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ABSTRACT

The goal of our study was to determine if the timing of the tissue plasminogen activator (tPA) administration influenced its effect on blood–brain barrier (BBB) permeability and the subsequent risk of hemorrhagic transformation.

Thirty spontaneously hypertensive male rats were subjected to a 90-minute unilateral middle cerebral artery occlusion. Six rats did not receive tPA treatment (vehicle control: Group 0), intravenous tPA was administered immediately after reperfusion (Group 1) or 4 h after reperfusion (Group 2). Dynamic contrast enhancement (DCE) and gradient-echo (GRE) MR sequences were used to assess the dynamic evolution of BBB permeability and hemorrhagic transformation changes at the following time points: during occlusion, and 3 h, 6 h, and 24 h post reperfusion.

In all groups, BBB permeability values in the ischemic tissue were low during occlusion. In Group 0, BBB permeability values increased at 3 h after reperfusion ($p = 0.007$, compared with the values during occlusion), and further at 6 h after reperfusion ($p = 0.004$, compared with those at 3 h post reperfusion). At 24 h post reperfusion, the values decreased to a level relative to but still higher than those during occlusion ($p = 0.025$, compared with the values during occlusion). At 3 h after reperfusion, BBB permeability values in the ischemic tissue increased, but to a greater extent in Group 1 than in Group 0 ($p = 0.034$) and Group 2 ($p = 0.010$). At 6 h after reperfusion, BBB permeability values in the ischemic tissue increased further in Group 2 than in Group 0 ($p = 0.006$) and Group 1 ($p = 0.001$), while Group 1 exhibited BBB permeability that were still abnormal but less than those observed at 3 h ($p = 0.001$). Group 2 tended to have a higher hemorrhage incidence (36.4%, 4/11) than Group 1 (10.0%, 1/10, $p = 0.311$) and Group 0 (0%), and hemorrhages occurred around 6 h after reperfusion when BBB permeability values were the highest. Mortality was higher in Group 2 (63.6%, 7/11) than in Group 0 (0%) and Group 1 (10.0%, 1/10, $p = 0.024$).

The findings suggest that the timing of tPA administration is of importance for its impact on BBB permeability and subsequent risk of hemorrhagic transformation.

1. Introduction

Hemorrhagic transformation (HT) is a feared complication of acute ischemic stroke. It can arise as the result of an ischemic damage to the

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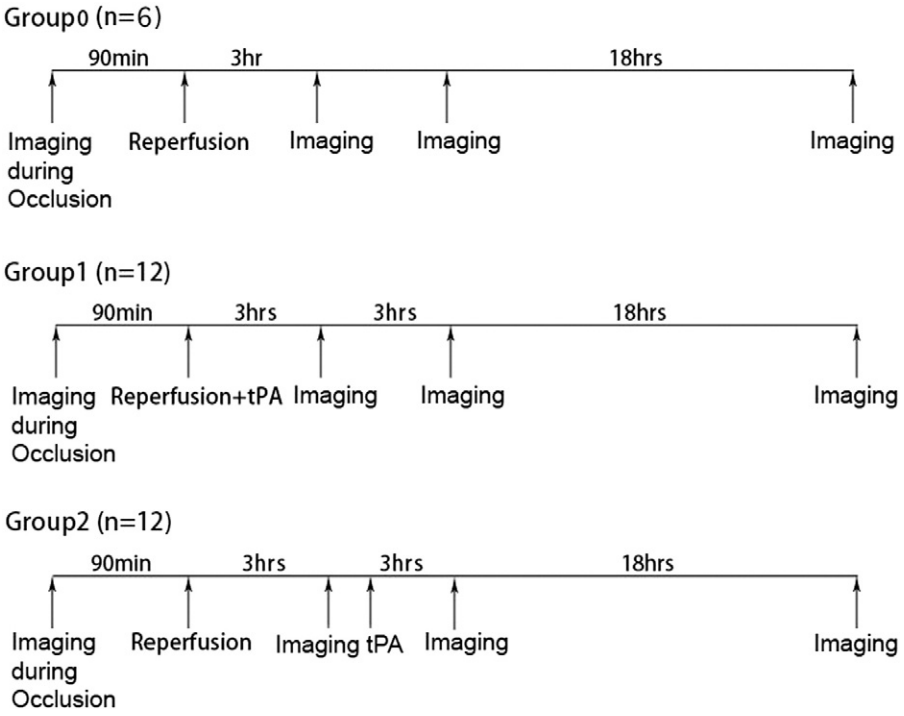

Fig. 1. Schematic representation of our study design and study groups. Group 0: vehicle control, without tPA administration. Group 1: tPA was administered immediately after reperfusion. Group 2: tPA was administered after the MR imaging at 3 h post reperfusion, and the imaging lasted approximately 1 h.

blood–brain barrier (BBB) with subsequent vascular leakage [7,26]. It is often triggered by reperfusion [32].

Tissue plasminogen activator (tPA) has been shown to be a successful thrombolytic drug in acute ischemic stroke patients [11,12] but significantly increases the risk of symptomatic HT [11,33,35]. In the National Institute of Neurological Disorders and Stroke (NINDS) tPA trial [1], the percentage of tPA-treated patients who developed significant HT following an ischemic stroke was 6.4% as compared with just 0.6% in the placebo group. One of the mechanisms by which tPA causes HT is its impact on the BBB permeability [20,27,32].

Reperfusion injury resulting from the thrombolytic effect of tPA involves reactive oxygen species and oxidative stress, which degrade protein and lipid components vital to the BBB function [14]. Independently of reperfusion, tPA activates matrix metalloproteinases (MMPs), which in turn alter the basal lamina of vascular endothelium, weaken vessels, and favors leakage and rupture [2,5,6,13,16,24,27,29,31,32].

The BBB permeability changes evolve in a dynamic process after reperfusion. In a 2-hour temporary middle cerebral artery occlusion (MCAO) model, Belayev et al. explored the time course and regional pattern of blood–brain barrier (BBB) opening after reperfusion, the quantitation of Evans' blue extravasation indicated some degree of BBB disruption occurring at 3–4 h after MCAO, maximal disruption at 5 h, and delayed BBB disruption at 48–50 h [3]. In a study with 3-h MCAO, the water content accumulated with time in the ipsilateral hemisphere within 12 h post MCAO (Slivka et al., 1995) [36]. In our study, we wanted to focus on the direct effect of tPA on the BBB permeability and on the risk of hemorrhagic transformation. We thus assessed the impact of tPA injected at various timepoints, while maintaining the duration of the occlusion constant.

2. Materials and methods

2.1. Study design

This animal study was approved by the Institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Spontaneously hypertensive (SHR) male rats (Charles River Laboratories, Inc., Wilmington, MA.), body weight 260–280 g, 11–13 weeks old, were subjected to 90 min of transient focal cerebral ischemia. The animals were then randomly assigned to one of the three treatment groups: (1) Group 0, controls: the rats were administered the same dose of saline vehicle as that of tPA (10 mg/kg, 10% administered as a bolus and the remainder as a continuous infusion over 60 min), (2) Group 1: tPA administered immediately after reperfusion, and (3) Group 2: tPA administered 4 h after reperfusion (Fig. 1). To determine whether the results would be influenced by the type of stroke model, we used two techniques to produce transient focal cerebral ischemia: (1) 3 vessel-occlusion technique (3VO) and (2) intraluminal filament occlusion technique (fMCAO).

Recombinant human tPA (Genentech, San Francisco, CA, USA) was reconstituted with the concentration of 1 mg/ml, and infused intravenously via the tail vein at a dose of 10 mg/kg. Ten percent was administered as a bolus and the remainder as a continuous infusion over 60 min, using a syringe infusion pump (New era pump system, Inc, Famingdale, NY, USA). This relatively high dosage was used because there is an approximately 10-fold difference in fibrin-specific enzyme activity between humans and rodents [23,31].

MR imaging was obtained at four time points: during occlusion, and at 3 h, 6 h, and 24 h post reperfusion. The MRI during occlusion and 3 h after reperfusion were used to confirm the inclusion and exclusion of the animals in the study. Exclusion criteria included: the diffusion-weighted imaging lesion during occlusion was too small (involved only subcortical regions, but not the cortex); the imaging before tPA administration (during occlusion or 3 h after reperfusion) showed intracranial hemorrhage.

2.2. Animal surgery

All surgery and MRI were conducted on the animals under anesthesia, which was induced by isoflurane at 4% and then maintained at 2%. During the procedures, the rats were intubated and ventilated

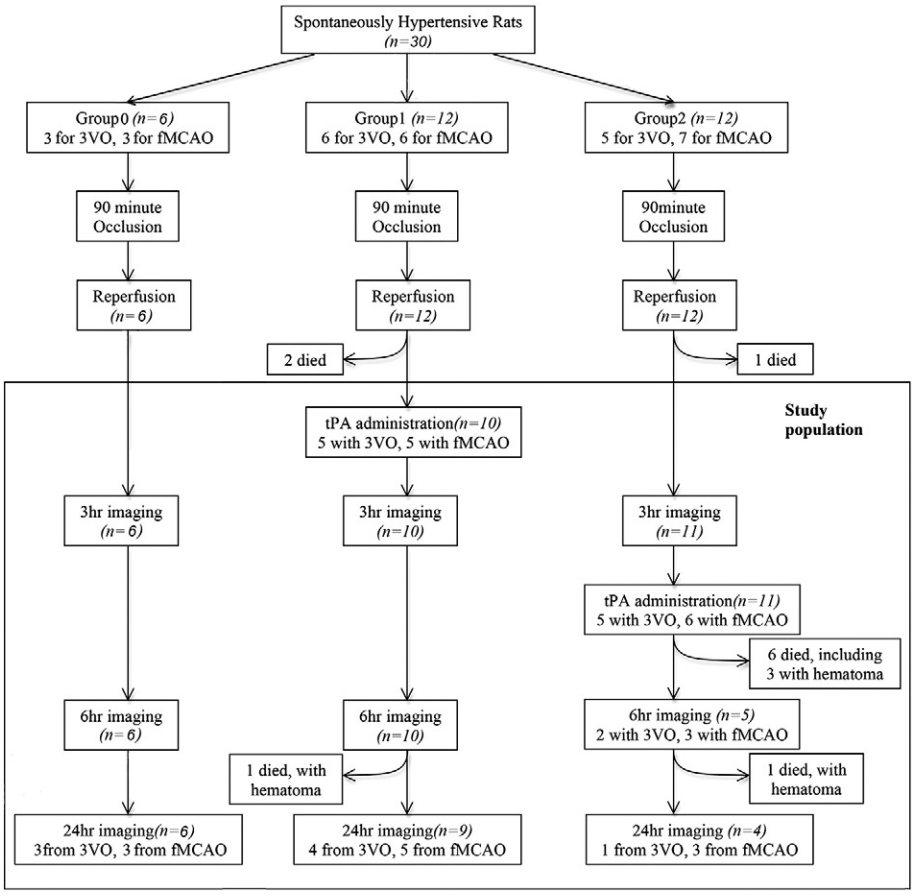

Fig. 2. Schematic representation of our study population.

mechanically. The tail artery will be cannulated for continuous blood pressure monitoring, and the mean blood pressure was kept at 110–120 mm Hg during surgery. Rectal temperature was monitored continuously and maintained at 37 °C using a heating blanket (during surgery) or thermostated circulating water (in MR imaging).

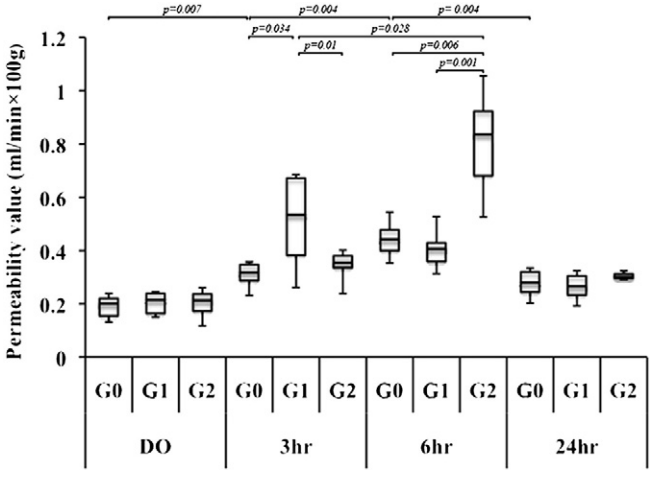

Fig. 3. Time-dependent changes in BBB permeability in the ischemic brain. Group 0 (G0): without tPA administration, Group 1 (G1): tPA administered immediately after reperfusion, Group 2 (G2): tPA given 4 h post reperfusion. During occlusion (DO), BBB permeability values in the ischemic area were low and there was no significant difference between the two groups ($p = 0.756$). At 3 h after reperfusion (3 h), BBB permeability in the ischemic tissue increased in all groups, but to a greater extent in Group 1 ($p = 0.0034$ and 0.01, compared with Group 0 and Group 2, respectively). At 6 h after reperfusion (6 h), BBB permeability was further increased in Group 2 ($p = 0.006$ and 0.001, compared with Group 0 and Group 1, respectively). At 24 h after reperfusion, BBB permeability in the ischemic tissue had decreased from the 6-hour levels, but remained higher than those observed during occlusion. The highest permeability values in Group 2 (at 6 h) were higher than those in Group 1 (at 3 h, $p = 0.028$).

Transient focal cerebral ischemia was obtained according to previously described techniques. For fMCAO [25], right middle cerebral artery (MCA) occlusion was achieved by advancing a 3–0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) through the external carotid artery into the internal carotid artery. Middle cerebral artery occlusion and associated ischemic injury were confirmed during occlusion by diffusion-weighted MRI and perfusion-weighted MRI. Reperfusion of the middle cerebral artery territory was established by withdrawal of the nylon suture 90 min after occlusion.

For 3VO, the procedure utilized has been described elsewhere [28]. The animals were placed in a decubitus position, with an incision between the left margin of the orbit and the tragus, the temporal muscle

Table 1
BBB permeability values at study time points.

Time points	Groups	Number of animals	Median (IQR) (ml/min × 100 g)
DO	G0	6	0.20 (0.15–0.22)
	G1	10	0.21 (0.16–0.24)
	G2	11	0.21 (0.17–0.24)
3 h	G0	6	0.30 (0.27–0.33)
	G1	10	0.53 (0.38–0.67)
	G2	11	0.35 (0.33–0.38)
6 h	G0	6	0.44 (0.40–0.48)
	G1	10	0.41 (0.36–0.43)
	G2	5	0.84 (0.68–0.92)
24 h	G0	6	0.28 (0.24–0.32)
	G1	9	0.26 (0.23–0.30)
	G2	4	0.30 (0.29–0.31)

Abbreviations: G0: Group 0; G1: Group 1; G2: Group 2; DO: during occlusion; 3 h: 3 h after reperfusion; 6 h: 6 h after reperfusion; 24 h: 24 h after reperfusion

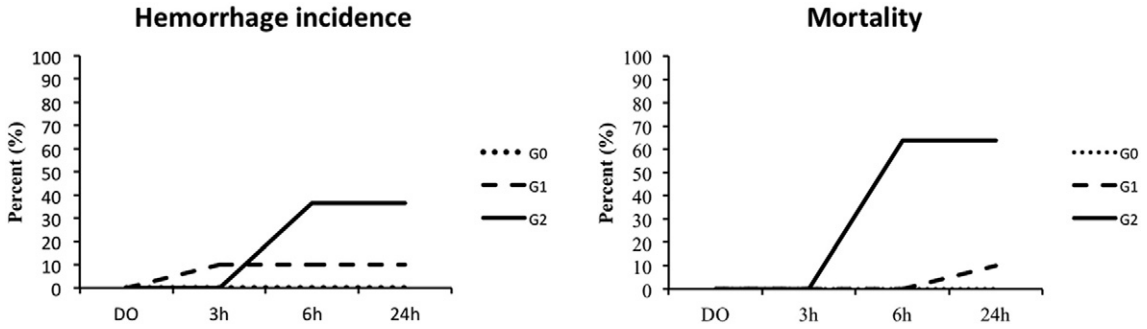

Fig. 4. Effect of the timing of tPA administration on the hemorrhage incidence and mortality rates. Group 2 (G2), which received tPA 4 h after reperfusion, showed a trend towards a higher rate of parenchymal hemorrhage (36.4%, 4/11) as compared with Group 1 (10%, 1/10, $p = 0.311$). The hemorrhages occurred at 6 h in Group 2 and at 3 h in Group 1. Mortality was higher in Group 2 (63.6%, 7/11) as compared with Group 1 (10.0%, 1/10, $p = 0.024$). No rat from Group 0 showed hemorrhage or died within 24 h post reperfusion. Abbreviations: G0: Group 0; G1: Group 1; G2: Group 2; DO: during occlusion; 3 h: 3 h after reperfusion; 6 h: 6 h after reperfusion; 24 h: 24 h after reperfusion.

was exposed and dissected from the cranium to expose the infratemporal bone. A craniectomy was made using an electric drill once the zygomatic arch had been removed. The left MCA bifurcation was then exposed by opening the dura carefully. At a point distal to the origin of the lenticulostriate arteries, MCA was clipped with a microclip (Sundt AVM microclip No. 1, Codman & Shurtleff, Inc.), and bilateral common carotid arteries were tied with polypropylene suture to complete the 3-vessel occlusion. Loss of blood flow was confirmed visually with the surgical microscope. The occlusion and associated ischemic injury were also confirmed by diffusion-weighted MRI and perfusion-weighted MRI during occlusion. Reperfusion of the middle cerebral artery territory was achieved by removing the microclip on the MCA and the sutures around common carotid arteries 90 min after the onset of ischemia.

2.3. MRI

MR imaging was performed on a 7 T Clinscan system (Bruker Biospin, Ettlingen, Germany) with a gradient strength of 600 mT/m/ms. The surface coil was centered over each rat's brain. During imaging, breathing was monitored using an MRI-compatible system (SA Instruments, Inc., Stony Brook, NY).

The MRI protocol at each time point included: diffusion-weighted imaging (DWI), T2-weighted imaging, gradient-echo imaging (GRE), dynamic contrast-enhanced (T1 DCE), and dynamic susceptibility contrast imaging (T2* DSC). All images were obtained in the coronal plane and consisted of 12 consecutive 1 mm slices with an image matrix size of 256 × 256 pixels (128 × 128 pixels for DCE and DSC).

DWI (TR/TE = 3000/32 ms, 6 b values of 250, 500, 750, 1000, 1500 and 2000/mm^2 applied along z-axis, averages = 2, FOV 40 mm^2, flip angle 90°–180°, ADC calculation) served to ensure the presence of an adequate ischemic lesion. A gradient echo sequence (GRE, TR/TE = 600/10 ms, averages = 2, FOV 40 mm^2) was used to detect intracranial hemorrhage, with a focus on parenchymal hematoma rather than petechial hemorrhage, as only parenchymal hematoma is associated with neurologic deterioration and worse outcome [15].

In order to achieve quantitative hemodynamic measurements of cerebral perfusion and permeability, two boluses of gadodiamide contrast (Omniscan, GE Healthcare AS, Oslo, Norway) were injected. The first bolus of contrast was administered to measure permeability (DCE, TR/TE = 10/1.08 ms, average = 1, FOV 40 mm^2, flip angle 90°) and served as a pre-load bolus for the DSC scan, performed with a second bolus of gadolinium contrast. Such contrast preloading minimizes the effect of contrast leakage for the perfusion T2* DSC imaging [34]. Occlusion and reperfusion were confirmed by DSC imaging (TR/TE = 345/11 ms, average = 1, FOV 40 mm^2).

For the rats in our study population that died within 24 h post reperfusion, T2, DWI, and GRE images were obtained after death.

2.4. Image processing

Perfusion maps (mean transit time — MTT, cerebral blood flow — CBF, cerebral blood volume — CBV) were computed using ImageJ and the plugin software DSCoMAN [4]. The areas with abnormal CBF (<66% of the contralateral CBF values [30]) was delineated on the DSC images obtained during occlusion and at 3 h after reperfusion, and the

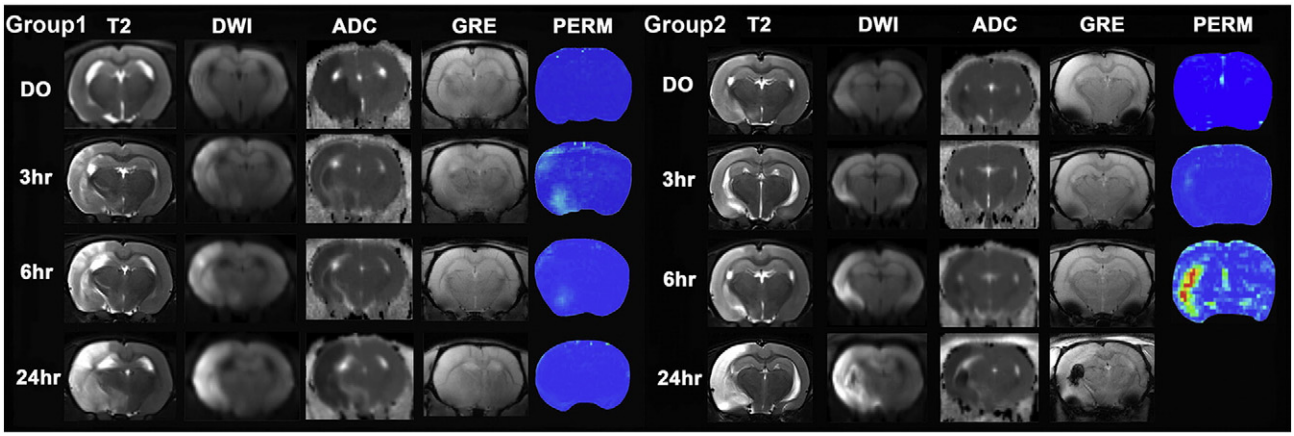

Fig. 5. Time course of changes in MRI imaging and BBB permeability. BBB permeability increased earlier in Group 1, at 3 h after reperfusion. Peak increase was however greater in Group 2 (at 6 h). Parenchymal hematomas were typically observed at 6 h in Group 2, and mortality was also higher in Group 2. Abbreviations: DO: during occlusion; 3 h: 3 h after reperfusion; 6 h: 6 h after reperfusion; 24 h: 24 h after reperfusion; T2: T2-weighted imaging; DWI: diffusion-weighted imaging, ADC: average diffusion coefficient; GRE: gradient-echo; PERM: BBB permeability.

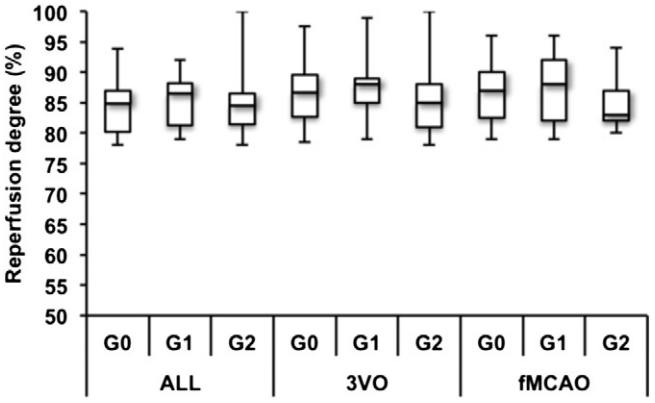

Fig. 6. Degree of post-ischemic reperfusion for the two surgical techniques and the three study groups. The degree of reperfusion was similar between the two experimental groups (Group 1 and Group 2) for all animals ($p = 0.567$), 3 vessel occlusion technique (3VO) ($p = 0.599$), and filament occlusion (filament) ($p = 0.675$).

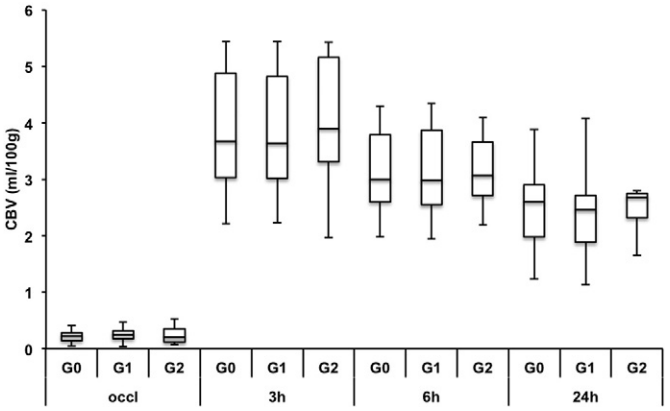

Fig. 7. Time course of CBV changes post-ischemia. CBV values in the ischemic brain increased significantly after reperfusion, with the highest values observed 3 h after reperfusion. No significant difference was observed between group 1 and group 2 ($p = 0.756$). Abbreviations: G0: Group 0; G1: Group 1; G2: Group 2; DO: during occlusion; 3 h: 3 h after reperfusion; 6 h: 6 h after reperfusion; 24 h: 24 h after reperfusion.

difference between these two areas was used to calculate the degree of reperfusion [30].

Permeability maps were computed using a Patlak analysis as previously reported [9,17,21,22]. One set of regions of interest (ROIs) were drawn to include the ischemic hemisphere, and another set of ROIs to include the contralateral nonischemic hemisphere. The ROIs were drawn on the anatomical images (T2) and then transferred to the co-registered BBB permeability maps. The blood-to-brain-transfer constants (referred to as BBB permeability values in the reminder of the text) in these ROIs were recorded.

2.5. Statistical analysis

We observed skewed distributions of BBB permeability and perfusion values and, therefore, used median and interquartile range (IQR) for our descriptive statistics.

We performed three analyses: (1) The first analysis compared the evolution of the BBB permeability values over the 4 different time points between the three groups (tPA administered immediately after reperfusion, and tPA administered 4 h after reperfusion). (2) We further compared the rate of parenchymal hemorrhage, as well as (3) the mortality rate between the three groups. The rate of reperfusion was considered as a possible confounder, as well as CBV values after reperfusion used as an indicator of luxury perfusion.

Nonparametric Kruskal–Wallis test was used to assess statistically significant differences for BBB permeability and CBV values across the different time points. Mann–Whitney U tests were used to evaluate each individual time point for significant differences between the three groups and to assess the reperfusion rate differences between the three groups. Fisher's exact tests were used to compare the parenchymal hemorrhage incidence and the mortality rate between the three groups. Probability values of less than 0.05 were considered statistically significant. We used the statistical software IBM SPSS statistics 22 for the statistical analyses.

3. Results

3.1. Study population

36 male SHR rats were subjected to a 90-minute fMCAO or 3VO, respectively. Of the 36 rats, 1 rat died right after surgery because of subarachnoid hemorrhage; 5 rats were excluded because of the inclusion/exclusion criteria; 3 rats died after reperfusion. For the remained 27 rats, 13 rats received 3VO (3 in Group 0, 5 in Group 1, 5 in Group 2), 14 rats received fMCAO (3 in Group 0, 5 in Group 1, 6 in Group 2). At 24 h post reperfusion, there were 6 rats left in Group 0, 9 rats left in Group 1(4 from 3VO, 5 from fMCAO), and 4 rats left in Group 2 (1 from 3VO, and 3 from fMCAO). A precise description of when the rats developed hemorrhage and/or died is graphically demonstrated in Fig. 2.

3.2. BBBP Evolution over time

In the three study groups, BBB permeability values were low in the ischemic area during occlusion ($p = 0.649$ between Group 0 and Group 1; $p = 0.746$ between Group 2 and Group 0; $p = 0.756$ between Group 1 and Group 2 (Fig. 3, Table 1)).

At 3 h after reperfusion, BBB permeability in the ischemic brain increased. This increase was greater in Group 1, in which tPA was administered immediately after reperfusion, than in Group 2, which had not received tPA at this time point ($p = 0.01$), and greater than in Group 0 ($p = 0.034$) (Fig. 3).

At 6 h after reperfusion, BBB permeability values in the ischemic brain were now higher in Group 2, which received tPA before this set of imaging, than Group 1 ($p = 0.001$), which had received tPA 6 h earlier, and greater than Group 0 ($p = 0.006$). Group 1 had BBB permeability values that were still abnormal but lower than those observed at 3 h (Fig. 3, Table 1).

At 24 h after reperfusion, BBB permeability values in the ischemic brain had decreased relative to earlier post-ischemic timepoints, but remained higher than those during occlusion ($p = 0.025$ in Group 0; $p = 0.010$ in Group 1; $p = 0.001$ in Group 2) (Fig. 3, Table 1).

The highest permeability values in Group 2, which were observed at 6 h after reperfusion, were higher than the highest permeability values in Group 1, which were observed at 3 h after reperfusion ($p = 0.028$).

3.3. Hemorrhagic incidence and mortality over time

Group 2, which received tPA 4 h after reperfusion, showed a trend towards a higher rate of hemorrhagic transformation (36.4%, 4/11, $p = 0.311$). The parenchymal hemorrhage typically developed 6 h after reperfusion when BBB permeability values were the highest (Figs. 4 and 5). In Group 1, only 1 of 10 rats (10%) demonstrated a parenchymal hematoma occurring at 3 h after reperfusion and tPA administration. (Fig. 4).

Mortality rate was higher in Group 2 (63.6%, 7/11) compared to Group 1 (10.0%, 1/10, $p = 0.024$).

In Group 0, no rat showed hemorrhagic transformation or died within 24 h post reperfusion.

We repeated the analyses described above separately for fMCAO and 3VO and did not find any significant differences in terms of BBB permeability value evolution between the two stroke models.

3.4. Reperfusion

The degree of reperfusion was similar between the two experimental groups (Group 1 and Group 2) ($p = 0.567$, 0.599, and 0.675, for animals with 3VO surgery or filament surgery, respectively) (Fig. 6), and therefore was not a confounding factor for the analyses reported above.

3.5. CBV evolution over time

CBV values in the ischemic brain increased significantly after reperfusion, with the highest values observed 3 h after reperfusion (median: 3.51 ml/100 g, IQR: 3.17–4.42 ml/100 g in Group 1; and median: 3.4 ml/100 g, IQR: 2.81–4.96 ml/100 g in Group 2) (Fig. 7). There was no significant difference between the two experimental groups (Group 1 and Group 2) ($p = 0.756$), indicating that luxury perfusion was not a confounding factor for the analyses reported above.

4. Discussion

The present study demonstrated that BBB permeability increased more when tPA was administered 4 h after reperfusion as compared to when it was administered at the time of reperfusion. In addition, hemorrhagic transformation and mortality were more frequent when tPA was administered late. Hemorrhagic transformation typically occurred when BBB permeability was the highest. The degree of reperfusion, luxury perfusion, and the type of surgery were not confounding factors as they did not differ significantly between the two experimental groups.

Our observations are in general agreement with previously published reports [9,17,22]. As shown in a Sprague–Dawley rat model of thromboembolic stroke, confluent hemorrhagic infarction was observed in 50% of the rats treated with tPA 6 h after onset, and in none of the rats that received tPA 2 h after onset [18]. In another study, tPA was administered 20 min and 3 h after occlusion in a mouse model of thromboembolic stroke. Again, a higher incidence of parenchymal hemorrhage and brain swelling (37.5%) was observed in the group with delayed administration of tPA [10].

A different set of studies assessed the dynamic evolution of the blood–brain barrier permeability over time in rodent models of stroke, using sequential MRI studies. In a study involving male Wistar rats subjected to a 90-minute MCA occlusion, tPA was administered intravenously 30 min after reperfusion, and sequential MR images with injection of Gd-DTPA to assess BBB permeability were acquired at the time of tPA administration and at 1 h and 24 h after tPA administration. BBB permeability was significantly increased following reperfusion and was exacerbated at 1 h after tPA administration, but decreased significantly after 24 h [19]. This study did not probe into hemorrhagic transformation following tPA administration. Dijkhuizen and coauthors intravenously injected tPA 6 h after stroke to spontaneously hypertensive rats with embolic stroke; they observed enhancement on postcontrast T1-weighted images in areas of subsequent bleeding, but did not investigate the exact timing of the hemorrhagic transformation compared to the evolution of the BBB permeability [8].

A few issues raised in the current study remain to be resolved. A trend towards a higher rate of hemorrhagic transformation in Group 2 as compared with Group 1 was observed. However, this difference was not significant. It is possible that a larger sample size in the experimental design would have revealed a significant change.

The use of imaging as the sole indicator of BBB permeability and HT was based on the fact that this approach was previously validated by comparison with histopathology and Evans blue studies [17].

Finally, we had had to use a relatively short duration of occlusion for all study groups, because prolonged occlusions are associated with an almost 100% rate of death and hemorrhage. This would have confounded our BBB permeability measurements and prevented the assessment of the impact of tPA administration and its timing on the BBB and the risk of hemorrhagic transformation.

5. Conclusion

In conclusion, this study demonstrates that tPA administered at different timepoints has different effects in terms of BBB permeability and subsequent risk of hemorrhagic transformation. This may be related to the fact that a BBB already affected by ischemia may be more vulnerable to the effect of tPA, resulting in even higher BBB permeability and a higher risk of hemorrhagic transformation.

Conflict of interest

On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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