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Original Article

# Effects of Ligustrazine on Ischemic Stroke by Photochemical Occlusion of Middle Cerebral Artery in Rat

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## ABSTRACT

**Background and Aim:** Stroke is a life-threatening disease leading to long-term disability in stroke survivors. In most acute ischemic strokes, the underlying cause is a thrombotic or embolic vascular occlusion. Therefore, one strategy for treating stroke is to restore blood flow within the ischemic area with a thrombolytic agent. This study was designed to investigate the effect of Ligustrazine on ischemic stroke induced by photochemical reaction in rat. **Methods:** Ischemic stroke model in rats was induced by the thrombotic occlusion of MCA based on the photochemical reaction between rose Bengal and green light, which causes endothelial injury followed by platelet activation and thrombus formation at the site of the photochemical reaction. Ligustrazine were injected i.v. 15 min (10 mL/kg) before rose Bengal injection and 2 hr (10 mL/kg) after injection of the dye. Neurological deficit, occlusive time, platelet aggregation, thromboxane $B_2$ and bleeding time were measured by conventional methods. **Results:** Ligustrazine decreased the neurological deficit about 24 hr after injection of rose Bengal, prolonged the occlusive time in middle cerebral artery and inhibited *ex vivo* platelet aggregation at 4 hr after injection of dye. **Conclusion:** Our results suggest that Ligustrazine used in the present study has potential effect on ischemic stroke induced by photochemical reaction.

**Keywords:** Ligustrazine, Ischemic stroke, Middle cerebral artery, Photochemical reaction, Rat model.

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## INTRODUCTION

Stroke constitutes a serious socioeconomic and health care problem because it is the principal cause of incapacity and the first cause of death in this section of the population in several countries. On average, every 40 seconds someone has a stroke and every 4 min someone dies of a stroke.[1]

Due to interruption of blood flow to the brain, stroke is typed as ischemic, intracerebral hemorrhagic, or subarachnoid hemorrhagic. Approximately 87% of strokes are ischemic.[2] Stroke survivors may not fully recover and risk long-term disability.[3] Therefore, in order to explore the pathologic processes and therapeutics for stroke many experimental models of ischemic stroke have been developed in a wide variety of animal species. Being pioneered by Watson *et al*.,[4] Photochemically Induced Thrombotic (PIT) stroke represents a general method that elicits various rodent models of focal cerebral ischemia. Previous studies have shown that the PIT model of proximal MCA occlusion is advantageous over other models for its reproducible larger ischemic lesion and the possibility of reperfusion. Therefore, it appears ideal for studying "tissue at risk" or ischemic penumbra and for evaluating novel anti-stroke drugs.[5,6]

Ligustrazine (Tetramethyl pyrazine) is one major efficient component from traditional medicine herb-Ligusticum chuanxiong Hort, which is currently widely used in Korea and China as a new kind of calcium channel antagonist for the treatment of coronary atherosclerotic cardiovascular disease and ischemic cerebrovascular disease.[7] Ligustrazine has been reported to inhibit the platelet aggregation,[8] to cause negative chronotropic and inotropic responses on isolated atria,[9] to inhibit vasoconstriction in isolated vascular strips,[10] and to act as a vasodilator, a free-radical scavenger, and anti-thrombosis and antihypertension agent. More recently, it has been found to be more effective in protection against injured vascular endothelial cell.[11] However, there are few reports regarding the effect of Ligustrazine on ischemic stroke by photochemical occlusion of middle cerebral artery in rat. The purpose of the present study was to further investigate the effect of Ligustrazine on ischemic stroke induced by photochemical reaction in rat.

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## MATERIALS AND METHODS

### Animals

Wistar male rats (7~8 weeks of age, 240~260 g) were provided by Laboratory Animal Centre of Medical Research Academia and adapted in a lab environment before experiments for a week. During the experiment, feed and water were available to rats at any time. The temperature was maintained at 20±2°C and the humidity was 55%.

40 rats were divided into two groups: control group ($n$=20): ischemic stroke model by photochemical reaction and Ligustrazine group ($n$=20): administration of Ligustrazine before and after ischemic stroke.

### Ischemic Stroke Model by Photochemical Occlusion of Middle Cerebral Artery

All rats were lightly anesthetized by intramuscular injection of 20 mg/kg ketamine hydrochloride. Endotracheal intubation was done and anaesthesia maintained with halothane at a concentration varying between 0.5% and 1.5%. The animals were placed in the supine position on a heating pad. The transorbital approach to the right middle cerebral artery was performed according to the method of Hudgins *et al.*[12] The orbital contents were dissected and excised. With the help of a Zeiss operating microscope, orbital craniotomy was performed using a dental drill to open an oval bony window. This permitted visualization of the intracranial dura and the right middle cerebral artery could be observed without cutting the dura mater. Thrombus was induced by photochemical reaction according to the classic method.[13] Briefly, the window was irradiated with green light (wave length 540 nm) achieved by use of a xenon lamp with a heat-absorbing filter and a green filter. The irradiation was directed by a 3 mm diameter optic fiber mounted on a micromanipulator. The probe of a pulsed doppler flowmeter was placed on the middle cerebral artery to measure middle cerebral artery blood flow. When a steady baseline flow was obtained, irradiation was started, and rose Bengal (20 mg/kg) was injected intravenously. Photo irradiation was continued for a further 20 min. Blood flow in the middle cerebral artery was continuously monitored for 3 hr after rose Bengal injection. The middle cerebral artery was considered to be occluded when the flow monitor indicated that blood flow had completely stopped. The time from injection of rose Bengal to the cessation of blood flow was recorded as the middle cerebral artery occlusion time. The dura was then covered with a moist gelatin sponge, the wound was closed, and the eyelids were sutured together. All animal treatments were approved by the Ethics Committee for Laboratory Animal Experiments at Pharmacy Institute, Medical Research Academia, DPR Korea.

### Ligustrazine and its Administration

Ligustrazine (purity>98%) was purchased from Mannyon pharmaceutical company, DPR Korea and identified by comparison with the voucher specimen deposited at National drug certification institute of Pyongyang, DPR Korea.

**Control group:** Rats were injected i.v. through a tail vein with 0.9% saline (10 mL/kg).

**Ligustrazine group:** rats were injected i.v. through a tail vein with Ligustrazine as following point: 15 min (10 mL/kg) before rose Bengal injection and 2 hr (10 mL/kg) after injection of the dye.

### Neurological Examination

About 24 hr after injection of rose Bengal, rats were examined for evidence of neurological deficit. Deficits were graded using a simple five-point scale according to the classic method.[14]

Grade 0: no deficit: normal climbing.

Grade 1: mild deficit: abnormal climbing, decreased dexterity of the contralateral hand.

Grade 2: moderate deficit: cannot climb, abnormal walking.

Grade 3: severe deficit: cannot walk, abnormal standing.

Grade 4: cannot stand.

Grade 5: death.

### Platelet Aggregation Assay

This assay was measured 15 min before injection of rose Bengal and 4 hr after injection of dye. Platelet-rich plasma and platelet-poor plasma was prepared by centrifugation of citrate-anticoagulated blood. Platelet counts in platelet-rich plasma were determined with an automatic cell counter and adjusted to a count of $3\times10^5$ platelets/μL with platelet-poor plasma. Platelet aggregation in platelet-rich plasma was measured using an aggregometer by recording the increase in light transmission through a stirred suspension of platelet-rich plasma maintained at 37°C for 5 min. Aggregation was induced by ADP at 20 mM.

### Bleeding Time

Bleeding time of the rats was measured 15 min before injection of rose Bengal and 4hr after injection of dye using a standard clinical procedure. A pneumatic cuff was inflated to 40 mm Hg on the bicep of the rats. Bleeding time was measured in the forearm with a spring-loaded blade system avoiding major subcutaneous veins. Blood coming from the incision was blotted with filter paper every 30 s until blood no longer stained the filter paper.

### Thromboxane $B_2$ Assay

This assay was also measured 15 min before injection of rose Bengal and 4hr after injection of rose Bengal. Released Thromboxane (TBX) $A_2$ was extracted from plasma samples as described elsewhere. Extraction yield was more than 80% in all experiments. The concentration of extracted thromboxane

$A_2$ was measured as the concentration of thromboxane $B_2$ by ELISA Measurement was done according to the manufacturer's instructions.

### Statistical Analysis of Data

Results were expressed as the mean and SEM. Data were analyzed by one-way Analysis of Variance (ANOVA) using SPSS 16.0 and the differences between the means assessed using Dunnet's multiple range test. A p value of <0.01 was taken as the level of statistical significance.

## RESULTS

### Behavioral Deficit

The results of neurological examination at about 24 hr after injection of rose Bengal are shown in Table 1. There were no deaths in two groups within 24 hr after injection of dye. All animals were much less combative than normal.

The neurological deficits of Ligustrazine -treated rats were milder than those in the control group.

### Occlusion Time

The time taken following injection of rose Bengal to the thrombotic occlusion of the artery was designated the occlusion time.

Table 2 showed that Ligustrazine significantly prolonged the MCA occlusion time compared with control group.

### Platelet Aggregation, Thromboxane Generation and Bleeding Time

The results for *ex vivo* platelet aggregation, thromboxane generation and bleeding time are shown in Table 3.

In Ligustrazine group, ADP-induced platelet aggregation was significantly inhibited compared with control group ($p<0.05$) at 4 hr after injection of rose Bengal. Ligustrazine inhibited $TXB_2$ generation, but this decrease was statistically no significant compared with control group. On the order hand, bleeding time by administration of Ligustrazine was prolonged, but there was no a significant difference between each group.

## DISCUSSION

Ligustrazine, a component contained in Ligusticum chuanxiong Hort, is widely applied in the treatment of vascular diseases in Korea. It blocks calcium channels, reduces the bioactivity of platelets and platelet aggregation, and inhibits free radicals. Ligustrazine has been demonstrated to have protective effects against kidney ischemia-reperfusion injury in rats and prevent acute myocardial infarction and cerebrovascular accidents in humans.[15,16]

Lots of the pharmacological action and mechanism of Ligustrazine show that it may be used as a potential therapeutic agent to treat cardiovascular diseases, but there has been no research to demonstrate the neuroprotective effect of Ligustrazine on ischemic stroke in rodents. In this study, we assessed the neuroprotective effect of Ligustrazine against ischemic stroke induced by photochemical reaction in rat. After photoirradiation, we

**Table 1: The effect of Ligustrazine on neurological examination.**

| Group | Grade | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Control (n) | 0 | 2 | 6 | 9 | 3 |
| Ligustrazine (n) | 11 | 7 | 2 | 0 | 0 |

**Table 2: The effect of Ligustrazine on occlusion time.**

| Group | Occlusion time (sec) | p value |
|---|---|---|
| Control (*n*=20) | 259.2±14.3 | <0.05 |
| Ligustrazine (*n*=20) | 513.8±21.8 | |

Each value represents the mean±SEM of 20 rats per group.

**Table 3: The effect of Ligustrazine on blood analysis and bleeding time.**

| Group | Platelet aggregation (% of pretreatment) | $TXB_2$ generation (% of pretreatment) | Bleeding time (fold of pretreatment) |
|---|---|---|---|
| Control (*n*=20) | 85.4±4.7 | 79.3±6.9 | 1.0±0.03 |
| Ligustrazine (n=20) | 39.5±6.1* | 71.1±8.4 | 1.7±0.29 |

Each value represents the mean±SEM of 20 rats per group. *p<0.05 as compared with Control group.

evaluated the effect of Ligustrazine, which can inhibit the platelet aggregation and vasoconstriction, and protect the endothelial cell injury in ischemic stroke. As a result, Ligustrazine decreased the neurological deficit in ischemic stroke model. Reduced ischemic cerebral damage by administration of Ligustrazine can lead to express a few neurological deficits. Pathogenesis of this model can be defined as occlusive thrombus formation in the rat middle cerebral artery by photochemical reaction. Thus, we investigated the protective effect of Ligustrazine on occlusive thrombus formation in middle cerebral artery. Ligustrazine prolonged the occlusive time in middle cerebral artery and inhibited *ex vivo* platelet aggregation at 4hr after injection of rose Bengal, and these changes led to statistically significant differences. These results suggest that Ligustrazine is a beneficial approach to preventing cerebral artery thrombosis. Even Ligustrazine significantly inhibited *ex vivo* platelet aggregation and prolonged the time to occlusive thrombus formation; these effects were not associated with an inhibition of $TXB_2$ generation and a prolongation in template bleeding time. To confirm the clinical therapeutic potential of Ligustrazine, an experiment on its dosage or administrated point may be needed in the future. We suggest that Ligustrazine has the positive effects on ischemic stroke induced by photochemical reaction in rat.

## CONCLUSION

In conclusion, our results suggest that Ligustrazine used in the present study has potential effect on ischemic stroke induced by photochemical reaction. Ligustrazine inhibited ADP-induced platelet aggregation in this model and this effect resulted in the prolongation of time to occlusive thrombus formation and improvement in Neurological deficit in this study. However, further studies can confirm positive dosage and also investigate administrated points that play a definite role in pathophysiology of ischemic stroke. It is however assumed that Ligustrazine may have therapeutic potential in the prevention of cerebral thrombosis and cerebral infarction.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**PIT:** Photochemically Induced Thrombotic; **MCA:** Middle Cerebral Artery.

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