論文の内容の要旨

Development of a mouse cerebral ischemia model and its utility for drug screening

マウス脳虚血モデルの開発とその薬剤スクリーニングへの応用

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平成 18 年 4 月入学

医学博士課程

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1. Introduction

Mouse intraluminal suture middle cerebral artery occlusion (MCAO) model, which provides ischemic lesion within 24 h and exposes large penumbra, is advantageous for studies of neuroprotective strategies. However, this suture MCAO model in mice is still associated with some issues, such as variability of infarction volume. To maintain the credibility of the research enterprise in stroke, efforts are still necessary to increase the stability of mouse MCAO model.

Injection into the cerebellomedullary (CM) cistern is one of the options for central administration. Although it has inherent advantages, intracisternal administration is very difficult to perform in mice because of its uncertainty and the technical demands related to the small size of

mice.

The research objectives are as follows:

1. To contribute to improving the reproducibility of mouse intraluminal suture MCAO model.

2. To develop an accurate and easy procedure to perform intracisternal administration of the drugs in mouse.

3. To assess and confirm the neuroprotective efficacy of some chemical substances for cerebral ischemia, using the above developed methodologies.

2. Materials and methods

2.1. Intraluminal suture MCAO model

Firstly, we developed the modified mouse MCAO model with high reproducibility. Secondly, this modified mouse MCAO model was applied for the assessment of neuroprotective drugs. The notable modification introduced in this study is that the pterygopalatine artery (PPA) was clipped through the whole duration of MCA occlusion (The effect of blood flow in the PPA on mouse MCAO model was evaluated at the first part of our whole study).

Prior to production of MCAO model, a laser Doppler probe was fixed on the left temporal skull surface. The occipital artery that arises from the proximal ICA and courses posterolaterally to enter the digastric muscle was isolated and divided. The left pterygopalatine artery (PPA), ICA (distal portion beyond the origin of the PPA) and CCA were closed with three miniature clips. Under cerebral blood flow (CBF) monitoring, a 6-0 silicone-coated monofilament was introduced into an

arteriotomy hole produced using a 30-gauge needle in the reflected ECA stump, and gently advanced into the left ICA until mild resistance was discerned. At this time, occlusion was confirmed as a sudden ≥80% drop in the CBF value (if not, the mice were ruled out). During occlusion, PPA blood flow was blocked by the clip, ICA blood flow was maintained by removing the ICA and CCA clips.

After 1 h of occlusion, the silicone-coated monofilament was withdrawn. The blood flow of CCA, ICA and PPA was maintained after reperfusion.

2.2. Evaluating the effect of blood flow in the PPA in the mouse MCAO model

The mice were randomly divided into three MCAO groups as follows: MCAO with blocked CCA blood flow (MCAO-C; n = 12), MCAO with blocked PPA blood flow (MCAO-P; n = 16) and MCAO with neither CCA nor PPA blood flow being blocked (MCAO-U; n = 12).

2.3. Development of a method of injection into cerebrospinal fluid via the CM cistern in mice

The long segment of a 27-gauge dental needle was curved (40°) at 2.5 mm from the tip, with the inclined plane of the tip facing laterally. The short segment of the needle was attached to the microsyringe via PE20 plastic tubing. The microsyringe was run by an infusion pump.

Anesthetized mice were placed in the prone position. The upper fore-tooth of the mouse was fixed by a wire of the facemask, and the head was bent, yielding nape elevation and distention. After skin's incision, the tip of the needle was inserted into the cleft between the occiput and the atlas vertebra through the muscles and ligaments in the midline. The puncture needle was kept on the line between the sagittal suture and midline of the nape during the entire period of injection.

2.4. Confirmation of the accuracy of the new developed method of injection into cerebrospinal fluid (CSF) via the cerebellomedullary cistern in mice

2.4.1. Checking the distribution of the dye in CSF after intracisternal dye injection

Each mouse was intracisternally injected with 6 μl 0.4% methylene blue aqueous solution. Mice were sacrificed 1 hour (n=8), 6 hours (n=6), or 24 hours (n=6) after injection. The location of injection and the intracranial distribution of methylene blue aqueous solution were examined.

2.4.2. Checking by intracisternal endothelin-1(ET1) injection

A 6-μl portion of each of the three doses (2 μg, 4 μg, or 6 μg) of ET1 or normal saline (NS) (4 groups, n=6 each group) was delivered into the CM cistern by the infusion pump (3 μl/min). Cerebral blood flow (CBF) was monitored for 60 minutes following injection. Respiratory condition was also recorded.

2.5. Evaluation of the therapeutic efficacy of ONO-1301, a new synthetic prostacyclin agonist

1ml Vehicle or ONO-1301 [0.3mg/kg; 1mg/kg; 3mg/kg or 10mg/kg] solution was injected subcutaneously immediately after occlusion of MCA.

Series of ONO-1301 experiments

Part 1. Mice were undergone 60minutes duration MCAO and sacrificed 3 days later; Part 2. Mice were undergone 60min duration MCAO and sacrificed 1 day later; Part 3. Mice were undergone 45min duration MCAO and sacrificed 3 days later.

2.6. Evaluation of the neuroprotective effect of dantrolene

2.6.1. Screening the effective dose of dantrolene in 45min MCAO model

4μl dantrolene solution [concentration: 0.25mM, 0.5mM, 2mM, 10mM] or Vehicle (DMSO) was injected intracisternaly 10min after reperfusion of 45min MCAO, at the speed of 2μl/min.

2.6.2. Confirming the neuroprotective effect of dantrolene in 60min MCAO model

4μl 0.5mM dantrolene solution or vehicle (DMSO) was injected intracisternaly 10min after reperfusion of 60min MCAO, at the speed of 2μl/min.

2.7. Determination of the infarct volume and edema

Infarct volume is described using three corrections.

The Leach correction was calculated as follows: lesion area of each section = (contralateral hemisphere area/ipsilateral hemisphere area) × ipsilateral lesion area. The lesion volume was calculated by summation of the lesioned areas of all sections and multiplying these by slice thickness.

The Swanson correction was calculated as follows: 100 × (contralateral hemisphere volume − non-lesioned ipsilateral hemisphere volume)/contralateral hemisphere volume.

The Pignataro correction was calculated using the following formula: 100 × (contralateral hemisphere volume − non-lesioned ipsilateral hemisphere volume)/ipsilateral hemispheric volume.

Edema= ipsilateral hemisphere volume-collateral hemisphere volume

Edema correction (%) = ipsilateral hemisphere volume/collateral hemisphere volume×100

3. Results

3.1. The effect of blood flow in the pterygopalatine artery (PPA) in the mouse MCAO model

3.1.1. CBF values

The range of CBF values during occlusion in MCAO-U was significantly wider than those in MCAO-C and MCAO-P ($p < 0.001$). During occlusion, the mean CBF value significantly differed between MCAO-U and the other two groups ($p < 0.001$).

3.1.2. Infarct volume and stability

The values for the Swanson and Pignataro corrections were considerably more stable in MCAO-P than in MCAO-U ($p < 0.05$). The values for the Leach correction tended to be more stable in the MCAO-P than in MCAO-U ($p = 0.07$). The values of the 3 corrections were much more stable in MCAO-P than in MCAO-U ($p < 0.05$) when we excluded mice in which the required CBF level of <30% of baseline was not maintained during 60 min of occlusion.

3.2. The accuracy of the new developed method of injection into cerebrospinal fluid via the cerebellomedullary (CM) cistern in mice

3.2.1. Success rate of puncture

In all mice (n=44), the puncture needle was successfully inserted into the CM cistern. All puncture procedures were completed with one attempt.

3.2.2. Temporal changes in dye distribution

At 1 hour after injection, a high concentration of dye was found in the CM cistern and the ventral cisterns. At 6 hours after injection, the color of the dye in the CM cistern and the ventral cisterns was lighter. At 24 hours after injection, no dye was observed in the intracranial space in any of the mice tested.

3.2.3. CBF values after intracisternal injection of ET1

Following 2-μg ET1 injection, CBF varied over time and did not significantly differ from control group values. The decrease in CBF in the 4-μg group was substantial and sustained, and CBF was significantly (10 and 40 minutes) or tended toward being significantly (30, 50 and 60 minutes) decreased at most time points compared with the control group. The 6-μg group exhibited the severest reduction of CBF.

3.2.4. Respiration impairment following intracisternal injection of ET1

No mouse in the NS group exhibited respiratory impairment. Mild sob-like respiration appeared immediately after ET1 injection in most mice (5/6) in the 2-μg group, but recovery from this was observed within 60 minutes. Obvious sob-like respiration appeared in all mice in the 4-μg group, but disappeared after resuscitation from anesthesia, except in 1 mouse that died during the 60-minute observation period. All of the mice receiving an intracisternal injection of 6 μg of ET1 died of respiratory insufficiency within 60 minutes after injection.

3.3. Evaluation of the therapeutic efficacy of ONO-1301

ONO-1301 increased the survival rate when mice were undergone 60minutes duration MCAO and sacrificed 3 days later. It also decreased the cerebral swelling when mouse were undergone 45minutes duration MCAO and sacrificed 3 days later.

There was no significant difference of infarct volume among groups in all parts of experiments about ONO-1301.

3.4. Evaluation of the neuroprotective effect of dantrolene

3.4.1. Screening the effective dose of dantrolene in 45min MCAO model

There was significant difference of infarct volumes between 0.5mM group and vehicle group while evaluated by Swanson correction and Pignataro correction ($p<0.05$).

3.4.2. Confirming the neuroprotective effect of dantrolene in 60min MCAO model

There was significant difference of infarct volumes between 0.5mM group and vehicle group while evaluated by Leach correction and Swanson correction ($p<0.05$).

4. Conclusion

This study provides 3 important findings relating to the mouse MCAO model. First, we have found out that collateral circulation from the pterygopalatine artery (PPA) to the brain significantly influences the mouse MCAO model, which seems to be one of main causes of variability of mouse MCAO model. Based on this finding, the advanced mouse MCAO model with modification by clipping PPA generates reproducible infarction in MCA territory with high consistency. Second, we have introduced the advanced procedure for injection of the drugs into cerebellomedullary cistern with accuracy and simplicity to perform in mice. The advantage of this method is that dye injection is not required in order to confirm adequate injection, because of the high degree of accuracy of this technique. In addition, we have confirmed the usefulness by the experiment that endothelin-1 injection induced dose-dependent reduction of cerebral blood flow and respiratory depression in mice. Third, these developed methodologies have wide applicability for the assessment of neuroprotective drugs prior to clinical trial.

ONO-1301 increased the survival rate in mouse severe ischemic model. It decreased the cerebral swelling in mouse mild ischemic model. ONO-1301 appears to deserve further

investigation regarding reduction of brain swelling early after stroke.

Dantrolene significantly decreased infarct volume in mice after transient MCAO. According to previous studies and our findings, there was scientific evidence in animal studies for the neuroprotective potential of dantrolene. Dantrolene might provide a promising new line of direction for neuroprotection.

Furthermore, the advanced mouse MCAO model and the accurate and easy-to-perform intracisternal injection in mice should be also useful to unveil the specific molecular mechanisms of cerebral ischemia in target gene modified mouse.