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EMBOLIC MIDDLE CEREBRAL ARTERY OCCLUSION MODEL USING THROMBIN AND FIBRINOGEN COMPOSED CLOTS IN RAT

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Abstract

Ischemic stroke accounts for over 80% in total human stroke which mostly affect middle cerebral artery (MCA) territory. Embolic stroke models induced by injection of homologous clots into the internal carotid artery and MCA closely mimic human stroke and have been commonly used in stroke research. Studies indicate that the size and composition of clots are critical for the reproducibility of the stroke model. In the present study, we modified the homologous clots formation by addition of thrombin and fibrinogen which produced even distribution of fibrin with tight cross linkage of red blood cells. We optimized the embolic MCA occlusion model in rats using different size of the mixed clots. A precise lodgment of the clots at the MCA bifurcation and highly reproducible ischemic lesion in the MCA territory were demonstrated in the embolic MCA occlusion model induced by injection of 10 pieces of 1-mm long mixed clots made in PE-60 catheter. We further tested the effect of recombinant tissue plasminogen activator (rtPA) in this embolic MCA occlusion model. rtPA induced thrombolysis, improved neurological outcome, and significantly reduced ischemic lesion volume when administered at 1 hour after embolism as compared with control. In summary, we have established a reproducible embolic MCA occlusion model using clots made of homologous blood, thrombin and fibrinogen. The mixed clots enable precise lodgment at the MCA bifurcation which is responsive to thrombolytic therapy of rtPA.

Keywords

middle cerebral artery; embolism; recombinant tissue plasminogen activator; ischemia; stroke

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1. INTRODUCTION

Stroke is one of the leading causes of death and the most common cause of long-term disability in the US and most of developed countries. Stroke patients must not only survive the acute stages of infarction, but they must then cope with significant mental, physical, and economic stresses associated with neurological impairment. Considering the cost in loss of life, physical and mental disability, and subsequent productivity, the need for effective therapeutic interventions is clearly urgent.

Ischemic stroke is the most frequent type of stroke, accounting for over 80% of all strokes, in which the middle cerebral artery (MCA) territory is mostly affected (Gillum, 2002). The translational research using the experimental ischemic stroke models provide indispensable insight not only for the biology of the ischemic stroke in human, but also the discovery of novel treatments for ischemic stroke. The successful translation of the experimental findings can only be expected if the models replicate the essential criteria of the clinical situation. Many ischemic stroke models have been established in rodent mostly through occlusion of MCA using either craniotomy or endovascular approach, which have been able to recapitulate many characteristics of human stroke (Carmichael, 2005). Occlusion of MCA through endovascular approach requires minimum surgical manipulation and simulates the clinical situation, hence, has been extensively used for translational research. A wide variety of materials have been used to induce ischemic stroke through endovascular approach in rats, including blood clots, microspheres, macrospheres, and monofilament suture (Hossmann, 2008; Traystman, 2003). Substantial differences in acute ischemic lesion evolution between suture MCAO and embolic MCAO has been demonstrated in rats (Henninger et al., 2006). Embolic stroke models using blood clots mimic human stroke more closely than other models of cerebral ischemia since most of human strokes are caused by thromboembolism (Durukan and Tatlisumak, 2007). Embolic stroke models have been most commonly induced by injection of homologous clots into the intracranial internal carotid artery (ICA) and MCA via external carotid artery (ECA). In addition, in situ thromboembolic stroke model has also been created by microinjection of thrombin to trigger local clot formation in mice (Orset et al., 2007).

In MCAO models, the lodgment of clots in the intracranial ICA and MCA leads to the occlusion of MCA, hence, induces ischemia in the MCA territory. Studies have demonstrated that reproducible ischemic lesions in the MCA territory could only be achieved when the obstructing clots are lodged in the intracranial ICA segment and MCA segment close to the MCA bifurcation (Busch et al., 1997; Zhang et al., 1997a). The size of the clots is critical for the precise lodgment of clots close to the MCA origin. In addition, the formation and composition of the clots have to be taken into consideration as they can affect the clots' elasticity, hence, their lodgment site in the cerebral arteries when injected into ICA. In the present study, we modified the current rat embolic MCA occlusion model by addition of thrombin and fibrinogen in the homologous blood and tested the embolic MCA occlusion model using various clots condition. In addition, the thrombolytic effect of recombinant tissue plasminogen activator (rtPA) was investigated in this model.

2. MATERIALS AND METHODS

2.1. Experimental animal

Male Sprague-Dawley rats (275–300 gram) were purchased from Charles River (Wilmington, MA). The rats were housed in a 12hr light and 12hr dark cycle and free accessed to water and food. All surgical procedures were approved by the Institutional Animal Care and Use Committee of University of North Texas Health Science Center.

2.2. Clots Preparation

Three types of clots were made by mixing different thrombotic factors with fresh arterial blood in different catheters. The blood donor rats were anesthetized with 1.5 % isoflurane. Fresh arterial blood was withdrawn from the heart by a 1 ml syringe with 21 gauge needle. For spontaneous clots, 0.4 ml fresh arterial blood was injected into PE 50 (ID: 0.58 mm, OD: 0.99 mm, Braintree Scientific Inc., Braintree, MA, USA) or 60 catheter (ID: 0.72 mm, OD: 1.22 mm). For thrombin added clots, 0.4 ml fresh arterial blood was mixed with 80 μ l of thrombin in a 1.5 ml microcentrifuge tube (50 U/ml, Sigma, St. Louis, MO, USA) and injected immediately into PE 50 or 60 catheter. For thrombin and fibrinogen added clots, 0.4 ml fresh arterial blood was mixed with 80 μ l of thrombin (50 U/ml, Sigma, St. Louis, MO, USA) and 0.4 ml of fibrinogen (10 mg/ml, Sigma, St. Louis, MO, USA) in a 1.5 microcentrifuge tube, and immediately injected into PE 50 or 60 catheter. All the blood were retained in the catheter at 37 °C overnight and subsequently stored at 4 °C. The clots were flushed out from the catheter by a syringe with normal saline and cut in small pieces in a petri dish. Images were taken under a dissecting microscope for diameter measurement (Zeiss Stemi DV4 microscope). Then, the clots were processed for paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining.

For embolic MCAO occlusion, a 3-cm long section was cut from a PE-60 clot-containing catheter and attached to two PE-160 catheters (ID: 1.14 mm, OD: 1.63 mm, Braintree Scientific Inc., Braintree, MA, USA), which was connected to a normal saline filled 1 ml syringe with 18 gauge needle (Figure 2A). The clot was washed in the tubing for 1 min by alternative pushing the two syringes. For the clot prepared in PE50 catheter, the clots were washed in PE50 catheter connected to PE60 catheter. The clot was flushed out of the catheter and cut into small cylindrical clots under microscope for the induction of embolic stroke model.

2.3. Embolic middle cerebral artery occlusion

Three different sizes of thrombin and fibrinogen added clots were used to induce embolic middle cerebral artery occlusion. Ten pieces of 1 mm-long thrombin and fibrinogen added clots (n=15) made in PE60 catheter were gently pushed one by one with a microforcep into a modified PE-50 catheter, of which out diameter is 0.72–0.75 mm. Animals were anesthetized with 1.5–3.0% isoflurane in air. Rectal temperature was monitored and maintained between 36.5 and 37.5 °C by a heating pad (Kaz Inc. Hudson, NY, USA). The breath rate, heart rate and oxygen saturation was monitored (MouseOx, Starr Life Science Corp, Oakmont, PA, USA) and be kept in the range of 45–65 bpm, 300–400 bpm, and >90% during the surgery, respectively (Table 3). With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA) and its branches of superior thyroid artery (STA) and occipital artery (Occi A), internal carotid artery (ICA) and its branch of pterygopalatine artery (PPA) were exposed through a midline cervical skin incision. The STA, distal ECA, Occi A were permanently cauterized, and other arteries were temporarily clipped. The PE-50 catheter containing 10 pieces thrombin and fibrinogen added clots was introduced into the lumen of ECA. After releasing the clip on ICA, the clots were quickly injected into the vessels with a short burst of strength. Then, the catheter was removed, and the clip on CCA and PPA were released and the skin incision was closed. As the same procedures, six or eight 2 mm and 3 mm long PE60 thrombin and fibrinogen added clots (n=10) and other sizes of PE50 thrombin and fibrinogen added clots (n=10) were also chose for embolic MCA occlusion. At 24 hrs after embolism, animals were sacrificed and the lodging site of clots was determined.

2.4. rtPA treatment

For thrombolytic intervention, rtPA (Genentech, Inc., San Francisco, CA, USA) was dissolved in saline and administered intravenously at a dose of 5mg/kg body weight with a 10% bolus and 90% continuous infusion over 30 min started at 1 hour after embolism using a Genie Plus Syringe Pump (Kent Sci. Corp., CT, US) via right jugular vein. L-arginine as a part of agent was used as control.

2.5. Near-infrared spectroscopy monitoring

To determine the efficacy of rtPA thrombolytic therapy, near-infrared spectroscopy (NIRS) was used to monitor the kinetics change of intracranial indocyanine green (ICG). Embolic MCAO was induced 11 male Sprague-Dawley rats by 10 pieces of 1 mm mixed clots prepared in PE 60 catheter. ICG was purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC, St. Louis, Mo) was dissolved and diluted with distilled water and administered via femoral vein at a bolus dosage of 1mg/kg body weight. The kinetics of ICG change was monitored by diffuse optical tomographic imaging system DYNOT (NIRx Medical Technologies, LLC, NY, USA) with absorbance of 760 nm and 830 nm. The fiber array consisted of 20 bifurcated source– detector probes were placed symmetrically on the scalp above the region of cerebrum (Figure 4A) with a nearest interoptode distance of 5 mm, as shown in Figure 4B. rtPA or L-arginine were administered intravenously via jugular vein (control group, n=5; rtPA group, n=6).

Diffuse optical images were reconstructed as described previously (Niu et al.). Briefly, the relative change of optical density for each source-detector pair due to presence of ICG can be calculated by:

$$\Delta OD = -\ln\left(\frac{I}{I_0}\right) \quad (1)$$

Where I represents the baseline intensity and I_0 is the intensity while ICG are presented. Eq. (1) can be also generalized in a matrix form and written as $\mathbf{y} = \mathbf{A}\mathbf{x}$, where matrix $\mathbf{y}$ represents measured changes in optical density from all the measurements, matrix $\mathbf{x}$ corresponds to a volumetric vector of the unknown perturbation in absorption coefficient in the volume under study, and matrix $\mathbf{A}$ describes the sensitivity of measurements to the change in absorption within each voxel in the medium.

In the current research field, to reconstruct a DOT image, the Tikhonov regularization method has been employed; an image of $\mathbf{x}$ can be given by:

$$\hat{\mathbf{x}} = \mathbf{A}^T (\mathbf{A}\mathbf{A}^T + \alpha \mathbf{S}_{\max} \mathbf{I})^{-1} \mathbf{y} \quad (2)$$

where $\hat{\mathbf{x}}$ is the reconstructed image of absorption perturbation, that is, the change of ICG concentration in this study since ICG absorbed the light; $\mathbf{I}$ is the identity matrix; $\mathbf{S}_{\max}$ is the maximal singular value of matrix $\mathbf{A}\mathbf{A}^T$, and α is the regularization parameter. Different time points of reconstructed images, which are the time before rtPA injection, and 20 to 110 minutes after rtPA received in increments of 10 minutes, were extracted and changes of ICG concentration (Δ ICG) for ischemic and normal side of brain were compared.

2.6. Neurologic deficits assessment and measurement of ischemic lesion volume

Before administrated of rtPA or L-arginine and 24 hours after onset of stroke, sensorimotor function was evaluated by the walk initiation and negative geotaxis tests. The animals were observed for up to 60s. Negative geotaxis was defined as the time in seconds required for a

rat placed head down on a 45-degree incline to turn 180° and began crawling up the slope. Walk initiation were defined as the time in seconds required for a rat placed on a horizontal surface in the center of circle with diameter of animal body to walk out of.

Animals were sacrificed at 24 hours after experimental embolism. Brains were harvested, the lodgment of the clots were investigated using a surgical microscope. Then the brains were sectioned coronally with 2.0 mm of thickness per section posterior to olfactory bulb. Slices were incubated for 30 min in a 2 % solution of 2,3,5-triphenyltetrazolium chloride at 37 °C and then fixed in 4 % formalin. The stained slices were photographed under a dissecting microscope (Zeiss Stemi DV4) and subsequently measured for ischemic lesion volume with Image-pro.

2.7. Statistic Analysis

All values are presented as means $\pm$ S.E.M. Data were analyzed using student *t* test and one-way ANOVA. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Histology and size of clots

Three different types of clots were assessed by histology. In both spontaneous formed pure blood clots and thrombin added clots, fiber component was mainly gathered in the center of the clots as demonstrated in the H&E staining (Figure 1). In the peripheral, red blood cells were loosely attached to the fibrin clot core, which could be easily washed off when rinse with normal saline. On the other hand, in the thrombin and fibrinogen added clots the fiber ingredient distributed evenly and red blood cells were tightly cross linked by the evenly distributed fibrin fiber, which did not dissociate from the clots when rinsing with saline. As a result, the fibrin rich clots prepared from the pure blood clots and thrombin added clots after washing in the tubing were heterogeneous in size and ingredients. We further assessed the size of the thrombin and fibrinogen added clots made in different tube. The diameter of thrombin and fibrinogen added clot made in PE60 catheter ($n=10$) was 0.64 ± 0.02 mm, significant bigger than the diameter of thrombin and fibrinogen added clot made in PE50 catheter ($n=10$), which is 0.47 ± 0.01 mm (Figure 2B).

3.2. Clots lodging sites and ischemic lesion volume induced by different sizes clots

The thrombin and fibrinogen added clots were chose for embolic MCAO in rats. We assessed the clots lodging sites and ischemic lesion volume in the embolic MCAO model induced by different size clots. Three groups of rats were assigned for embolic MCAO: rats received ten pieces of 1 mm-long thrombin and fibrinogen added clots made in PE60 catheter with diameter of 0.64 mm (PE60/1 mm * 10 mode, $n=15$); rats received six to eight 2–3 mm long thrombin and fibrinogen added clots made in PE60 catheter with diameter of 0.64 mm (PE60/2–3mm*6–8 mode, $n=10$); and rats received thrombin and fibrinogen added clots made in PE50 catheter with diameter of 0.47 mm (PE50 mode, $n=10$). Ischemic lesion was identified in 4 of the 10 animals received clots made in PE/50. In only one case, the clots were found to lodge in the bifurcation of ICA, while clots lodgment either could not be identified or in the distal branches on MCA and PCA (Table 1, Figure 3A). In the PE60/2–3mm*6–8 model, clots were found mainly in the distal ICA in all 10 animals. However, clots were not reach the bifurcation and no lesion were induced in 5 rats (50%). In 3 rats, clots were lodged in the main ICA and bifurcation close to MCA, and large ischemic lesion were observed. As a result, all the 3 animals (30%) died within 24 hrs after stroke and intracerebral hematoma occurred in 1 rat. In remain 2 rats, clots lodged in the ICA and anterior choroid artery (Ach. A) bifurcation and ischemic lesion was found in the basal ganglion (Table 1, Figure 3B). In the PE60/1 mm*10 model, clots were lodged in the MCA

bifurcation region in 13 rats (86.7%) and produced consistent ischemic lesion involving both ganglion and frontoparietal cortex. In the remaining 2 rats, clots were lodged in the main ICA and MCA bifurcation which induced a large ischemic lesion. One animal died of intracerebral hematoma (Table 1, Figure 3C). As the result, significant differences in ischemic lesion volume were observed between groups. The PE50 model has the smallest lesion volume of $31.09 \pm 23.08 \text{ mm}^3$, of which no lesion was found in 6 of the 10 rats. The PE60/2–3 mm*6–8 model has ischemic lesion of $142.53 \pm 68.51 \text{ mm}^3$, in which no lesion was found in 50% of rats. For the PE60/1mm*10 model, a reproducible ischemic lesion of $344.48 \pm 27.03 \text{ mm}^3$ was induced (Figure 3D).

3.3. rtPA-induced reperfusion decreases infarct volume, enhance cerebral perfusion and improve neurological deficit

To test the effect of rtPA on this model, animals were intravenously infused either with vehicle (L-arginine, n=8) or rtPA (5 mg/kg, n=11) via jugular vein (10% bolus, 90% perfusion during 30 minutes) started at one hour after stroke. We examined the change of cerebral perfusion before and after rtPA treatment by near-infrared spectroscopy monitoring of indocyanine green (ICG). Before rtPA treatment, higher optical density of ICG was observed on the normal (right) hemisphere as compared to the ischemic (left) hemisphere (figure 5A, C, E), suggesting a lower cerebral blood flow on the ischemic side. About 20 minutes after rtPA treatment (n=6), the Δ ICG peak began to decrease, suggesting that the cerebral blood flow gradually increase in left hemisphere. At ~60 minutes after rtPA administration, a total recovery of cerebral blood flow was observed in the left hemisphere cerebral blood flow (Figure 5E). In control group (n=5), no difference of Δ ICG peak was found after L-arginine treatment (Figure 5E).

Physiological variables (temperature, heart rate, breath rate, and oxygen saturation) were monitored during the surgery and no different were found between each group (Table 3). We examined the sensorimotor function by walk initiation and negative geotaxis before and after intravenous administration of rtPA (Figure 6A & B). Before treatment, no significant difference of neurological conditions was found between rtPA treatment and control group. At 24 hours after stroke, survival animals received rtPA (n=8) took significant less time both in walk initiation ($p=0.0106$) and turning 180° to complete negative geotaxis ($p=0.0156$) as compared with control animals (n=6). In addition, rtPA significantly reduced the ischemic lesion volume as compared with control ($p<0.0001$, Figure 6D). The infarct volume was $331.56 \pm 28.01 \text{ mm}^3$ and $60.25 \pm 23.77 \text{ mm}^3$ at 24 hours after stroke in the control and rtPA treatment group, respectively.

4. DISCUSSION

Therapies for acute ischemic stroke have achieved several important successes during the past decade, primarily related to thrombolysis. Intravenous recombinant tissue plasminogen activator (rtPA) treatment has been shown to significantly increase the number of stroke patients with no or minimal deficit when treated within 3 hours of symptom onset. rtPA has been recommended for selected patients within 3 hours after ischemic stroke (del Zoppo, 2000). However, nearly two decades after the approval of rtPA, its clinical use remains very limited, in large part, because ischemic tissue injury becomes irreversible within 2–4 hours post infarction, giving rise to a short time window (~3 hours) for thrombolytic intervention. Overall, rtPA has had only a modest impact in the overall burden of ischemic stroke because of its short therapeutic window (del Zoppo, 2000; del Zoppo et al., 1992; Wang et al., 2004; Zivin, 1999). There is a clear need for reproducible animal models to mimic the embolic stroke in human, to delineate the pathophysiology of the ischemic stroke in human, and to discover novel treatment for ischemic stroke.

Although embolic stroke models with homologous blood clots may not be as convenient as other methods such as MCA occlusion with nylon suture or foreign bodies. It definitely better mimic the clinical situation. The embolic stroke models using homologous clots could be used to test thrombolytic agents to evaluate the ischemic damage after thrombolysis. In addition, it can be used to study combined therapies with thrombolytic agents, hence, provide many advantages over other ischemic stroke models. The embolic MCA occlusion models closely mimics human ischemic stroke, but produces infarcts of more variable size and location (Carmichael, 2005). In embolic animal models, the clot introduction, stability and lodgment are difficult to manage, which leads to variations in infarct size and affected brain regions (Kaneko et al., 1985; Kudo et al., 1982; Overgaard et al., 1992). Two major techniques have been reported to improve the reproducibility and uniformity of the embolic stroke models in rats, of which fibrin-rich clots were selected for embolization. Zhang et al. have been using a modified PE 50 catheter, which could reach the origin of MCA endovascularly, to deliver a fibrin-rich clot to selectively occlude MCA (Zhang et al., 1997a; Zhang et al., 1997b). This method was latterly improved by DiNapoli et al. utilizing simultaneous laser Doppler flowmeter monitor (Dinapoli et al., 2006). However, the modification of the PE50 catheter requires extensive training, and strict quality control procedure has to be adopted during the manufacture of the catheters to ensure the tips of the catheter less than 0.3 mm in out diameter (Beech et al., 2001). The stretching of the polythene tube made the wall very thin and easily damaged when handled with forceps. As a consequence a new catheter is needed for each experiment. Busch et al. have been able to produce reliable clot occlusion of the proximal part of MCA by using thrombin added fibrin-rich clots (Busch et al., 1997). In this method, arterial blood is mixed with thrombin and clot is formed in PE 50 tube. The red blood cells are removed with the rinse of saline and the fibrin-rich segments are selected under a microscope. Our histological study demonstrated that the thrombin added clots and spontaneous formed pure blood clots have similar structure with fibrin component localized in the center of the clots and loose attachment of red blood in the peripheral. Therefore, red blood cells could be easily washed off when rinse with saline. Especially for pure blood clots, they could completely disperse and disappear. Even the fibrin core still exist, the size of the remaining fibrin-rich core after washing could be very different because of the irregular shape of the fibrin-rich core. For the thrombin added clots, clot formation is similar to the pure blood clots with fibrin-rich cores and lack of elasticity. On the other hand, the red blood cells were tightly glued together with the evenly distributed fibrin network in the fibrinogen and thrombin added clots, which enable the clots stand for extensive washing without fragmentation and maintain good elasticity.

Similar as the endovascular suture MCA occlusion model (Tsuchiya et al., 2003), the size of the clots is the most important factor for the success of the embolic ischemic stroke model. Injection of microsphere/macrosphere has been used for the ischemic stroke models. The macrosphere technique involves installation of large 0.3 to 0.4 mm diameter spheres into the internal carotid artery, which lodge in the MCA and produce an infarct of similar size and location as the endovascular suture MCAO models (Gerriets et al., 2003). While the microsphere model utilizes 50 μ m diameter sphere, that produce smaller, multifocal infarctions throughout the brain (Mayzel-Oreg et al., 2004). Consistently, endovascular injection of clots also produces different size infarcts in different location when different size of blood clots was used. When smaller size of clots (0.1 to 0.3 mm in diameter) were used, lodgment of clots could be found in a much wider range of cerebral vasculatures including proximal and distal MCA, ACA, and PCA. In addition, contralateral embolic clots were found in 30% of the rats (Kaneko et al., 1985). On the other hand, most of clots were found localized in the distal end of the ICA and extended into the proximal MCA and ACA, when a bigger size (0.35 in diameter) of fibrin-rich clots were used (Busch et al., 1997).

In the present study, three different sizes of thrombin and fibrinogen mixed clots were used for embolic stroke: 6 to 10 pieces 2–3 mm long clots made in PE50 catheter; 6 to 8 pieces 2–3 mm long clots made in PE60 catheter; and 10 clots 1-mm long made in PE60 catheter. We observed different clot lodgment site and ischemic lesion volume in embolic MCAO model using different type of clot. The diameter of thrombin and fibrinogen added clot made in PE60 catheter is $0.64 \pm 0.02\text{mm}$, which is significant bigger than that of the thrombin and fibrinogen added clot made in PE50 catheter ($0.47 \pm 0.01\text{mm}$). The elasticity of fibrinogen and thrombin mixed clots enable them to contract during injection and with the pulsatile blood pressure and occlude the MCA. For the PE50 prepared clots, the diameter of the clots was apparently too small which enable them to pass through the much small vessels and lodge in the distal MCA and PCA. For 2–3 mm long PE60 prepared clots, the larger size of the clots lead to the occlusion of the main branch of the ICA with or without the occlusion of MCA, hence, result in a larger variation of the ischemic lesion. On the other hand, the 1-mm long clots enable them lodge precisely in the intracranial ICA and proximal MCA, could induce reproducible ischemic lesion in the MCA territory. In addition, the appropriate strength of injection was another important factor for the embolic MCAO model. In our experience, a distortion of ICA in subject to pressure induced by a short burst of clots injection indicates the precise lodgment of clots in the ICA segment closed to the MCA bifurcation.

Elevated endogenous tPA have been found at the acute phase in ischemic stroke patients (Lindgren et al., 1996). In an experimental study, activation of the fibrinolytic system as indicated by significantly elevated tPA antigen concentrations was observed in thromboembolic stroke model (Stehling et al., 2008). The activated endogenous tPA could lead to thrombolysis, hence, spontaneous reperfusion. The higher concentration of fibrin in the thrombin and fibrinogen added clots could make the clots less vulnerable to the endogenous fibrinolytic system (Niessen et al., 2003). In the present study, this improved embolic stroke model is stable and resistant to the endogenous fibrinolytic system. On the other hand, this embolic MCAO model does response to rtPA thrombolytic therapy. The kinetics of intracranial ICG change was monitored by diffuse optical tomographic imaging system which provided 3-D tomographic images that detail the dynamics of the cerebral vascular response to thrombolytic therapy. Reperfusion was induced by rtPA treatment initiated at 1 hour after stroke. Consistently, the rtPA induced reperfusion, identified by NIRS, was associated with the improvement of neurological deficit and reduction of ischemic lesion volume. However, the current embolic MCAO model using the thrombin and fibrinogen composed artificial clots might have unique feature as compared with the previous embolic stroke models. Future studies are needed to determine the dose-dependent therapeutic window of rtPA and the rtPA associated intracerebral hemorrhagic transformation.

In summary, our present study established a novel embolic MCAO in rats using thrombin and fibrinogen composed clots, which have even distribution of fibrin with tight cross linkage of red blood cells. When formed in PE60 tube, thrombin and fibrinogen added clots have an optimal size and elasticity, which enable them to precisely lodge to the MCA bifurcation, hence, induce reproducible lesion in the MCA territory. This embolic MCAO model overcomes the limitation of the uniformity in the infarct size in the previous models using fibrin-rich embolus made from thrombin added clots. In addition, the current embolic MCAO model is relatively easy to produce which does not need endovascular placement of a special modified catheter to the MCA bifurcation. This embolic model may be useful for future studies of novel fibrinolytic agents and other combined therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A novel embolic middle cerebral artery occlusion model using thrombin and fibrinogen composed clots in rats.
- Precise clots lodgment at the MCA bifurcation and reproducible ischemic lesion.
- Responsive for recombinant tissue plasminogen activator thrombolytic therapy.

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Figure 1.

H&E staining of the three types clots (20×). **A–B**: Cross and longitudinal sections of the spontaneous formed clot. **C–D**: Cross and longitudinal sections of the thrombin added clot. **E–F**: Cross and longitudinal sections of the thrombin and fibrinogen added clot. Fibrin component gathered in the center of the clots in both spontaneous formed and thrombin added clots. While, fibrin fiber distribute evenly and tightly crosslink the red blood cells in the thrombin and fibrinogen added clots.

Figure 2.

A. Clot washing in a PE60 catheter connected to 2 PE160 catheters by continuous alternative pushing the two connected syringes at the end of PE160. **B.** Diameter of PE60 prepared clots (n=10) is 0.64 ± 0.02 mm, which is significant larger than that of clots made in PE50 catheter (0.47 ± 0.01 mm, n=10). ***p<0.0001.

Figure 3.

A–C: Distribution of clots (in red) in the carotid artery system induced by injection of different sizes of clots. ICA: internal carotid artery, MCA: middle cerebral artery, ACA: anterior cerebral artery, PCA: posterior cerebral artery, AchA: anterior choroidal artery, PcoA: posterior communicating artery. **D:** Ischemic lesion volume induced by embolic MCAO using three different sizes of clots. Embolic MCAO using 10 pieces of 1 mm long PE60 prepared clots results in reproducible larger lesion volume with smaller variance than that using 2–3 mm long PE60 prepared clots or PE50 prepared clots. ** $p < 0.01$, *** $p < 0.0001$.

Figure 4.

A: Photograph of experimental setup where the bifurcated fibers (indicated in white arrow) were placed on the scalp. **B:** Illustration the schematic arrangement of bifurcated fibers for DYNOT imager.

Figure 5.

A–D: Horizontal (A,B) and coronal (C,D) reconstructed tomographic images for optical density (ΔOD) after ICG injection. A,C: Representative images before rtPA treatment. B,D: Representative images at 60 minutes after rtPA treatment. Higher ΔOD represents higher ICG concentration (ΔICG) since more light was absorbed. Higher ΔOD of ICG was observed on the non-ischemic hemisphere (right) as compared to the ischemic side (left) before rtPA treatment (A and C). No significant difference of ΔOD of ICG was observed between the ischemic and non-ischemic hemisphere after rtPA treatment (B and D). “R” represents right hemisphere (non-ischemic) and “L” represents left hemisphere (ischemic). **E:** ΔICG peak in the ischemic and non-ischemic hemisphere recorded in the white dashed line-indicated section (A, B) at multiple time points before and 20 to 80 minutes after rtPA treatment. Blue dashed line represents the polynomial fit for data points from vehicle treatment group (control, $n=5$) and red dashed line are fitted trend line for the rtPA treatment group (rtPA, $n=6$). Every data point is presented as mean $\pm$ SEM.

Figure 6.

A–B: Walk initiation and negative geotaxis test before and after the treatment of rtPA or L-arginine. No significant difference in time of walk initiation and time taken to complete negative geotaxis was observed before rtPA or L-arginine treatment. At 24 hours after embolic MCAO, rtPA treatment group (n=8) took significant less time for walk initiation and completion of negative geotaxis as compared with control (n=6). * p<0.05. **C:** rtPA treatment (n=11) significantly decreased the ischemic lesion volume as compared with control (n=8). *** p<0.0001.

Table 1

Outcome of embolic MCAO induced by different clots.

	lesion	hematoma	death
PE50/1mm *6–8 (n=10)	4	0	0
PE60/2–3mm*6–8 (n=10)	5	1	3
PE60/1mm*10 (n=15)	15	1	3

PE50/1mm *6–8: 6 to 8 pieces 1 mm-long clots made in PE50 catheter; PE60/2–3mm*6–8: 6 to 8 pieces 2–3 mm-long clots made in PE60 catheter; PE60/1mm*10: 10 pieces 1 mm-long clots made in PE60 catheter.

Table 2

Outcome of rtPA and vehicle treatment in the embolic MCAO induced by 10 pieces 1-mm long clots made in PE60 catheter.

	Lesion	SAH*	Hematoma	Death
rtPA (n=11)	7	2	1	3
L-arginine (n=8)	8	0	0	2

(SAH*: subarachnoid hemorrhage)

Table 3

Physiological parameters in rtPA and vehicle treatment groups.

	Temperature (°C)		Heart Rate (bpm)		Breath Rate (bpm)		Oxygen Saturation (%)	
	Before	During	Before	During	Before	During	Before	During
rtPA (n=11)	36.73±0.14	36.73±0.07	336.4±11.22	372.5±9.99*	57.38±2.47	56.00±2.65	92.21±0.81	94.08±0.66
L-arginine (n=8)	36.90±0.19	36.96±0.08	328.3±10.17	337.3±11.52	51.33±3.98	57.17±3.37	93.18±1.14	92.02±1.07

During rtPA administered intravenously, the heart rate significantly increased compared with before treatment. (*P=0.0306)