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Original article

Ellagic acid-induced thrombotic focal cerebral ischemic model in rats

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ABSTRACT

Introduction: Ischemic stroke is a common cause of human disability and death. Animal models of focal cerebral ischemia are widely utilized to mimic human ischemic stroke. Although models of focal cerebral ischemia have been well established, very few evidence is based on triggering the intrinsic coagulation system to induce focal cerebral ischemia. Ellagic acid (EA) has been identified to trigger the intrinsic coagulation system via activating coagulation factor XII. However, it remains unknown whether EA can serve as a novel pharmacological approach to induce a new model of focal cerebral ischemia in rats. **Methods:** EA was used for inducing focal cerebral ischemia in adult rats. The dose- and time-dependent effects of EA were characterized. The cerebral infarction ratio was determined with triphenyltetrazolium chloride staining, and the histopathological analysis of the brain tissue was performed under light microscopy. The neurological deficit score was evaluated by a modified method of Bederson. Malondialdehyde (MDA) level and lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activities in serum were determined by spectrophotometry. **Results:** Injection of EA into the middle cerebral artery of rats was able to generate focal cerebral infarction and increased the neurological deficit score and the brain weight to body weight ratio in dose- and time-dependent manners. Furthermore, EA raised serum LDH activity and MDA level and decreased serum SOD activity in a dose-related fashion. **Discussion:** This is the first evidence to show that EA induces focal cerebral ischemia in rats, which is similar to human ischemia stroke in pathogenesis. This model holds promise for pathological, pharmacological and clinical studies of ischemic stroke.

1. Introduction

Stroke is a heterogeneous disease with a complex pathophysiology and causes death and disability of patients as well as a significantly economic burden. With the fact that life expectancy increases, stroke is projected to be a more challenging disease in the future (Mukherjee & Patil, 2011). Ischemic stroke which is caused by an embolic or thrombotic occlusion of an artery supplying a specific territory of brain accounts for 80% of all strokes. Animal models of focal cerebral ischemia have been developed to mimic human stroke and play a major role in the experimental study of stroke (Durukan & Tatlisumak, 2007). Over the past years, animal models have largely advanced our knowledge on the pathophysiology and etiology of ischemic stroke (Mergenthaler, Dirnagl, & Meisel, 2004). However, due to the heterogeneity of human stroke, most translational stroke trials that aim to introduce basic experimental findings into clinical therapeutic strategies have failed, which is mainly attributed to ischemic stroke models not simulating pathogenesis of human stroke.

The focal cerebral ischemia model commonly used in the animal experiment is the middle cerebral artery occlusion (MCAO) model via intraluminal suture, which is developed in rats by Koizumi et al. and, is subsequently modified (Belayev, Alonso, Busto, Zhao, & Ginsberg, 1996; Longa, Weinstein, Carlson, & Cummins, 1989). By controlling the suture, both transient ischemia and permanent ischemia are performed using this model. However, the intraluminal suture in MCAO model varies largely from most human focal cerebral ischemia because it is actually a mechanical occlusion model. Thromboembolism is one of the most frequent causes of ischemic stroke in humans. Therefore, development of thromboembolic focal cerebral ischemia models will serve as a unique approach to mimic human stroke, which exhibits an advantage over other stroke models. Although thromboembolic models have previously been reported (Kudo, Aoyama, Ichimori, & Fukunaga, 1982; Zhang et al., 1997), few models are developed based on triggering the intrinsic coagulation system to induce thrombosis in vivo.

Ellagic acid (EA), 2,3,7,8-tetrahydroxy-chromeno [5,4,3-cde] chromene-5, 10-dione, is a plant phenol found in various fruits. It has been confirmed that EA triggers the intrinsic coagulation via activating coagulation factor XII, and promotes platelet aggregation by thrombin (Damas, Remacle-Volon, & Adam, 1989; Ratnoff & Crum, 1964), which have been applied in inducing the hypercoagulable state (Botti, 1966; Girolami, Cella, Burul, & Zucchetto, 1976; Iomhair & Lavelle, 1996; Ratnoff & Saito, 1982; Shiozaki et al., 1994). Hara et al. first established the global cerebral ischemia by injection of EA suspension into the common carotid artery of rats, and conducted a primary evaluation

Abbreviations: MCAO, middle cerebral artery occlusion; EA, ellagic acid; TTC, 2,3,5-triphenyltetrazolium chloride; MDA, malondialdehyde; LDH, lactate dehydrogenase; SOD, superoxide dismutase; MCA, middle cerebral artery; BBB, blood brain barrier.

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(Hara, Iwamoto, Ishihara, & Tomikawa, 1994). However, there is no study to identify whether EA induces focal cerebral ischemia and the comprehensive evaluation. The aim of the present study was to develop a stable EA-induced focal cerebral ischemia model similar to human thrombotic ischemia stroke in rats.

2. Materials and methods

2.1. Chemical reagents

EA was purchased from Sigma (St. Louis, MO, USA), and dissolved in 1 M NaOH, and then pH in the solution was adjusted to 7.4 with 1 M HCl. 2,3,5-Triphenyltetrazolium chloride (TTC) was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Assay kits for detecting malondialdehyde (MDA), lactate dehydrogenase (LDH) and superoxide dismutase (SOD) were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animals

Male Sprague–Dawley rats weighing 280–320 g were purchased from the Experimental Animal Center of Xi'an Jiaotong University School of Medicine, Xi'an, China. Animals were housed at 20–25 °C with a relative humidity of 50%–60% and a 12 h light/dark cycle. Food and water were provided ad libitum. Rats were fasted for 24 h before the experiment. All the experimental procedures carried out in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Xi'an Jiaotong University, and approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

2.3. Establishment of the focal cerebral ischemia model in rats

The surgical process of the focal cerebral ischemia model induced by EA was modified according to Zhang's thrombotic focal cerebral ischemia model (Zhang et al., 1997) and Longa's MCAO model (Longa et al., 1989). Rats were anesthetized with intraperitoneal injection of chloral hydrate (300 mg/kg). The left common carotid artery was exposed at the level of the external and internal carotid artery bifurcation under the sterile condition. Catheter (0.3 mm, O.D.) filled with EA or saline was inserted into the left common carotid artery and gently advanced into the internal carotid artery for a length of about 20–21 mm until a slight resistance was felt. Such resistance indicated that the catheter just passed over the proximal segment of the anterior cerebral artery. Then, the catheter was pulled back 2 mm and at this point, the intraluminal catheter was about 2 mm to the origin of the middle cerebral artery (MCA). Next, 0.1 mL EA was slowly injected into MCA of rats through the catheter in the model group (EA-treated group), while 0.1 mL normal saline was given to rats in the sham group in an identical fashion to the EA-treated group instead. Finally, the catheter was pulled out 5 min after the injection. During the whole process of animal surgery, the rat rectal temperature was maintained at 37.0 °C with a heat lamp.

2.4. Experimental protocol

In order to present a comprehensive evaluation on the model, we examined dose- and time-dependent characteristics of EA-induced focal cerebral ischemia. In the dose–effect experiment, rats were randomly divided into the sham group and three EA-treated groups (0.5, 1.0, 1.5 mg/mL; $n = 8$–10). The neurological deficit score, the cerebral infarction size, the brain weight to body weight ratio, LDH, SOD, MDA in serum and morphological changes were evaluated at 24 h after the injection of EA or saline. In the time-effect experiment, rats were randomly divided into the sham group and EA-treated group (1.5 mg/mL; $n = 8$–10). The above-described measurements were analyzed 3, 6, 12, 24 h after the injection of EA or saline.

2.5. Evaluation of neurological deficit

The neurological deficit score was recorded 6 h after the injection of EA in the dose-effect experiment, and at the indicated time after the injection of EA in the time-effect experiment. The neurological status of each rat was carefully evaluated by an observer who was blinded to the experimental procedure following a modified method of Bederson (Bederson et al., 1986). ① Rats were gently held by the tail, suspended one meter above the floor, and observed for forelimb flexion. Normal rats extended both forelimbs toward the floor. Rats that consistently flexed the forelimb contralateral to the injured hemisphere were scored as 4, otherwise scored as 0. ② Rats were pushed in the contralateral direction and scored as: 0 (resistance to lateral push), 1 (initially reduced but progressive resistance), 2 (reduced resistance), or 3 (lateral down fall). ③ Rats were then allowed to move freely to observe circling behavior. The movement was scored as: 0 (straight movement), 1 (movement to the right), 2 (circling movement), or 3 (no movement). The scores of three tests were summed and represented the neurological deficit score (0 to 10).

2.6. Assessment of the brain weight to body weight ratio

In the end of each experiment, rats were sacrificed by decapitation under the deep anesthesia with chloral hydrate. Brains were removed, and weighed immediately. The brain weight to body weight ratio was calculated according to the following formula:

$$\text{Brain weight to body weight ratio}\,(\%) = (\text{brain weight}/\text{body weight}) \times 100$$

2.7. Detection of cerebral infarction size

Brains were removed and sliced into six 2-mm-thick coronal slices (first to sixth from rostral to caudal). The brain slices were incubated in 2% phosphate buffer solution of TTC for 30 min at 37 ºC, and immersed in 10% formalin overnight. The unstained area (pale) was the infarcted area, while the stained area (red) was the normal area. The slices were photographed with a digital camera and the infarction volume was calculated by summing the infarction volume of sequential sections with a commercial image processing software program (Photoshop, Adobe Systems; Mountain View, CA, USA). The investigator who analyzed the image was blinded to the experimental groups. The cerebral infarction size was presented as the infarction ratio, which was calculated according to the following formula:

$$\text{Infarction ratio}\,(\%) = (\text{infarction volume}/\text{whole brain volume}) \times 100$$

2.8. Determination of MDA, LDH and SOD

Blood was collected via the abdominal aorta in the end of the experiment. Then, the separated serum was used to spectrophotometrically determine LDH activity at 450 nm, SOD activity at 550 nm and MDA concentration at 532 nm following the manufacturer's instructions.

2.9. Histopathological analysis

Brains were removed and fixed in 10% formalin in the end of the experiment. Then, the brain tissue was embedded in paraffin and sectioned at 4 μm thickness in the coronal plane. The sections were stained with haematoxylin and eosin, and examined under light microscopy.