Hallenbeck, J.M., 2003. Ischemic tolerance and endogenous neuroprotection. Trends Neurosci. 26 (5), 248–254.

Fan, Zhang, Xiaolei, Zhang, Mingsan, Miao, 2012. Effect of total flavonoid of ilex pubesceus on rat model of blood stasis combined with cerebral ischemia. Chinese J. Exper. Traditional Med. Formulae 18 (22), 187–191.

Huiqing, Liu, Wenbin, Li, Rongfang, Feng, Qingjun, Li, Xiaoling, Chen, Aimin, Zhou, Honggang, Zhao, Jie, Ai, 2003. Effect of nitric oxide synthase inhibitor L-NAME on the induction of cerebral ischemic tolerance in rats. Acta Physiologica Sinica 02, 219–224.

Jing, Lv, Huiping, Jiang, Tengfei, Wang, Jianqiang, Yu., 2015. Progress of research on the main pathogenesis of cerebral ischemia reperfusion injury. J. Binzhou Med. Univ. 38 (04), 291–293.

Jing, Zhang, Y'uan, Zou, Qian, Xue, Xiaohua, Dong, 2017. Effect of ischemic preconditioning on advanced glycation end receptor and interleukin-1β in the cerebral cortex of rats. The Chinese. J. Clin. Pharmacol. 33 (05), 439–442.

Koizumi, J., Yoshida, Y., Nakazawa, T., et al., 1986. Experimental studies of ischemic brain edema, I:a new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn. J. Stroke 8, 1–8.

Nagasawa, H., Kogure, K., 1989. Correlation between cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. Stroke 20 (8), 1037–1043.

Simon, R.P., Nitro, M., Gwinn, R., 1993. Prior ischemic stress protects agsinst experimental stroke. Neurosci. Lett. 163, 135.

Xiao, Cheng, Xiaolei, Zhang, Mingsan, Miao, 2012. Effect of total flavonoid of ilex pubesceus on cerebral ischemia rat models. Traditional Chinese Drug Res. Clin. Pharmacol. 23 (06), 640–643.

Xiaoyan, Fang, Ting, Zuo, Jingyi, Qiao, Suxiang, Feng, Mingsan, Miao, 2016. Study on the cerebral ischemic tolerance of rats by the total flavonoid of ilex pubesceus. J. Nanjing Univ. Traditional Chinese Med. 32 (05), 442–446.

Yiming, Jiao, Jianling, He, Shengnan, He, 2017. Expression of Nogo-A and NgR after focal cerebral ischemic preconditioning of cerebral ischemia reperfusion rats. Chinese J. Gerontol. 37 (02), 299–301.

Zea Ionga, E., Weinsten, P.R., Carlson, S., et al., 1989. Reversible middle cerebral artery occlusion without craniectomyin rats. Stroke 20, 84–91.