**Effect of Ligustrazine Liposomes on Apoplexy of Focal Cerebral Ischemia in Rats**

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**ABSTRACT**

**Objective:** To study the efficacy of Ligustrazine liposomes in the treatment of focal cerebral ischemic stroke of rats. **Method:** In this study, a rat model with focal cerebral ischemia was replicated by suture embolization. The efficacy of Ligustrazine liposomes was studied by testing neurological scores, brain index, cerebral infarction index, combined with anti-thrombotic experiments in rats and platelet aggregation inhibition experiments. **Result:** According to the experiment, Ligustrazine liposome can ease the neurological symptoms, and decrease the cerebral infarction index in model animals, and can slow down thrombosis, reduce the severity of thrombosis, and reduce the maximum platelet aggregation rate. To some extent, Ligustrazine liposomes have anti-cerebral ischemic stroke in rats. **Conclusion:** This experiment proved the therapeutic effect of Ligustrazine liposomes on cerebral ischemic stroke in rats, and provided a theoretical basis for the clinical use of Ligustrazine liposomes for the treatment of ischemic stroke. **Keywords:** Ligustrazine; liposome; stroke; efficacy

**INTRODUCTION**

Stroke is a clinically common ischemic cerebrovascular disease with high morbidity, high mortality, high disability, high recurrence, and multiple complications[¹]. It is one of the leading causes of human death and disability worldwide[²]. Cerebral thrombosis is the main pathological basis of stroke disability and high mortality. Thrombosis is caused by vascular thickening and lumen stenosis and occlusion caused by arteriosclerosis of cerebral artery or cortical branch. Also, thrombosis can result in decreased blood flow to the brain or interruption of blood supply. Cerebral ischemia and hypoxia can possibly lead to softening and necrosis of brain tissue, resulting in focal neurological symptoms. Effective anti-platelet aggregation in ischemic cerebrovascular disease can reduce the adhesion and aggregation of thrombus, which can significantly slow the rate of thrombus occlusion. It plays a crucial role in fighting thrombosis [³], thus reducing the harm of stroke.
Ligustrazine (chemical name: tetramethylpyrazine) is the main active ingredient of the traditional Chinese medicine Chuanxiong. It was isolated from the rhizome of Ligusticum chuanxiong. It is an Amide alkaloids. It has a variety of pharmacological activities, such as anti-ischemic reperfusion injury, anti-platelet aggregation, expansion of arterioles and clear microcirculation. Ligustrazine is widely used in clinical treatment of cerebral thrombosis and ischemic cerebrovascular diseases\textsuperscript{[4-6]}. Ligustrazine injections are currently mainly divided into ligustrazine phosphate injection and ligustrazine hydrochloride injection, which have been widely used in the treatment of cerebrovascular diseases\textsuperscript{[7]}.

Ligustrazine liposome as a new form of ligustrazine is initially applied in cerebrovascular treatment\textsuperscript{[8]}. To prove the effectiveness of the new formulation of ligustrazine, this experiment established the rat model of focal ischemia (MCAO) to investigate the effects of ligustrazine lipids, to explore whether Ligustrazine liposomes have anti-thrombosis and inhibit platelet aggregation.

**EXPERIMENTAL MATERIALS AND METHODS**

**Experimental reagents**

Ligustrazine Phosphate for Injection, purchased from Yangzhou Pharmaceutical Co., Ltd. Ligustrazine liposome test material, preparation room provided (according to the following proportion: soybean lecithin / tetramethylpyrazine phosphate / mannitol / soybean oil / purified water/ VE/lactose / sucrose 0.2~1/ 0.25/ 0.2~1/ 0.02~0.1/ 10/ 0.05/ 0.5~1.5/ 0.5~1). Chlorine-2,3,5-triph-enyl tetrazole, purchased from Beijing Boaotuo Technology Co., Ltd.

**Experimental design**

Wistar rats, male, weighing 220±20g, were obtained from the Academy of Military Medical Science Animal Laboratories (Beijing, China, certificate number SCXK 2014-0004). All animals were handled according to the Principles for Care and Use of Experimental Animals from Hebei University and approved by the institutional committee of animal care. All animals were maintained under standard environmental conditions (23±2°C, 55±5% humidity and 12h/12h light/dark cycle). All animals are not fasted and standard laboratory rat food.

Focal cerebral ischemia was divided into sham operation group, model group, blank matrix group, Ligustrazine group for injection (25mg/kg), Ligustrazine liposome high dose group (50mg/kg), Ligustrazine liposome medium dose group (25mg/kg), Ligustrazine liposome low dose group (10mg/kg). Anti-thrombosis and platelet aggregation inhibition experiments were divided into control group, Ligustrazine for injection group (25mg/kg), Ligustrazine liposome high-dose group (50mg/kg), Ligustrazine liposome medium dose group (25mg/Kg), Ligustrazine liposome low-dose group (10mg/kg). The rat tail vein was administered continuously for 3 days and the administration volume was 0.5 mL/100 g. The sham operation group, model group, and control group were given physiological saline.

**Establishment of Focal Cerebral Ischemia Model in Rats**

30 min after the last administration of the rats, intraperitoneal injection of 10% chloral hydrate (350 mg/kg), the rats were immobilized on the anatomical plate in a supine position, disinfected, and cut off the median muscle and bluntly separate the neck muscles and the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. ECA and CCA were ligated to the end of the heart, at the end of CCA, an artery clamp was placed at the proximal end of ICA, a 0.2mm ‘V’ incision was cut at the near branch of CCA, and the depth (18.5 ± 0.5) mm was inserted into the plug line, and the bolt line entered ICA to reach the initial part of the anterior cerebral artery (ACA), blocking the blood supply of
the middle cerebral artery. The sham operation control group did not insert the embolus line, and the other steps were the same as the model group. During the operation, the body temperature of the rats was maintained at 37.0±0.5°C. After the operation, the animals were stored in a well-ventilated room at 25±3°C, providing food and water.

**Evaluation of the degree of neurological impairment**
The improved Zea Longa standard was used to evaluate the degree of neurological impairment in rats for all animals, which was divided into the following criteria: 0 points, no defects; 1 point, unable to extend the lateral forelimb; 2 points, bending the lateral forelimb; 3 points, slightly to the side turn; 4 points, serious to the side loop; 5 points, lateral paralysis.

**Determination of brain index**
The rats were weighed at 24 h of cerebral ischemia, executed, taken brain, removed the olfactory bulb, cerebellum and the lower brain stem, called the brain wet weight, got the brain index, the brain index = the brain wet weight / rat weight *100%

**Determination of cerebral infarction index**
The brain was frozen at -20 degrees centigrade for about 10-20min, and the coronal plane was cut into 5 slices from the optic intersection. The thickness of each slice was about 2mm. It was placed in 2% TTC (Triphenyltetrazolium chloride) solution, 37 °C dark stain 15min, the normal brain tissue stained rose red, while the infarcted area was white, digital photography, carefully picking the infarct area. The cerebral infarction index refers to the ratio of infarct zone weight to brain mass.

**Experimental thrombus formation of Ligustrazine liposomes in rats**
At 30 minutes after the last administration, rats were injected intraperitoneally with 10% chloral hydrate (350 mg/kg). Rats took supine position and were fixed on animal anatomy, then the left common carotid artery was detached and the length was about 13 mm. The next step was use a filter paper to blot the body fluid around the blood vessels. Then the YLS-14B small animal thrombosis instrument thrombus probe upper platen was opened. Afterward the stripped common carotid artery was hooked into the probe groove (the probe indicates the direction of blood flow was consistent with the direction of blood flow of the common carotid artery of the animal being tested), and the pressure plate was gently put down. Hereafter, put the upper end of the cord into the clip of the fixing bracket, then adjust the height and direction. The thrombus production was completed with a current of 1.8mA for 3 minutes. Thrombus formation when the host shows that the obstruction rate rose to 95%. Next record thrombus generation time. If the host shows that the blocking rate does not increase to 95% within 3 minutes, it will be deleted. After 3 minutes of electrical stimulation, the thrombotic blood vessels were ligated and fixed in 4% paraformaldehyde. The blood vessels of thrombus section were sent to the Department of Pathology of the Chinese People's Liberation Army Air Force General Hospital for thrombosis and HE examination.

**Ligustrazine liposomes inhibit platelet aggregation**
At 30 minutes after the last administration, rats were injected intraperitoneally with 10% chloral hydrate (350 mg/kg). Rat abdominal aorta took blood and placed blood in heparin sodium anticoagulant centrifuge tube. Platelet-rich plasma (PRP) was obtained after rat anticoagulated whole blood was centrifuged at 1500 r/min for 10 min. After taking quantitative PRP, the remaining PRP was again centrifuged at 4,000 rpm for 10 min to obtain its own control platelet-poor plasma (PPP). The concentration of PPP was adjusted to make the concentration of each concentration close to each other. The PRP was preheated in a constant
temperature well at 37°C, and the inducer ADP (final concentration 5 μmol/L) was added to induce platelet aggregation, then record the maximum aggregation rate.

**Analytical method**

Statistically processed data is presented in the form of mean±standard deviation (x±S), the single factor analysis of variance (one-way ANOVA) was used in the SPSS19.0 statistical analysis software, and the t-test was used to compare the difference between the two groups, P < 0.05 was considered significant.

**RESULT**

**Effects of Ligustrazine liposomes on neurological scores in rats with focal cerebral ischemia:**

Compared with the sham group, the neurological function scores of the model group increased significantly, P<0.05, and the difference was statistically significant; Compared with the model group, there was a significant difference in the low-dose group, P<0.05, and the difference was statistically significant; No statistical difference was found in other groups. However, the high and middle doses of Ligustrazine liposomes all decreased the neurological score. (Figure 1)

![Figure 1](image)

*Figure 1* (Note:* compared with the sham group, P<0.05, the difference is significant; # compared with the model group, P<0.05, the difference is significant)

**Effect of ligustrazine liposomes on cerebral index of focal cerebral ischemia in rats:**

Compared with the sham group, the brain index of the model group increased significantly, P<0.05, and the difference was statistically significant; Compared with the model group, all dose groups did not significantly reduce the brain index. (Figure 2)

![Figure 2](image)

*Figure 2* (Note:* compared with the sham group, P<0.05, the difference is significant.)
Effect of ligustrazine liposomes on cerebral infarction index of focal cerebral ischemia in rats:
Compared with the sham group, the cerebral infarction index in the model group increased significantly, P<0.05, and the difference was statistically significant; Compared with the model group, there was no statistical difference in each group, however, the high-dose Ligustrazine liposome group and the ligustrazine injection group had a significant decrease in the cerebral infarction index. (Figure 3)

![Figure 3](image)

Figure 3 (Note:* compared with the sham group, P<0.05, the difference is significant.)

Effect of Ligustrazine Liposomes on Thrombosis in Rats:

Effect on thrombus formation time: Compared with the control group, the Ligustrazine liposome medium dose group can significantly slow down the time of thrombus formation, P<0.05, the difference was statistically significant. (Figure 4)

![Figure 4](image)

Figure 4 (Note:* compared with the sham group, P<0.05, the difference is significant.)

Effect on pathological staining score of thrombus: Pathological staining results (Figure 5), the results of pathological staining score were divided into 0, 1, 2 and 3 grades according to the severity of thrombus. According to HE test results, compared with the control group, the middle and low doses of Ligustrazine liposomes and ligustrazine for injection could significantly reduce the severity of thrombosis, P<0.05, and the difference was statistically significant. (Figure 5)
Effect of Ligustrazine liposome on the maximum platelet aggregation rate

The maximum platelet aggregation rate showed that compared with the control group, tetramethylpyrazine injection did not significantly reduce the maximum platelet aggregation rate, and all the dose groups of Ligustrazine liposomes significantly decreased the maximum platelet aggregation rate, $P<0.05$, the difference was statistically significant. (Figure 6)

Figure 6 (Note:* compared with the sham group, $P<0.05$, the difference is significant.)

Figure 7.(Note: A:control group, B:Ligustrazine group for injection, C:Ligustrazine liposome high-dose group, D:Ligustrazine liposome medium dose group, E:Ligustrazine liposome low-dose group)
Ligusticum wallichii is the dry root of the umbrella plant Ligusticum wallichii. It is spicy, warm. It is commonly used traditional Chinese medicine for promoting blood circulation and removing stasis. Its main active ingredient is tetramethylpyrazine, widely used in clinical treatment and prevention of ischemic cardiovascular and cerebrovascular diseases\(^9\), and it can inhibit apoptosis triggered by cerebral ischemia\(^10\), and can down regulate TNF-α and cells. Intercellular adhesion molecules are expressed, thus reducing the inflammatory response\(^11\).

Liposomes refer to tiny vesicles formed by encapsulating drugs in lipid-like bilayers. They are often used for the preparation of drugs. The use of liposomes can fuse with cell membranes to deliver drugs into cells. By adjusting the proportion of the hydrophilic/hydrophobic part of the lipid molecule, the morphology of the vesicles can be altered, thereby increasing the loading on different drugs\(^12\). As a non-degradable, non-toxic and non-immunological drug carrier in vivo, it can improve the therapeutic index, reduce the toxic and side effects of drugs, and reduce the dose, etc. It is an ideal carrier for increasing the concentration of drugs in the brain\(^13\), and lipids the body has a high degree of lipophilicity and can be transported to the brain parenchyma by, for example, passive transport, membrane fusion with the cerebrovascular endothelial cell membrane, or endocytic pathway\(^14\). Therefore, the liposome is a delivery method that is very suitable for penetration of the blood-brain barrier (BBB), and can effectively increase the bioavailability and brain targeting the reagent components. Ligustrazine injections is difficult to penetrate the blood-brain barrier and the utilization rate of ligustrazine is low. The combination of liposomes and tetramethylpyrazine will help to solve the problem that the low usage of ligustrazine injections.

In the experimental observation of focal cerebral ischemia model in rats, compared with the sham group, the neurological function score, brain index and cerebral infarction index of the model group increased significantly; compared with the model group, there was a significant difference in the low dose group. Ligustrazine liposome high and middle dose groups all had a decreasing trend in neurological function scores; There was no significant decrease in brain index in all drug delivery group; Ligustrazine liposome and Ligustrazine for injection group had a decreasing trend in cerebral infarction index. It showed that it had a certain degree of anti-cerebral ischemic stroke in rats.

In the anti-rat thrombosis experiment, the medium-dose group of Ligustrazine liposome can significantly slow down thrombus formation. Compared with the control group, the low and medium doses of Ligustrazine and Ligustrazine liposomes for injection can significantly reduce the thrombosis severity. In platelet aggregation inhibition experiment, the results of platelet aggregation showed that compared with the control group, tetramethylpyrazine for injection did not significantly reduce the maximum platelet aggregation rate, and each dose group of ligustrazine liposome significantly reduced the maximum platelet aggregation rate.

Therefore, the experimental results show that Ligustrazine liposome is effective in treating cerebral ischemic stroke. Compared with Ligustrazine for injection, there are obvious advantages. This experiment highlights the feasibility and effectiveness of liposomes as a carrier of ligustrazine. To a certain extent, it can improve the utilization rate of ligustrazine and reduce unnecessary losses, and provides experimental basis for the clinical use of Ligustrazine liposome in the treatment of cerebral ischemic stroke.


