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Investigation of Linarinic acid and one of its derivatives against cerebral ischemia in mice

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ABSTRACT
The study aims to investigate the effects of (-)-Linarinic acid (LA) and one of its derivatives (LAd) on brain injury induced by ischemia. Malonaldehyde (MDA) is determined as an index for lipid peroxidation both in vitro and vivo. Mice were pre-treated with LA and LAd for 3 d. Thereafter, they were induced to have incomplete cerebral ischemia with both bilateral carotid artery occlusion and hypotension (BCAOH). In the first part of the in vivo experiment, mice were divided into four groups: sham (control), ischemia, ischemia + LA (200 mg/kg, i.g.) and ischemia + LAd (200 mg/kg, i.g.). In the second part, the dose-response of LAd was investigated at 100, 200 and 400 mg/kg i.g., respectively. A modified neurological severity score was developed for evaluating behavioral deficits of the mice with ischemia. Brains of the mice were excised in order to determine MDA after ischemia for 6 h. Survival time, survival rate, neurological injury score and MDA level in brains were observed. Results were: 1) The data in vitro showed that both LA and LAd could inhibit the generation of MDA. IC50 values obtained by Probit analysis were 2.9 mM for LAd and 4.88 mM for LA; 2) BCAOH could significantly shorten the survival span, reduce the survival rate and cause neurological deficits, which were associated with high level of lipid hydroperoxide production in cerebral tissues; 3) LAd decreased lipid peroxidation and improved the neurological outcome more than LA. It is concluded that LAd offers a better neuroprotection than LA against brain damage caused by cerebral ischemia.

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1. Introduction

With the aged population greatly increasing worldwide, more and more cerebrovascular events have taken place and threaten the elders’ health [1]. Academic and pharmaceutical investigators have been engaging in drug discovery and preclinical or clinical research in order to achieve optimal approaches to the treatment for stroke. Many studies showed that the major pathogenic mechanisms of cerebral ischemia contained excitotoxicity, peri-infarct depolarization, oxidative stress, inflammation and delayed cell death [2–4]. Oxygen free-radicals in cell damage associated with stroke play an important role and they also serve as signaling molecules that trigger inflammation and apoptosis [5,6]. However, there is still no effective treatment for brain damage caused by ischemia. Therefore, it is urgently necessary to explore effective drugs for the treatment of cerebral ischemia.

Natural products is a main source of new drugs, which have been studied for a long time [7]. Linarinic (Linaria vulgaris Mill.), a traditional Chinese medicine plant which is distributed in Northeast and Inner Mongolia, China, has functions of detoxification and detumescence. Linarinic acid (LA, Fig. 1) isolated from the ethanol extract of Linarinic herbs is an optimally active tricyclic quinazoline alkaloid. Results from computer-assisted procedures used to predict the activity of the compound demonstrated that the Linarinic acid could relax blood vessel, inhibit phosphodiesterase V and activate potassium channels. The properties of (-)-Linarinic acid have been observed in preliminary investigation by means of different methods. Therefore, further study found that some of the (-)-Linarinic acid derivatives synthesized by modifying the basic structure of LA have better pharmacological activities [8]. In the current study, the anti-ischemia action of (-)-Linarinic acid and its semi-synthetic derivatives, LA₄ (Fig. 1), and whether the administration of the new compounds reduces oxidative reaction in mice with global cerebral ischemia, are investigated.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and drugs

(-)-Linarinic acid and LA₄ (ee% > 99%) were provided by the laboratory of pharmaceutical engineering department in Shenyang Pharmaceutical University. Trichloroacetic acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China). 2-Thiobarbituric acid (TBA) was from Sigma. Breviscapine was obtained from First Biochemical Pharmaceutical Co., Ltd. of Shanghai.

2.1.2. Animals

The subjects are male Kunming adult mice and Wistar rats obtained from Animal Center of Shenyang Pharmaceutical University. The weight of each Kunming mouse ranged from 22 g to 25 g and the weight of each Wistar rat ranged from 200 g to 300 g. They were group-housed in standard environmental conditions (22 ± 1 °C, humidity 60 ± 5%, 12 h light:12 h dark cycle) with free access to a standard commercial diet and water ad libitum. They were allowed at least one week to acclimatize before application.

2.2. Methods

2.2.1. In vitro determination of malonaldehyde (MDA) and drug intervention

The preliminary study indicated that the level of MDA in liver tissues of rats was mostly abundant among brain and liver tissues of 4 species of animals such as rabbits, rats, guinea pigs and mice. The liver tissues of rats could provide enough MDA samples to perform in vitro screening research to compare the biological activities among different compounds. Therefore, elderly rats were used to get the in vitro tissue samples in the present study. It is initially needed to adjust the concentration of liver homogenates according to the former results in order to ensure the MDA level of the control at the range of 110.6 – 356.5 nM/g (wet tissue). All the tested samples were divided into 4 groups, i.e. the control, the (-)-Linarinic acid (LA) treated, the LA₄ treated, and the breviscapine (B.S) treated. Two mM aqueous solution of LA or LA₄ was respectively prepared. Levels of MDA were determined with the TBA colorimetric method. Tetraethoxypropane as the standard substance was used to make a standard curve. Production of lipid peroxide in per-gram wet tissue was calculated. The IC₅₀ value (half maximal inhibitory concentration of a substance) of each compound was made out by means of Probit analysis.

2.2.2. Protocols for induction of incomplete global cerebral ischemia and pharmaceutical intervention

The mice were randomly divided into groups of sham (control), ischemia, ischemia plus LA and ischemia plus LA₄ solution (suspended in 0.5% CMC-Na) was intragastrically (i.g.) and daily administered for 3 d to mice (200 mg/kg body weight) before the ischemia was induced. The surgery of bilateral common carotid artery occlusion plus hypotension was performed according to literature [9,10] and our preliminary experiment. Briefly, after lowering the mean arterial blood pressure by eye venous exsanguinations to 30% of total blood volume [11], the mice were placed on an isothermic customized operating table with the neck extended. The skin was opened with a midline vertical incision and the underlying submandibular gland bluntly dissected in the midline to produce left and right lobes. The omohyoid muscle was divided, and then the right sternocleidomastoid muscle was dissected to expose the common carotid artery (CCA), on which the vagus nerve was separated. Then two sutures were used to ligate the CCA at both ends. The same procedure was performed to the

![Fig. 1 – Chemical structure of LA (the left) and LA₄ (the right).](image-url)
CCA on the other side. Begin to time when the CCA on the other side was ligated. Finally, the middle point between the two sutures of each CCA was cut, respectively [12]. The skin was closed with clinical suture and animals were allowed to recover in pre-warmed cages. Sham control was treated with the same volume of vehicle and underwent similar surgical procedures but without BCAOH. In order to determine the dose-effect response of LA₄, another population of mice was used and divided into 5 groups: sham (control), ischemia, and ischemia plus LA₄ (grouped into 100, 200, and 400 mg/kg body weight). They were individually treated with drugs for 3 d. The animal protocol was approved by the Shenyang Pharmaceutical University Animal Care and Use Committee. The experiments were in accordance with the Guidelines for the Care and Use of Laboratory Animals.

2.2.3. Neurological evaluation
After the operation of cerebral ischemia, the mice were tested for neurological deficits according to the following scoring table (Table 1), which was established on the basis of both the previous work in laboratory and available literature [13–17]. An investigator blinded to the experimental groups conducted the behavioral assessment. Measurements were performed in the duration from the end of the surgery to the deadline, 6 h after the ischemia. Deficits were various in mice, but primarily consisted of flexion of forelimbs, head moving more than 10° to the vertical axis, and the disabled limbs in placing test.

2.2.4. Determination of lipid peroxidation in brain of mice
MDA, an indicator of lipid peroxidation, was applied as previously described [18–20]. The mice were randomly divided into 4 groups and drugs were administered i.g. once a day for 3 d before ischemia, as described above. Brain tissues were removed after 6-h ischemia and the level of MDA was determined with a general spectrophotometric measurement. Briefly, brains were homogenized in normal standard saline. And then 1.2 milliliters of homogenate was incubated in HH-S11.2 type electric water bath pot (Jiangsu Dongtai Electrical Appliance Factory, China) under 37 °C and stirred slightly for 1 h. In addition, 0.6 ml of 20% trichloroacetic acid was mixed with them on TDL80-2B type centrifuge (Shanghai Anting Scientific Instrument Factory, China). Supernatant was collected and mixed with 0.6 ml of 0.67% 2-thiobarbituric acid, and boiled at 100 °C for 10 min. MDA was colorimetrically determined at 532 nm with a spectrophotometer (Shanghai Analytical Instrument Factory, China). Levels of MDA in the tested samples were calculated against standards and expressed as nM per-gram wet tissue.

2.2.5. Statistical analysis
Data were expressed as means ± SD and subjected to a one-way ANOVA and followed by LSD multiple comparison test (in homogeneity of variance) or Dunnett’s T3 (in heterogeneity of variance) with means of SPSS program (Version 19.0). Significant differences of survival rate among the groups were analyzed with χ²-test. A P value less than 0.05 represents statistically significant.

3. Results and discussion
3.1. LA and LA₄ inhibit lipid peroxidation in vitro
Results showed that both LA and LA₄ effectively inhibited in vitro the generation of MDA; the effect of structurally modified LA₄ was better than that of LA (Fig. 2). IC₅₀ values were obtained from Probit analysis so that the compounds’ potencies were easily compared and they were 0.008 mM for B.S, 2.9 mM for LA₄ and 4.88 mM for LA.

| Behavioral test                  | Score |
|----------------------------------|-------|
| Placing the mouse on a floor     | 1     |
| Inability to walk straight       | 1     |
| Circling or flexion of lateral forelimb toward tail | 2 |
| Rolling seizure                  | 2     |
| Falling down to one side at rest | 3     |
| Abnormal movement                |       |
| Myodystony, seizures, myoclonus  | 1     |
| Irritability (leaping)           | 1     |
| Keep rest with gasping (leaning to one side at rest if it takes place) | 2 |
| Die just after operation (within 10 minutes) | 4 |
| Areflexia                       |       |
| No pinna reflex (a head shook when touching the auditory meatus) | 1 |

Note the highest score if various situations take place at the same time. Try to push the mice slightly on its hip and give it a little stimulation if it does not make any movement at all.
3.2. Suppression of LA and LA₄ on BCAOH-induced ischemia

The number of dead mice, their individual survival time and neurological score were recorded. As shown in Fig. 3A/Fig. 4A and Fig. 3B/Fig. 4B, BCAOH could significantly reduce the survival time and the survival rate when compared to the control (*P < 0.05). LA₄ (200 mg/kg) had an increased tendency with reference to the indexes (Fig. 3A/Fig. 4A and Fig. 3B/Fig. 4B). Neurological scores illustrated that the mice with ischemia had neurological and functional deficits due to the ischemic insult (Fig. 3C/Fig. 4C, **P < 0.01). Mice treated with LA (200 mg/kg, Fig. 3C) and LA₄ (200 mg/kg, Fig. 3C/Fig. 4C) showed an improvement trend in the behavioral test but there was no statistic significance between the drug treatment group and the model group.

Brains were dissected in an ice bath after 6 h ischemia and used to determinate the levels of MDA. As shown in Fig. 3D and Fig. 4D, BCAOH could induce a significantly increased level of MDA compared to the group of control (*P < 0.05). LA₄ treatment (200 mg/kg) suppressed the ischemia-induced increase of lipid peroxidation significantly (compared to the ischemia

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**Fig. 3 – Effects of LA and LA₄ on survival time, survival rate, neurological deficits and the level of MDA in brain in the mice subjected to incomplete global ischemia. LA (200 mg/kg, i.g.) or LA₄ (200 mg/kg, i.g.) was daily given to mice for 3 d before ischemia. The number of the dead mice (survival rate, B) and their individual survival time (A) were recorded. Neurological deficit scores were measured after 6-hour ischemia by an observer who knew nothing about the groups (C). Brains were dissected after 6 h of BCAOH for the measurement of MDA (D). Data were expressed as means ± SD (n = 10 for the control group, n = 12 for the ischemia, n = 11 for the ischemia + LA, n = 12 for the ischemia + LA₄). Values were significant between the control group and the ischemia (*P < 0.05 and **P < 0.01) or the ischemia + LA₄ and the ischemia (*P < 0.05). Difference among the groups were analyzed by one-way ANOVA, followed by LSD multiple comparison tests by SPSS 19.0.**
LA treatment (200 mg/kg), however, was not significantly effective (Fig. 3D). In order to further test the dose-response of LA, more doses of the drug (100, 200 and 400 mg/kg) were administered to the mice. Results were illustrated in Fig. 4. Mice treated with 200 mg/kg of LA showed an improvement tendency in survival time (Fig. 4A), survival rate (Fig. 4B) and neurological deficit score (Fig. 4C), which was similar to the results in Fig. 3A, Fig. 3B and Fig. 3C. Data from Fig. 4D showed that a high dose (400 mg/kg) of LA significantly suppressed the ischemia-induced increase of lipid peroxidation (*P < 0.05), while the dose of 200 mg/kg did not, which was quite dissimilar to the result in Fig. 3D. The probable reason would be discussed in the following text.

In recent years, stroke becomes one of the major reasons of mortality and has a high incidence of morbidity [21], but relatively few treatment options to minimize tissue death following a stroke are available at present. The thrombolytic drug, tissue plasminogen activator (t-PA), is one of the most biologically effective treatments for acute ischemic stroke now, but it is only effective in the circumstance when the treatment is provided within 3 h of stroke onset. Its limited application is due to the narrow therapeutic time coupled with the risk of cerebral hemorrhage and cerebral edema [22,23]. Therefore, focus...
is on the development of new therapeutic agents of anti-cerebral ischemia. Oxidative stress mediated through active oxygen species is enhanced in both the ischemic core and penumbra following stroke injury, which is believed to cause much damage in these regions [24,25].

(-)-Linarinic acid is a substance obtained from the extract of Linarinic herbs. Previous data showed that it was a powerful anti-oxidant agent in vitro, but whether it has anti-ischemia activity in vivo is still unknown. We also wonder if its chemical derivatives have stronger pharmacological activity than that of LA after being structurally modified by medicinal chemistry. New drugs for potentially treating stroke might be developed with the activity of (-)-Linarinic acid derivatives being discovered. Some studies have demonstrated that (-)-Linarinic acid, a novel tricyclic quinazoline alkaloids, could inhibit the formation of peroxides and suppress cytotoxicity, and also has significant anti-inflammatory activity [26,27]. The precise mechanism of the actions, however, is not clear.

The present results show that LA₄ can slightly improve neurological function, increase survival rate and significantly reduce lipid peroxidation (as determined by the levels of MDA) in the cerebral tissues. MDA is known as an index for the evaluation of lipid peroxidation. Its level in tissue or serum indirectly reflects the balance of oxidant and anti-oxidant and the degree of tissue damage due to peroxidation [28]. Therefore, determining the content of MDA and understanding the extent of lipid peroxidation in tissue or serum is helpful and useful in the study of the pathophysiology of some diseases such as brain injury from ischemic stroke and the therapeutic effect of drugs. Based on the theory, the determination of MDA is regarded as a very primary index in this study to evaluate the anti-ischemic effect of the tested drugs. Results showed that LA₄'s anti-oxidant effect is better than that of LA. As shown in Fig. 1, a halogen group is added at site seven of the basic structure of LA, which leads to an increased lipophilicity. The modification also makes LA₄ easily go through blood brain barriers, which is very vital for the evaluation of an anti-ischemia drug. Moreover, it is found that the suppressive effects of LA₄ on MDA at the dose of 200 mg/kg is statistically significant in the first part of the in vivo experiment, but at the same dose, it shows just an effective tendency in the dose-dependent experiment, in which only high dose of LA₄ (400 mg/kg) significantly suppressed the increased level of MDA. The discrepancy may be caused by individual differences which can usually be observed in animal experiments, especially in those relevant to the in vivo study of cerebral ischemia [29]. And also it is likely that the dose of 200 mg/kg is some threshold one under the tested condition.

The study showed that (-)-Linarinic acid had the effect of inhibiting peroxidation in vitro, but its pharmacological effects are not obvious in mice in vivo. In contrast, as one of its semi-synthetic derivatives, LA₄ has significant effect on the mice with cerebral ischemia when it is evaluated with the level of MDA. As described above, (-)-Linarinic acid is a new compound with a sinistral property, which was extracted and isolated from Linarinic herbs. LA₄ was chemically modified into a dextral type and was also added a new halogen group to its original structure. Therefore, it is inferred that the changed optical type and the newly added halogen group might be involved in affecting the in vivo anti-ischemic activity in mice. This finding might be helpful for medicinal chemists to explore new compounds with greater pharmacological properties in future.

4. Conclusion

In conclusion, the findings in vitro and in vivo from LA and LA₄ give support to researchers to study the potential of the new compounds to prevent and treat cerebral ischemic injury based on the theory of oxidative mechanism. Since an increase of oxidative stress exerts bad influence in the pathophysiology of a number of neurodegenerative diseases, a series of LA compounds might be potential to treat stroke as well as other neurodegenerative diseases.

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