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Research article

A modified mouse model of haemorrhagic transformation associated with tPA administration after thromboembolic stroke

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ABSTRACT

Objective: To establish a new mouse model of haemorrhagic transformation associated with delayed tissue-type plasminogen activator (tPA) treatment to provide a novel tool to study therapeutic strategies for haemorrhagic transformation.
Methods: Male C57BL/6 mice were subjected to carotid artery thrombosis stimulated with ferric chloride. The thrombus was then mechanically detached to induce migration toward the intracranial circulation. To induce haemorrhagic transformation, mice were intravenously injected with 10 mg/kg tPA 4.5 h after the onset of ischaemia and were sacrificed 24 h after tPA treatment.
Results: In this new model, administration of tPA 4.5 h after stroke exacerbated the risk of intracerebral haemorrhage. Thrombolysis with tPA also exacerbated cerebral infarction, brain oedema, blood–brain barrier breakdown, and neurological deficits. However, cerebral blood flow was not significantly affected.
Conclusion: The present model is reproducible, easy to perform, and mimics the clinical situation of haemorrhagic transformation after tPA treatment in humans. This modified model can be used as a new tool to test experimental drugs for haemorrhagic transformation associated with delayed tPA administration after an ischaemic stroke.

1. Introduction

Currently, induction of early intravenous thrombolysis with tissue-type plasminogen activator (tPA) is the only US Food and Drug Administration (FDA) approved drug for acute ischaemic stroke treatment [1,2]. However, tPA must be administered intravenously within 4.5 h of ischaemic stroke onset owing to the increased risk of haemorrhagic transformation [3,4]. Only a small percentage of patients with ischaemic stroke benefit from thrombolytic therapy with tPA because of its narrow therapeutic window [5]. Therefore, it is necessary to decrease the risk of haemorrhagic transformation caused by delayed tPA treatment and extend the therapeutic time window for thrombolysis therapy [6].

Many animal models to investigate haemorrhagic transformation caused by tPA treatment after ischaemic stroke have been established, including mechanical and thrombosis models [7]. In the mechanical model, cerebral ischaemia is induced by intraluminal

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occlusion of the middle cerebral artery with an inserted nylon filament [8]. The filament is then removed to cause reperfusion after a certain period of time. This model is characterised by large infarct volume, high reproducibility, and definite reperfusion injury after ischaemia. However, it does not mimic the clinical situation of thromboembolic stroke, as there are no thrombi. In the thrombosis model, clots are either injected into the middle cerebral artery from the carotid artery or formed in situ using thrombin, electrocoagulation, or photochemical methods [9]. This process is similar to the clinical situation of human thromboembolic stroke. However, subtemporal craniotomy is required in this model, and surgery may influence the status of the middle cerebral artery [10]. Therefore, a new, simple, and practical animal model for tPA thrombolysis after ischaemic stroke is needed.

In this study, we modified a previously reported large-vessel thromboembolic stroke model [11]. At 4.5 h after tPA thrombolysis,

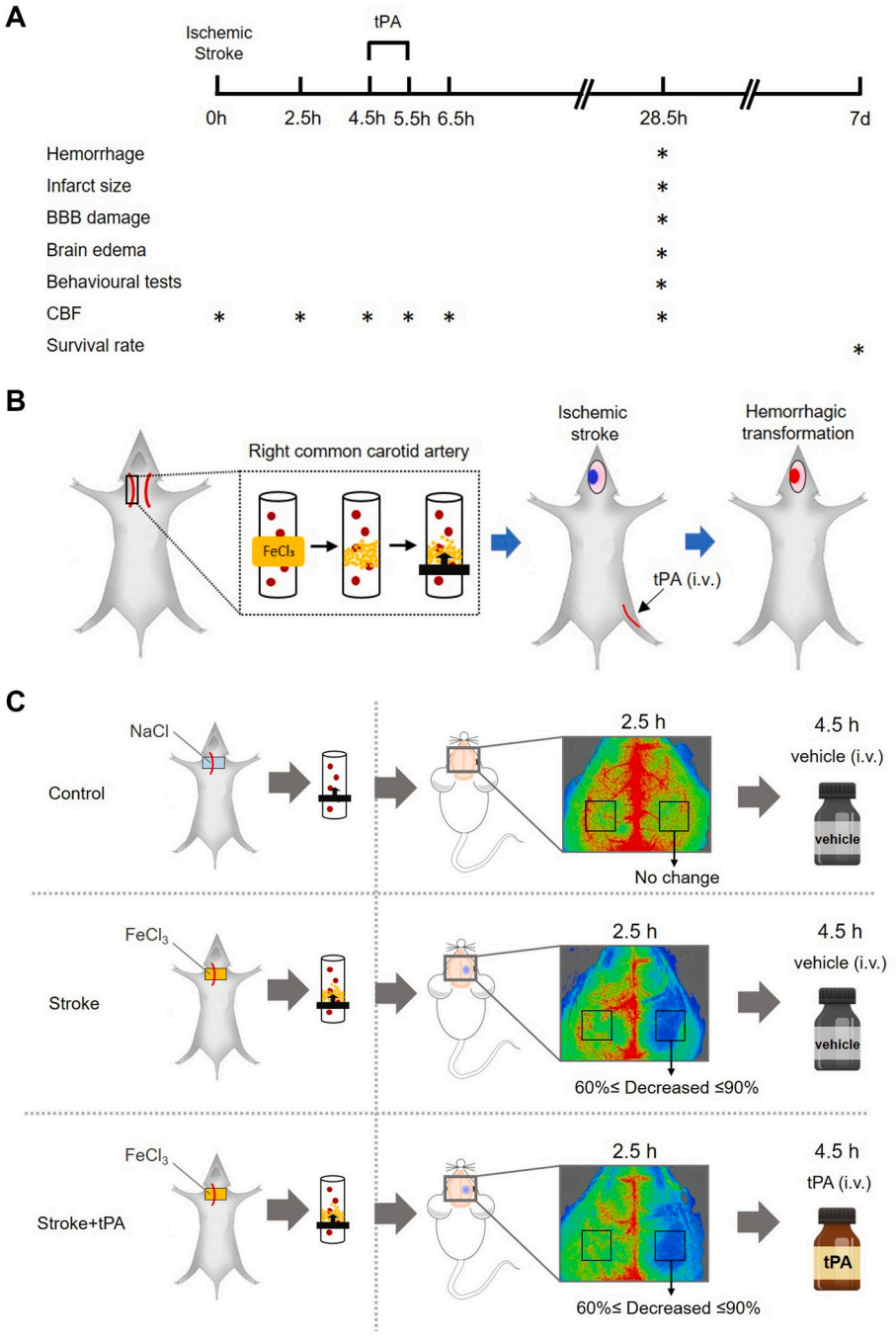

Fig. 1. Schematic representation of the experimental design. (A) Flow chart of the study. (B) Schematic drawing of the establishment of the animal model. (C) Experimental animal grouping.

we assessed the recanalization of blood vessels, blood-brain barrier (BBB) permeability, brain water content, and cerebral haemorrhage in the new model. Moreover, cerebral infarction, neurological deficits, and survival rates were evaluated to comprehensively assess this model. This study aimed to establish a new animal model of haemorrhagic transformation associated with delayed tPA treatment. This novel model may be used to study the pathophysiological mechanism of haemorrhagic transformation associated with tPA administration, and to develop novel therapeutics to inhibit intracerebral haemorrhage.

2. Materials and methods

2.1. Animals

A total of 181 male C57BL/6 mice weighing 20–24 g (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Certificate No. SCXK 2016-0006) were used for this study. All animal experiments were performed in accordance with the guidelines of the Peking University Health Science Center Animal Research Committee.

2.2. Experimental groups

The experimental design is illustrated in Fig. 1. Mice were randomly assigned to three groups: (1) Control, comprising sham mice were treated with vehicle. (2) Stroke, comprising thromboembolic mice treated with the vehicle. (3) Stroke + tPA, comprising thromboembolic mice treated with tPA. The mice were randomly assigned to each group using random numbers (Fig. 1C). The number of animals in each group is shown in Table S1. A total of 72 mice were excluded from the study.

2.3. Surgical procedures and tPA administration

Cerebral ischaemia was induced by ferric chloride stimulation of the carotid artery, followed by mechanical detachment of the thrombus, as described previously [11]. Briefly, the mice were anaesthetised with 2% sodium pentobarbital (45 mg/kg, I.P.) and observed under a dissecting microscope. The body temperature of the mice was maintained at 37 ± 0.5 °C using a heating plate during and after surgery. The right common carotid artery was gently exposed after making an incision in the neck, and the isolated right common carotid artery was wrapped with filter paper strips (2 mm × 5 mm) soaked in ferric chloride solution (20%, Fuchen, China) for 5 min (Fig. 1B). Then, the filter paper was removed, and the residual ferric chloride was gently washed with a 37 °C saline solution. After 10 min, the thrombus was examined under a dissection microscope. If the common carotid artery wrapped with a filter paper strip was filled with a yellow thrombus, the thrombus was detached using microforceps to induce migration toward the brain. The right common carotid artery was then returned to its original position, and the incision was sutured. Only mice whose percent cerebral blood flow fell by 60–90% compared to the baseline at 2.5 h were used in the ischaemic groups (Fig. 1C). Mice in the control group underwent the same operation, except that the ferric chloride solution was replaced with saline. To induce haemorrhagic transformation, tPA (10 mg/kg, Boehringer Ingelheim) was intravenously administered (left femoral vein, 10% bolus, 90% continuous infusion for an hour) at 4.5 h after ischaemia onset. Mice in the other two groups received the same volume of vehicle under identical conditions.

2.4. Measurement of cerebral infarction

Mice were euthanatized 24 h after tPA administration. The brains were removed immediately, and brain tissue was sliced into five serial 2-mm-thick coronal sections. Sliced brain tissues were immersed in a solution of 2,3,5-triphenyltetrazolium chloride (TTC, 2%, Sigma-Aldrich, United States) at 37 °C for 20 min [12]. The brain slices were then immersed in saline solution to stop the TTC reaction. The slices were photographed, and the infarction volume was calculated using the Image J software.

2.5. Determination of brain water content

Brain water content was evaluated using the dry-wet weight method. Mice were euthanised 24 h after tPA administration. The brains were quickly removed and weighed to obtain the wet weight. The brain tissue was then dried in an oven at 60 °C for 72 h and re-weighed to obtain the dry weight. Brain water content was calculated as 100% × (wet weight – dry weight)/wet weight.

2.6. Measurement of intracerebral haemorrhage

At 24 h after tPA treatment, mice were anaesthetised with sodium pentobarbital and perfused transcardially with ice-cold saline until the outflow fluid was colourless. Brain tissues were removed quickly and sliced into five 2-mm-thick serial coronal sections. The slices were photographed, homogenised in PBS, and centrifuged (12,000×*g*). The haemoglobin content was measured using a colorimetric assay kit (No. 700540, Cayman Chemical, United States). The severity of haemorrhage was classified into four levels: no haemorrhage (NH), haemorrhagic infarction type 1 (HI–I), revealing a single petechia in the ischaemic area; haemorrhagic infarction type 2 (HI-II), 2–3 petechiae in the ischaemic area; and haemorrhagic infarction type 3 (HI-III), more than 3 petechiae in the ischaemic area [13].

2.7. Evaluation of blood brain barrier permeability

Evans blue (2%, 4 ml/kg, Sigma-Aldrich, United States) was intravenously injected 3 h before sacrifice to assess the BBB permeability [14]. After 3 h, the mice were deeply anaesthetised and transcardially perfused with cold saline. Brain tissues were isolated and sliced into 2-mm coronal sections. The slices were photographed and then homogenised in PBS, followed by centrifugation (12, 000×*g*). Subsequently, the supernatants were collected, and the absorbance was quantified using a spectrometer at 620 nm. External standards were used to calculate the Evans blue level.

FITC-labelled albumin leakage has also been used to assess BBB permeability [15]. The skull was thinned carefully using an electric cranial drill. At 24 h after tPA treatment, FITC-labelled albumin (50 mg/kg, Sigma-Aldrich, United States) was infused into the femoral vein. Thirty minutes later, leakage of FITC-labelled albumin was observed under intravital fluorescent microscopy (BX51WI, Olympus, Japan). The fluorescence signal was acquired using a 420–490 nm wavelength for excitation and 520 nm wavelength for emission. Quantification of the fluorescence intensity was performed using the Image J software. The results are presented as I/V (I, fluorescence intensity in interstitial tissue; V, fluorescence intensity within the cerebral venule).

2.8. Monitoring of cerebral blood flow

Cerebral blood flow was determined using laser-Doppler flowmetry (moorFLPI-2, Moor Instruments, UK) [15]. Mice were

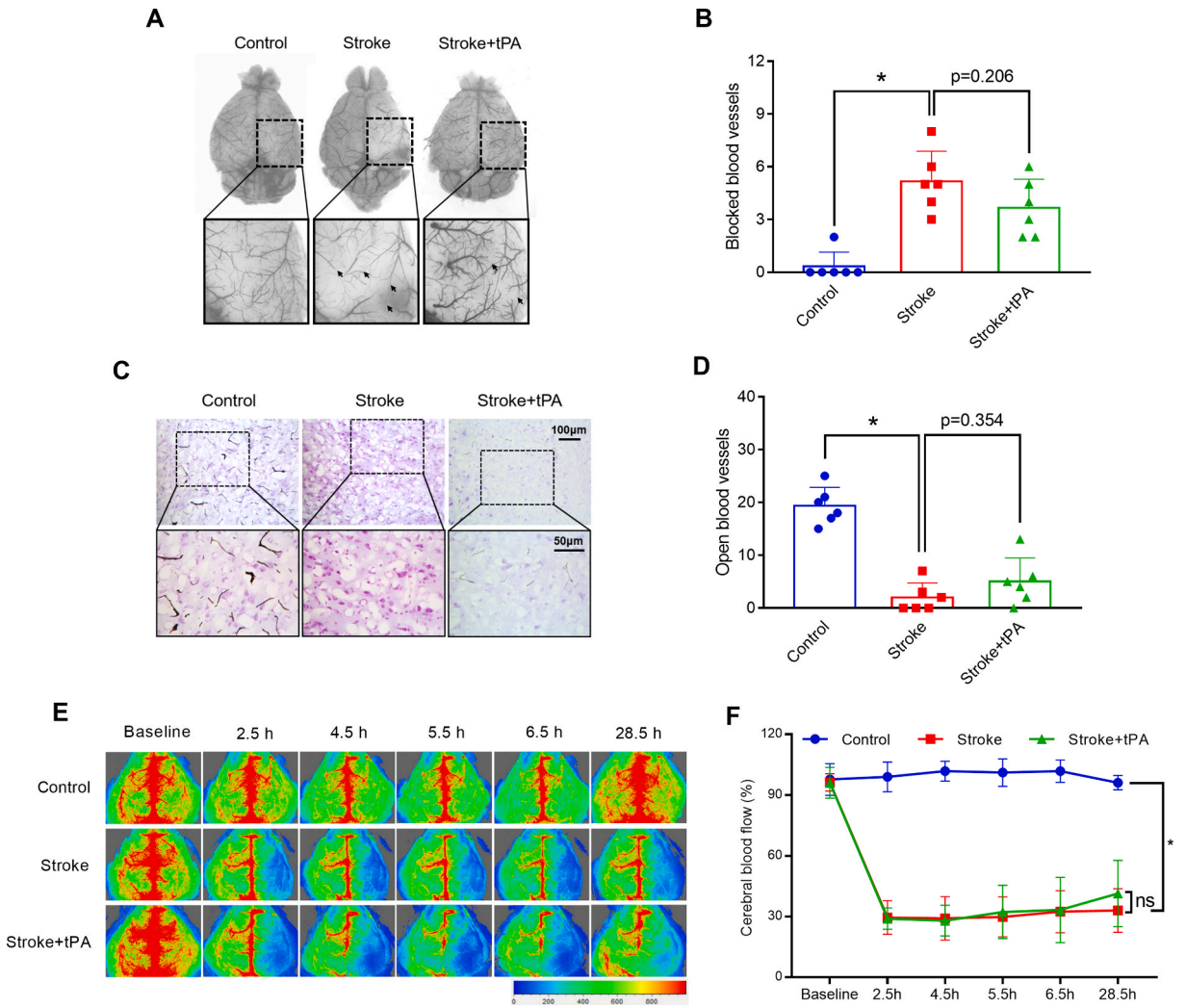

Fig. 2. Blood vessels and cerebral blood flow. (A) Representative images and (B) quantitative analysis of blocked blood vessels. Black arrows indicate blocked blood vessels. (C) Representative images and (D) quantitative analysis of open blood vessels. n = 6 per group. Data were compared by a one-way ANOVA followed by Tukey's post hoc test. (E) Representative images and (F) quantitative analysis of cerebral blood flow. n = 6 per group. Data were compared with a two-way ANOVA followed by Tukey's post hoc test. *P < 0.05, compared as indicated, ns indicates no significant difference.

anaesthetised with 2% sodium pentobarbital (45 mg/kg, I.P.). The skull was then exposed after making a skin incision on the head. A laser-Doppler flowmetry probe was used to scan the brain and record the cerebral blood flow. Images were obtained at baseline, and at 10 min, 2.5 h, 4.5 h, 5.5 h, 6.5 h, and 28.5 h after ferric chloride stimulation. Cerebral blood flow changes were analyzed using the moorFLPI Review software.

2.9. Nissl staining and Indian ink perfusion

Nissl staining was performed as previously described [16]. After fixation with 4% paraformaldehyde for 48 h, the brain tissues were dehydrated in gradient sucrose solution (10%, 20%, 30%, 48 h for per concentration). Optimal cutting temperature (OCT) compounds were used to embed brain tissues. Frozen brain tissues were sectioned coronally using a freezing microtome (CM 1900, Leica, Germany) at a thickness of 8–10 μm. The sections were hydrated in absolute ethyl alcohol, 95%, and 80% gradient of ethanol. After washing with distilled water for 10 s, the sections were stained with Nissl staining solution (M20171, Saint-bio, China) at 55 °C for 40 min. They were then washed with distilled water, differentiated with 95% ethanol, vitrified with dimethylbenzene, and sealed with neutral gum. Images were captured using a light microscope (BX512DP7, Olympus, Japan). For Indian ink perfusion, mice were transcardially perfused with saline followed by Indian ink (25%, 30 ml, Phygene, China) prepared in 6% gelatine and saline [17]. The brain was then cooled at 4 °C for 2 h to allow gelatine solidification. Brain tissues were sliced into five 2-mm serial coronal sections and immersed in saline solution. Images were captured using a digital camera and a light microscope. Quantification of blood vessels was performed using the Image J software.

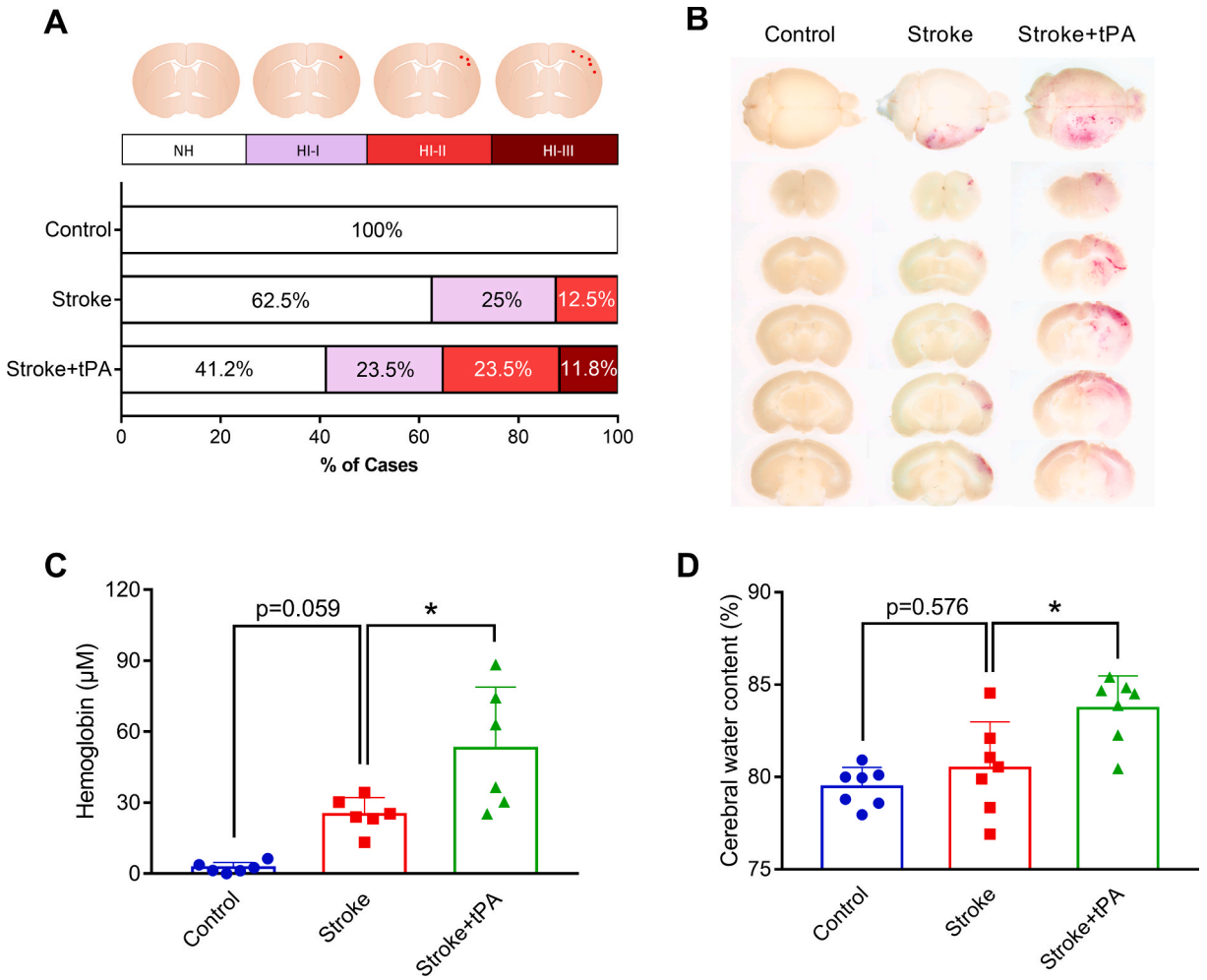

Fig. 3. Brain haemorrhage and oedema. (A) Distribution of each haemorrhagic severity. (B) Representative slices and (C) quantitative analysis of brain haemorrhage. n = 6 per group. (D) Quantitative analysis of the cerebral water content. n = 7 per group. Data were compared with a one-way ANOVA followed by Tukey's post hoc test. *P < 0.05, compared as indicated, ns indicates no significant difference.

2.10. Survival rate and neurological deficits

The survival rate was assessed every day for 7 days after stroke onset and calculated as the percentage of each group of mice. The Neurological Evaluation Scale (NES) was used as previously described (Table S2) [18]. At 24 h after tPA treatment, neurological deficits in the mice were assessed by a blinded investigator.

2.11. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). GraphPad Prism software was used for data analysis, and all endpoints were evaluated in a blinded manner. The normality of data distribution was assessed using the Shapiro-Wilk test. For normally distributed variables, one-way or two-way ANOVA followed by Tukey's post-hoc test was applied to assess differences between groups. For non-normally distributed variables, the Kruskal–Wallis test was performed for statistical analysis. The log-rank test was used to analyse the survival curves. Differences with $P < 0.05$ were considered statistically significant.

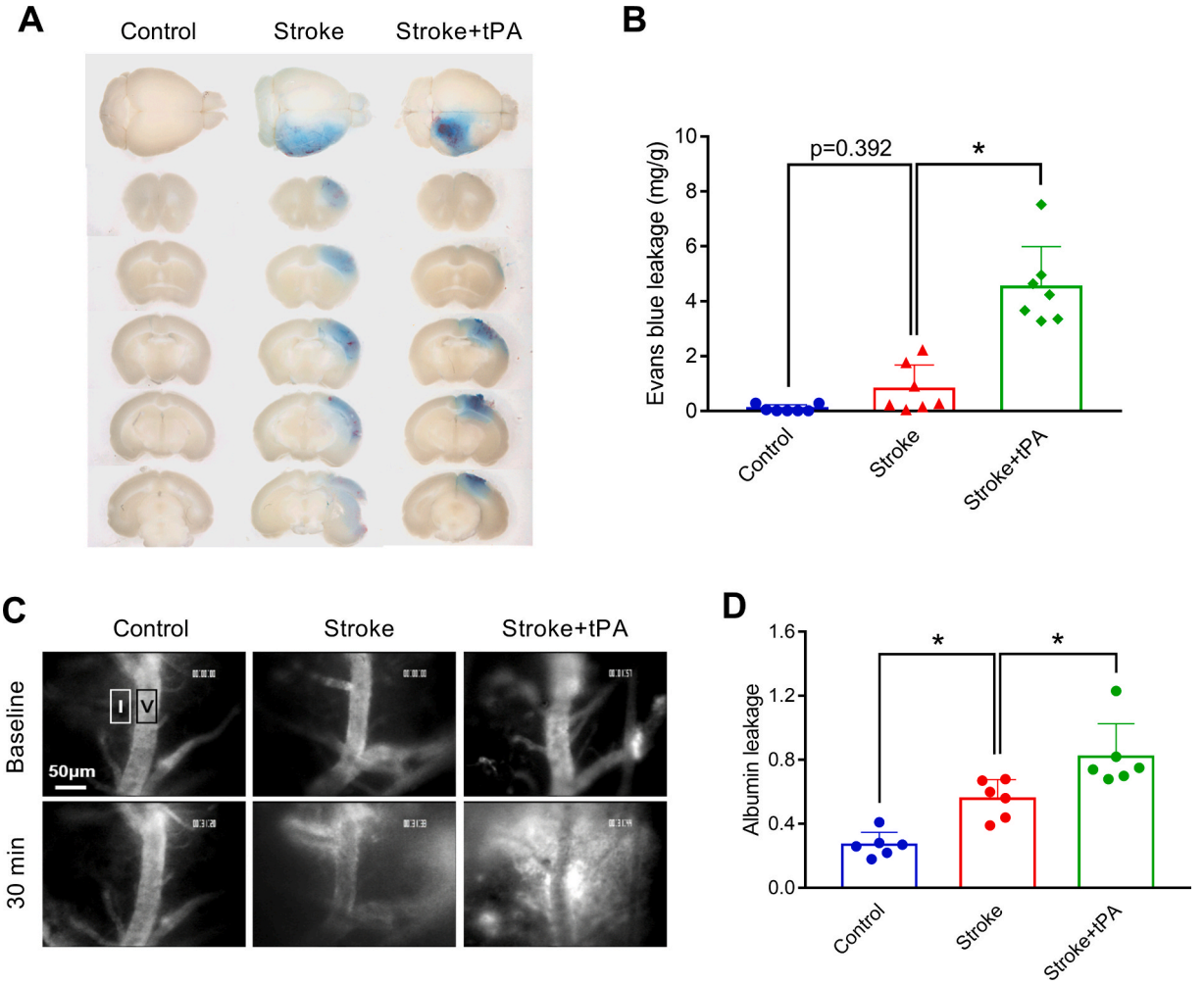

Fig. 4. Blood brain barrier (BBB) permeability. (A) Representative images and (B) quantitative analysis of Evans blue extravasation. n = 7 per group. (C) Representative images and (D) quantitative analysis of albumin leakage. n = 6 per group. I, the fluorescent intensity in interstitial tissue; V, the fluorescent intensity within cerebral venule. Data were compared with a one-way ANOVA followed by Tukey's post hoc test. *P < 0.05, compared as indicated, ns indicates no significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Number of blood vessels

A diagram of the experimental design is shown in Fig. 1. We first quantified the blocked and open blood vessels using the India ink perfusion method (Fig. 2A–D). Compared to the control group, blocked blood vessels were significantly increased in stroke mice ($P < 0.001$, F [2,15] = 17.49, $R^2 = 0.7$); This increase was partly prevented by tPA administration, but the difference was not statistically significant ($P = 0.206$; Fig. 2A and B). Open blood vessels were significantly decreased in stroke mice compared to the control group ($P < 0.001$, F [2,15] = 38.75, $R^2 = 0.84$). Treatment with tPA partly reversed the decrease in open blood vessels, although this improvement was not statistically significant ($P = 0.354$; Fig. 2C and D).

3.2. Cerebral blood flow

We monitored the cerebral blood flow in the new model using the laser Doppler technique (Fig. 2E and F). Cerebral blood flow was expressed as a percentage of that in the contralateral hemisphere (uninjured hemisphere). No significant alterations in cerebral blood flow were observed in control mice. As expected, ferric chloride stimulation plus thrombus detachment significantly decreased cerebral blood flow (28.5 h, $P < 0.001$). However, there was no statistically significant difference in cerebral blood flow with or without tPA administration following cerebral ischaemia (28.5 h, $P = 0.219$). This means that delayed tPA administration did not significantly improve cerebral blood flow compared with ischaemia alone.

3.3. Intracerebral haemorrhage and brain oedema

We subsequently evaluated haemorrhagic transformation in the new animal model. The haemorrhage rate of stroke alone was 37.5%, which was increased with tPA administration (58.8%; Fig. 3A). Compared to the stroke group, tPA treatment increased the percentage of severe haemorrhage (HI-II and HI-III). The volume of intracerebral haemorrhage was quantified using a photometric

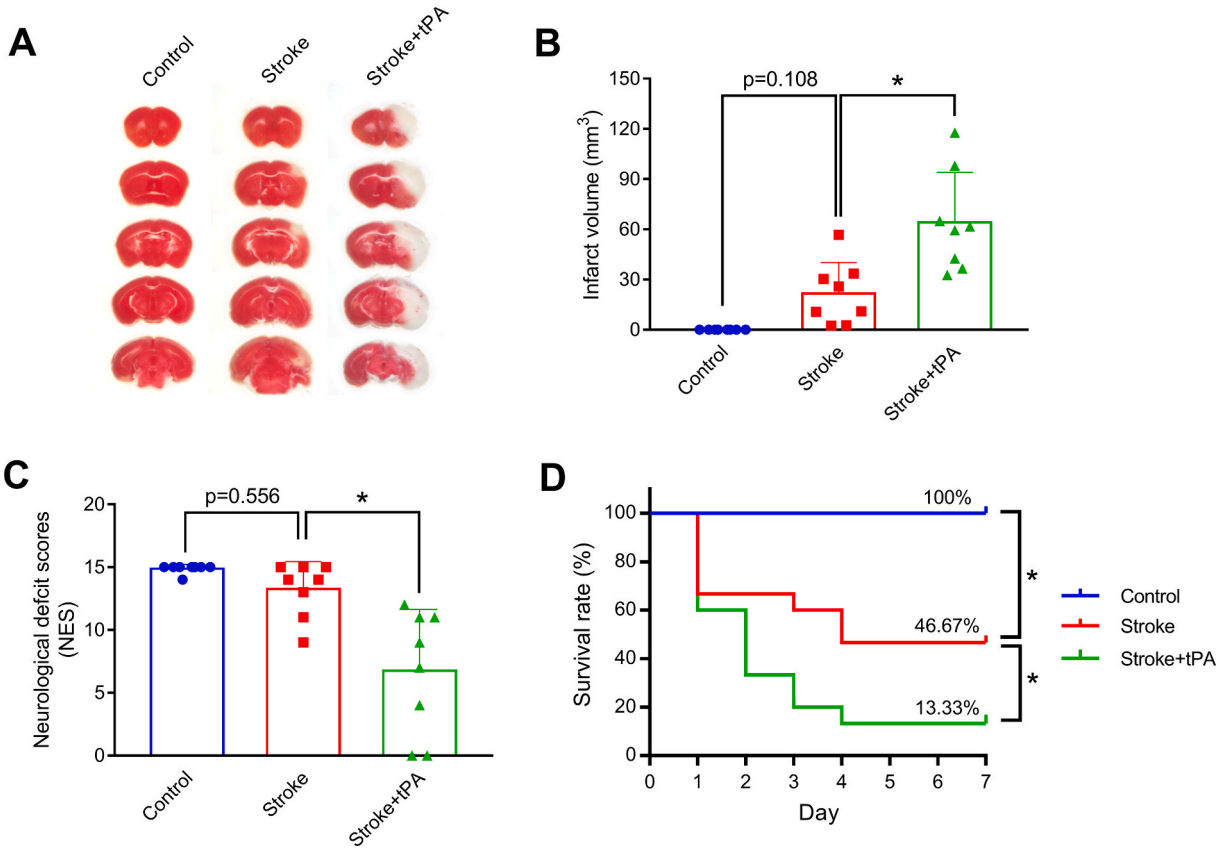

Fig. 5. Infarction and neurological scores. (A) Representative images and (B) quantitative analysis of infarct volume. n = 8 per group. Data were compared with a one-way ANOVA followed by Tukey's post hoc test. Neurological deficits were assessed by (C) neurological evaluation scale (NES). n = 8 per group. Data were compared with a Kruskal–Wallis test. (D) Survival rate. *P < 0.05, compared as indicated, ns indicates no significant difference. n = 13–15 per group. Data were compared with a Log-rank (Mantel-Cox) test.

haemoglobin assay (Fig. 3B and C). No significant cerebral haemorrhage was detected in any of the mice in the control group. However, ferric chloride stimulation plus thrombus detachment induced intracerebral haemorrhage in comparison to the control group ($P = 0.059$, F [2,15] = 15.78, $R^2 = 0.68$). Delayed tPA treatment after ischaemia significantly increased cerebral haemorrhage compared with ischaemia alone ($P = 0.019$). The cerebral water content in the stroke group was increased compared to the control group, although the difference was not statistically significant ($P = 0.576$, F [2,18] = 9.96, $R^2 = 0.53$). However, we found obvious brain oedema in tPA-treatment group compared to the stroke group ($P = 0.012$; Fig. 3D).

3.4. BBB permeability

Next, we quantified BBB permeability by measuring the extravasation of Evans blue dye into the brain tissue (Fig. 4A and B). The data showed that there was no significant Evans blue extravasation in the mice from the control group. Brain ischaemia slightly increased extravasation of Evans blue ($P = 0.392$, F [2,18] = 40.44, $R^2 = 0.82$), while occlusion in combination with tPA treatment remarkably exacerbated Evans blue extravasation compared with stroke alone ($P < 0.001$). Similar results were obtained using the FITC-labelled albumin leakage method (Fig. 4C and D); albumin leakage was higher in the stroke group than in the control group ($P = 0.01$, F [2,15] = 21.61, $R^2 = 0.74$), and treatment with tPA significantly exacerbated albumin leakage ($P = 0.017$).

3.5. Infarct volume

We evaluated infarct volume using TTC staining (Fig. 5A and B). No infarction was observed in any of the control mice. Cerebral ischaemia alone increased infarct volume ($P = 0.108$, F [2,21] = 20.66, $R^2 = 0.66$). Compared with ischaemia alone, delayed tPA treatment after ischaemia significantly increased the infarct volume ($P = 0.001$).

3.6. Neurological deficits and survival rate

We subsequently evaluated neurological deficits in the different groups (Fig. 5C). The present data showed that neurological deficits were not detected in control mice. Mice in the stroke group presented with neurological dysfunction, as assessed using the Neurological Evaluation Scale ($P = 0.556$, F [2,21] = 15.38, $R^2 = 0.59$). Cerebral ischaemia with tPA treatment significantly increased neurological deficits compared to the stroke alone ($P = 0.001$). The survival rate of mice was observed daily until day 7 (Fig. 5D). No mice in the control group died during the study period. Compared to the control group, mice in the stroke group presented significantly reduced survival rate ($P = 0.004$), and treatment with tPA further reduced the survival rate ($P = 0.044$).

4. Discussion

Thrombolytic treatment with tPA remains the sole therapy for ischaemic stroke but has a high risk of haemorrhagic transformation [19]. To date, there is no perfect animal model of haemorrhagic transformation associated with delayed tPA administration. Here, we present a novel mouse model of haemorrhagic transformation caused by tPA treatment after ischaemic stroke. This model is easy to perform and reproducible. Most importantly, it mimics the clinical situation of haemorrhagic transformation induced by tPA after ischaemic stroke.

Rupture of atherosclerotic plaques in the carotid arteries is the main cause of acute ischaemic stroke [20,21]. Recently, Lizarrondo et al. [11] described a new thromboembolic stroke model that mimicked the pathophysiology of human carotid atherosclerotic stroke. In this model, the carotid thrombus caused by ferric chloride was artificially detached to induce intracranial thrombus embolization. This new model mimics carotid atherothrombotic stroke in humans, and is easy to perform. Inspired by this study, we explored whether this stroke model could be used to induce haemorrhagic transformation with delayed tPA administration.

TPA is a glycosylated protein that binds to fibrin and directly converts plasminogen into plasmin. Several pathophysiological mechanisms are involved in the process of tPA-induced haemorrhagic transformation, including the proteinase activity of tPA itself, the activation of MMPs, and ischaemia reperfusion injury caused by tPA [22]. All of these links lead to BBB hyperpermeability, which plays an important role in the development of haemorrhagic transformation in ischaemic stroke [23]. In this model, administration of tPA did not significantly restore blood flow in ischaemic tissue. This may be because tPA can dissolve only fibrin-rich thrombi. However, ferric chloride-induced thrombi are platelet-rich and tPA resistant [11]. Delayed thrombolytic therapy with tPA was associated with deteriorated brain infarction and neurological deficit [24,25]. In agreement with previous studies, our results showed

Table 1
Comparison between the present model and existing tPA-induced hemorrhagic transformation models.

Method	Strength	Weakness
tPA + filament MCAO	high reproducibility, high recognition degree	no thrombus
tPA + embolic MCAO	well mimic the clinical situation	complicated to operate
The present model	easy to perform, well mimic the clinical situation	no obvious reperfusion injury, locations of the thrombi can not be accurately controlled

MCAO, middle cerebral artery occlusion.

that tPA administration 4.5 h after stroke onset increased infarct volume and aggravated neurological scores. We further found that tPA treatment disrupted the integrity of the BBB and caused intracranial haemorrhage, which may eventually lead to a decrease in the survival rate of mice.

The results of our animal models were quite different from those in clinical situations. For example, the rate of haemorrhagic transformation in stroke patients with tPA treatment is about 10% [26], while the rate of haemorrhagic transformation in our study was 58.8%. In addition, the mortality rate in our study reached up to 86.7%, whereas the rate in humans is only approximately 5% [26]. Similar pathological characteristics between animal models and clinical situations are the most important factors in translational research. From this perspective, our model is suitable for translational studies.

Our model has potential advantages over other existing models (Table 1). The classic filament middle cerebral artery occlusion (MCAO) model is the most commonly used procedure in tPA-induced haemorrhagic transformation studies [7]. In the filament MCAO model, a nylon filament is inserted into the circle of Willis to block the middle cerebral artery, after a few hours, the filament is removed to induce reperfusion injury [27]. The severity of brain injury depends on the duration of ischaemia. This model is highly reproducible, and may be used to induce ischaemia-reperfusion injury. However, the filament MCAO model does not have any thrombi therefore thrombolysis with tPA in this model does not strongly mimic the clinical situation. Thrombi can be induced by various methods (such as thrombin administration, electrocoagulation, photochemical stimulation) in situ embolic MCAO models [9]. This model is similar to the process of human ischaemic stroke and can respond to tPA thrombolysis. However, the in situ embolic MCAO model is relatively complicated as it requires craniotomy. In contrast, the proposed model is relatively easy to perform and has good repeatability. Furthermore, our model mimicked the clinical situation of human thromboembolic stroke. Recently, we used this model to explore the efficacy and mechanisms of QiShenYiQi (QSYQ), a compound Chinese medicine, in tPA-induced brain oedema and haemorrhage [15]. These results are exciting, and the model has proven to be practical for pharmaceutical research. An important weakness of our study design is that the present model was not compared with other existing models. This makes it impossible to determine the optimal model for each outcome. Experimental evidence comparing this model with others will be provided in future work.

The present study has some limitations (Table 1). First, thrombi caused by ferric chloride mainly consist of platelets, which is different from thrombi caused by thrombin. The thrombi did not dissolve well in this model. Therefore, the present model is inapplicable to studies investigating reperfusion injury following an ischaemic stroke. Second, the locations of the thrombi could not be accurately controlled, and the infarct positions were not the same between the animals. Third, we did not reserve the brains of mice that were used for the survival rate at day 7 or dissect the brains of dead mice. Therefore, the relationship between haemorrhagic transformation and mortality remains unclear. Furthermore, we did not perform power calculations to identify the minimum number of animals required per group in the present study. The number of animals used was determined according to the results of previous studies [7,18].

5. Conclusion

In summary, we presented here a new model of haemorrhagic transformation induced by tPA administration after stroke in mice. This model is reproducible, easy to perform, and mimics the clinical situation in humans with haemorrhagic transformation after tPA treatment. We believe that the present model may be useful for investigating the pathophysiological mechanisms and exploring new drugs for intracerebral haemorrhage caused by delayed tPA treatment after ischaemic stroke.

Author contribution statement

Yang Ye: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xi-Yan Xin: Analyzed and interpreted the data; Wrote the paper.

Hao-Lin Zhang; Yu-Tian Zhu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Rui-Wen Fan: Contributed reagents, materials, analysis tools or data.

Dong Li: Conceived and designed the experiments.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13102.

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