Peroxynitrite Decomposition Catalyst Reduces Delayed Thrombolysis-induced Hemorrhagic Transformation in Ischemia-reperfused Rat Brains

Han-Sen Chen,^{1,2} Xing-Miao Chen,^{1,2} Jing-Han Feng,^{1,2} Ke-Jian Liu,³ Su-Hua Qi⁴ & Jian-Gang Shen^{1,2}

¹ School of Chinese Medicine, The University of Hong Kong, Hong Kong, China

² The University of Hong Kong-Shenzhen Institute of Research and Innovation (HKU-SIRI), Shenzhen, China

³ Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM, USA

⁴ Research Center for Biochemistry and Molecular Biology and Provincial Key Laboratory of Brain Disease Bioinformation, Xuzhou Medical College, Xuzhou, China

Keywords

Hemorrhage; Matrix metalloproteinases; Peroxynitrite; Tissue plasminogen activator.

Correspondence

Prof. Jian-Gang Shen, School of Chinese Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hong Kong, China. Tel.: +852-2589-0429;

Fax: +852-2168-4259;

E-mail: shenjg@hkucc.hku.hk.

and

Prof. Su-Hua Qi, Research Center for Biochemistry and Molecular Biology and Provincial Key Laboratory of Brain Disease Bioinformation, Xuzhou Medical College, Xuzhou 221002, China. Tel.: +86-516-8583-4231;

Fax: +86-516-8583-4231;

E-mail: qisuhua9999@163.com

Received 26 March 2015; revision 17 April

2015; accepted 22 April 2015

SUMMARY

Aim: Hemorrhagic transformation (HT) is a major complication of delayed tissue plasminogen activator (t-PA) treatment in ischemic stroke. We aimed to explore whether peroxynitrite decomposition catalyst (PDC) could prevent such complication. **Methods:** Male Sprague-Dawley (SD) rats were subjected to middle cerebral artery occlusion (MCAO) with t-PA (10 mg/kg) or t-PA plus FeTMPyP (3 mg/kg, a representative PDC) at MCAO for 2 or 5 h and reperfusion for 22 or 19 h, respectively. HT was assessed with hemoglobin assay. Neurological deficit was evaluated with Modified Neurological Severity Score (mNSS). Peroxynitrite formation was examined by detecting 3-nitrotyrosine (3-NT) formation. The expression and activity of MMP-9/MMP-2 were assessed by Western blotting and gelatin zymography. **Results:** t-PA treatment at 2 h of MCAO did not induce HT but attenuated neurological deficit, whereas treatment at 5 h significantly induced HT and worsened the neurological outcome. Such complications were prevented by FeTMPyP cotreatment. Early t-PA treatment inhibited 3-NT and MMP-9/MMP-2 expression, whereas delayed treatment induced 3-NT and MMP-9/MMP-2 expression and activity. FeTMPyP cotreatment downregulated 3-NT and inhibited MMP-9/MMP-2 in both time points. **Conclusion:** Peroxynitrite decomposition catalyst could prevent hemorrhagic transformation and improve neurological outcome ischemic rat brains with delayed t-PA treatment via inhibiting peroxynitrite-mediated MMP activation.

doi: 10.1111/cns.12406

Introduction

Tissue plasminogen activator (t-PA) remains the only FDA-approved therapy for acute ischemic stroke, with a restrictive treatment time window of 4.5 h [1–4]. Treatment beyond this time window significantly increases risk of hemorrhagic transformation (HT) and mortality [5–8], which worsens the patients' outcome [9]. With the narrow therapeutic window and potential severe complications, t-PA treatment is only applied for <5% stroke patients practically [10]. Developing novel adjuvant therapeutic strategy to extend t-PA's therapeutic time window and

reduce the risk of HT is critical for improving outcome of stroke treatment.

Activating matrix metalloproteinases (MMPs), a proteolytic zinc-containing enzyme family, could degrade neurovascular matrix, disrupt tight junction proteins and induce the BBB opening and HT in ischemic stroke [11,12]. During thrombolytic treatment, t-PA promotes the expression of MMP-9 [13,14] and the release of MMP-9 from neutrophils, contributing to HT [15]. Inhibition of MMP-9 reduced incidence and severity of HT in focal ischemic rat model with t-PA treatment [16,17]. Plasma MMP-9 level becomes a biomarker for predict-

ing the risk of HT in ischemic stroke patients receiving t-PA treatment [18,19]. Meta-analysis on 22 clinical trials with 3289 patients showed that MMP-9 level was highly correlated with infarct volume, stroke severity, and functional outcome [20]. MMP-2 activation also contributes to the BBB leakage at the early stage of cerebral ischemia [21,22] as well as the t-PA-mediated HT [23]. MMP-2 knockout mice revealed less BBB damage and HT than wild-type mice after focal cerebral ischemia–reperfusion [24].

After t-PA infusion, recanalization causes cerebral ischemia–reperfusion injury, producing large amounts of reactive oxygen and nitrogen species including superoxide, nitric oxide (NO), and peroxynitrite (ONOO[−]). Peroxynitrite is derived from the superoxide and NO, but it is much more active than those precursors [25]. Peroxynitrite exerts strong cytotoxic effects via inducing protein tyrosine nitration, lipid peroxidation and DNA damage [26]. Peroxynitrite could activate MMPs and disrupt tight junctions, leading to damage of neurovascular unit in ischemic brain [27,28]. Scavenging peroxynitrite with NAC or peroxynitrite decomposition catalyst FeTMPyP significantly inhibited MMP-9 activity, reduced infarct volume, and improved neurological function during cerebral ischemia–reperfusion injury [29–33]. Increased nitrosative oxidative stress was found in ischemic stroke patients [34]. Thus, we raised the hypothesis that peroxynitrite decomposition catalyst (PDC) could prevent hemorrhage transformation through inhibiting peroxynitrite-mediated MMP activation in ischemic brain with delayed t-PA treatment and subsequently extend therapeutic window of t-PA.

Materials and Methods

Middle Cerebral Artery Occlusion (MCAO) Model

Male Sprague-Dawley (SD) rats weighing 260–290 g were obtained from Laboratory Animal Unit, The University of Hong Kong. All animal experiment protocols were approved and regulated by the Committee on the Use of Live Animal in Teaching and Research. Rats were randomly divided into the following groups: sham control, 2 h of MCAO plus 22 h of reperfusion (M2/R22), 5 h of MCAO plus 19 h of reperfusion (M5/R19), M2/R22 plus t-PA, M5/R19 plus t-PA, M2/R22 plus t-PA and FeTMPyP, M5/R19 plus t-PA and FeTMPyP. Cerebral ischemia–reperfusion MCAO model was conducted as we previously described [35]. Briefly, MCAO rats were anesthetized with 4% isoflurane and maintained with 2% isoflurane via inhalation. A silicon-coated suture (diameter 0.38 mm) was inserted from the external carotid artery (ECA) into the internal carotid artery (ICA). Sham control rats were subjected to similar surgical operation without occlusion. At designed time window of MCAO ischemia, the suture was removed to induce reperfusion. During the operation, rats were kept on warming pads until recovered from anesthesia. Success of MCAO was confirmed by TTC staining of a 1-mm-thick coronal slice that is 6 mm away from the frontal tip. Brains without infarction should be excluded for study. All MCAO models were successful and included in this study.

Drug Treatment

We compared the effects of FeTMPyP, a representative PDC, on HT in the brains receiving t-PA treatment at 2 and 5 h of cerebral ischemia. At 1.5 and 4.5 h of MCAO ischemia, recombinant t-PA (Actilyse, Boehringer Ingelheim, 10 mg/kg) was injected (10% bolus, 90% continuous infusion for 0.5 h) via femoral vein according to previously described methods [36]. Accordingly, the time points of t-PA treatment were counted as 2 and 5 h after cerebral ischemia. In parallel experiments, same volume of saline was used as control. Right after t-PA injection, the suture was immediately removed to induce reperfusion. With t-PA treatment at 2 and 5 h, we designed the reperfusion period for 22 and 19 h, respectively, to keep consistent timeframe to 24 h of ischemia–reperfusion. To investigate the effects of PDC on preventing t-PA-induced HT, FeTMPyP (3 mg/kg, Cayman, 75854) was given via femoral vein just before t-PA treatment. We chose the dosage of 3 mg/kg FeTMPyP for this study which was based previous reports by us and others [32,33,37].

Hemoglobin Assay

Hemoglobin assay was used to examine the severity of hemorrhage as previously reported [38]. Briefly, rats were transcardially perfused with cold PBS and hemispheres were homogenized with cold PBS. The homogenized samples were collected and centrifuged at 15 000 g for 15 min. Supernatants were collected and the level of hemoglobin was detected using QuantiChrom hemoglobin assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacture's protocol. O.D. value at 400 nm was detected with a microplate reader.

Western Blot Analysis

After homogenization, brain samples were collected and proteins were extracted with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). Proteins were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), loaded for electrophoresis, and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with diluted primary antibodies including anti-MMP-9 (Merck, IM72), anti-MMP-2 (Merck, IM33), anti-3-nitrotyrosine (3-NT, Abcam, ab61392), and anti-GAPDH (Millipore, abs16) overnight at 4°C and followed by incubating secondary antibody at room temperature for 2 h. After washed with TBST buffer, signal was detected using ECL advance Western blotting detection reagents (BD biosciences, San Jose, CA, USA).

Gelatin Zymography

MMP activity was measured using gelatin zymography protocol as previously described with minor modifications [28]. Briefly, the same amount of total proteins was electrophoresed in 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis, gels were collected and washed with 2.5% Triton-100 for 1 h and then incubated with developing buffer at 37°C for 48 h.

After stained with Coomassie blue, the gels were washed with ddH₂O until clear bands appeared.

Neurological Deficit Measurement

Modified Neurological Severity Score (mNSS) test was adopted to evaluate the animal neurological deficit according to previous publication [39]. Neurological function was graded on a series of scales from 0 to 18 (normal score, 0; maximal deficit score, 18). The mNSS includes motor, sensory, reflex, and balance tests. Tests were carried out by an investigator who was blinded to the experiments.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 statistical programs (SPSS, Chicago, IL, USA). For multiple groups designed experiments, comparisons were made by one-way analysis of variance (ANOVA) and followed by least significant difference test for two-group comparison within the multiple groups. Significance levels were set at $P < 0.05$. Data were presented as means $\pm$ SEM.

Figure 1 Effects of PDC (FeTMPyP, 3 mg/kg) on hemorrhagic transformation in ischemic rat brains with early and delayed t-PA treatments. Rats were treated with t-PA (10 mg/kg) at 2 and 5 h of MCAO ischemia following 22 and 19 h of reperfusion, respectively. M2/R22, 2 h of MCAO plus 22 h of reperfusion; M5/R19, 5 h of MCAO plus 19 h of reperfusion; (A) brain samples in M2/R22 plus t-PA groups with or without PDC treatment; (B) brain samples in M/R22 plus t-PA groups with or without PDC; (C) brain hemoglobin content in M2/R22 groups with t-PA or t-PA plus PDC treatments; (D) brain hemoglobin content in M5/R19 groups with t-PA or t-PA plus PDC treatments (* $P < 0.05$, compared to M5/R19 group; # $P < 0.05$, compared to M5/R19 + t-PA group). Data are expressed as mean $\pm$ SEM ($n = 6-11$).

Results

PDC Treatment Abolished Hemorrhagic Transformation in Ischemia-reperfused Brains with t-PA Infusion at 5 h

We first investigated the different effects of early and delayed t-PA treatments on HT in ischemic brains. T-PA was intravenously injected into the rats at 2 and 5 h of cerebral ischemia, and then, the brain samples were collected at the time points of 22 and 19 h of reperfusion, respectively, for accessing HT. Early t-PA infusion (10 mg/kg) at 2 h did not induce HT, whereas delayed treatment at 5 h resulted in HT in the MCAO ischemic brains (Figure 1A and B). Hemoglobin assay confirmed that HT only occurred in the ischemic brain with t-PA treatment at 5 h. (Figure 1C and D). Notably, cotreatment of FeTMPyP (3 mg/kg, a representative PDC) abolished delayed t-PA-induced HT in the ischemic brains. Those results indicate that PDC could prevent HT in ischemic brains with delayed t-PA treatment.

PDC Treatment Reduced Neurological Severity Scores in Ischemic Brains with t-PA Infusion at 5 h

We then investigated the mNSS scores for neurological functions. Interestingly, t-PA treatment at 2 h remarkably improved neurological functions whereas at 5 h worsened the neurological outcome in the MCAO ischemia-reperfused rats, indicating

Figure 2 Effects of PDC (FeTMPyP, 3 mg/kg) on neurological severity scores (NSS) in MCAO ischemic rats with early and delayed t-PA treatments. Rats were treated with t-PA (10 mg/kg) at 2 and 5 h of MCAO ischemia following 22 and 19 h of reperfusion, respectively. M2/R22, 2 h of MCAO plus 22 h of reperfusion; M5/R19, 5 h of MCAO plus 19 h of reperfusion. A: t-PA treatment at 2 h after MCAO ischemia with or without PDC cotreatment (*** $P < 0.001$, VS. sham control; ## $P < 0.01$, compared to M2/R22). B: t-PA treatment at 5 h after MCAO ischemia with or without PDC cotreatment (*** $P < 0.001$, compared to sham control; ## $P < 0.01$, compared to M5/R19; &&& $P < 0.001$, compared to M5/R19 + t-PA). Data are expressed as mean $\pm$ SEM ($n = 6-11$).

that early and delayed t-PA treatments have opposite therapeutic outcome in neurological functions (Figure 2A and B). Co-treatment of FeTMPyP not only abolished t-PA-induced

Figure 3 Effects of PDC (FeTMPyP, 3 mg/kg) on 3-NT expression in MCAO ischemic rat brains with early and delayed t-PA treatments. Rats were treated with t-PA (10 mg/kg) at 2 and 5 h of ischemia plus 22 and 19 h of reperfusion, respectively. M2/R22, 2 h of MCAO plus 22 h of reperfusion; M5/R19, 5 h of MCAO plus 19 h of reperfusion. I, ipsilateral; C, contralateral. **(A)** Western blot results and statistical results in M2/R22 groups received t-PA or t-PA plus PDC (ipsilateral) ($***P < 0.001$, compared to sham control; $^{\#}P < 0.05$, $^{###}P < 0.001$, compared to M2/R22; $^{\&\&}P < 0.01$, compared to M2/R22 + t-PA). **(B)** Western blot results and statistical results in M5/R19 groups received t-PA or t-PA plus PDC treatments (ipsilateral) ($***P < 0.001$, compared to sham control; $^{##}P < 0.01$, compared to M5/R19; $^{\&}P < 0.01$, compared to M5/R19 + t-PA). Data are expressed as mean $\pm$ SEM. ($n = 3$).

Figure 4 Effect of PDC (FeTMPyP, 3 mg/kg) on MMP-9 activity and MMP-9/MMP-2 expression in MCAO ischemic rat brains with early and delayed t-PA treatments. Rats were treated with t-PA (10 mg/kg) at 2 and 5 h of MCAO ischemia following 22 and 19 h of reperfusion, respectively. M2/R22, 2 h of MCAO plus 22 h of reperfusion; M5/R19, 5 h of MCAO plus 19 h of reperfusion. I, ipsilateral; C, contralateral. **(A)** Representative gelatin zymography and statistical results in M2/M22 groups (ipsilateral) ($**P < 0.01$, $***P < 0.001$, compared to sham control; $^{##}P < 0.01$, compared to M2/R22). **(B)** Representative gelatin zymography and statistical results in M5/M19 groups (ipsilateral) ($***P < 0.001$, compared to sham control; $^{###}P < 0.001$, compared to M5/R19; $^{\&\&}P < 0.001$, compared to M5/R19 + t-PA). **(C)** Representative Western blot and statistical analysis for MMP-9/MMP-2 expressions in M2/R22 groups ($**P < 0.01$, compared to sham control; $^{\#}P < 0.05$, $^{##}P < 0.01$, compared to M2/R22). **(D)** Representative Western blot and statistical analysis for MMP-9/MMP-2 expressions in M5/R19 groups ($***P < 0.001$, compared to sham control; $^{###}P < 0.001$, compared to M5/R19; $^{\&\&}P < 0.001$, compared to M5/R19 + t-PA). Data are expressed as mean $\pm$ SEM ($n = 3-4$).

neurological deficiency in the MCAO rats with t-PA treatment at 5 h but also had better neurological functions than the MCAO ischemic rats with saline treatment as control (Figure 2B), indicating that PDC treatment could improve the recovery of neurological functions in cerebral ischemia-reperused rats with delayed t-PA treatment.

PDC Treatment Downregulated 3-NT Expression and Inhibited MMP-9/MMP-2 Expression/activity in Ischemic Brains with Delayed t-PA Infusion at 5 h

We finally investigated whether the underlying mechanisms of t-PA's opposite effects and the protective effect of PDC are related to peroxynitrite and MMP activation in the ischemic brains. As showed in Figure 3, t-PA treatment at 2 h remarkably downregulated the formation of 3-NT, a peroxynitrite biomarker, whereas the treatment at 5 h upregulated 3-NT level in the MCAO ischemic brains, indicating that delayed t-PA treatment could promote peroxynitrite production. Cotreatment of FeTMPyP (3 mg/kg) significantly inhibited the expression of 3-NT in the ischemic brains with t-PA treatment at both 2 and 5 h.

Meanwhile, t-PA treatment at 2 hours significantly downregulated the expression of MMP-9/MMP-2 in the ischemic brains, while treatment at 5 h significantly upregulated the expression of MMP-9/MMP-2 and MMP-9 activity (Figure 4). Cotreatment of FeTMPyP significantly downregulated the expression of MMP-9/MMP-2 and inhibited MMP-9 activity in the ischemic brains with t-PA treatments at both 2 and 5 h after cerebral ischemia. Those results indicate that early t-PA treatment could reduce the production of peroxynitrite and the expression of MMPs, while delayed t-PA treatment could exacerbate peroxynitrite production and the expression and activity of MMPs in ischemic rat brains, and PDC treatment could inhibit 3-NT formation and the expression and activity of MMPs in the ischemic brain with delayed t-PA treatment.

Discussion

This is the first report that PDC could prevent hemorrhagic transformation and promote neurological functions after cerebral ischemia with delayed t-PA treatment. PDC has the potentials to be an adjunct therapy to expend t-PA's therapeutic time window and prevent hemorrhagic transformation in post-stroke treatment.

MMPs play critical roles in inducing the BBB disruption and HT during cerebral ischemia–reperfusion injury [16–19,23]. PDC could prevent MMP activation and neurovascular injury in rat brains after prolonged cerebral ischemia [32,40,41]. Therefore, we tested the hypothesis that PDC could be effective to reduce HT through inhibiting peroxynitrite-mediated MMP activation in ischemic brains with delayed t-PA treatment. HT was only presented in the rats with t-PA treatment at 5 h rather than that with

t-PA treatment at 2 h of MCAO cerebral ischemia–reperfusion. Furthermore, early t-PA treatment at 2 h of MCAO cerebral ischemia improved neurological function scores, whereas delayed t-PA treatment at 5 h worsened the therapeutic outcomes. These results are consistent with previous reports [42–44]. Interestingly, early t-PA treatment at 2 h significantly reduced 3-NT formation, while delayed t-PA treatment at 5 h oppositely induced 3-NT formation, indicating that early recanalization could reduce the formation of peroxynitrite in the ischemic brains. Cotreatment of FeTMPyP prevented HT and improved neurological function in the rats with t-PA treatment at 5 h of MCAO cerebral ischemia following 19 h of reperfusion. Those results indicate that peroxynitrite plays an important role in HT and neurological deficiency in ischemia–reperused brains with delayed t-PA treatment. In line with those results, early and delayed t-PA treatments had opposite effects on MMP-9/MMP-2 expression and MMP-9 activity in the ischemic brains. Cotreatment of FeTMPyP revealed to inhibit the expression of MMP-9/MMP-2 and the activity of MMP-9 at both early and delayed t-PA treatments. Those results suggest that peroxynitrite-mediated MMP activation could be the underlying mechanism of HT in ischemia–reperused rat brains with delayed t-PA treatment.

It is notable that only 3 mg/kg PDC was used in the study. We designed the dosage according to our previous study that PDC treatment at 3 mg/kg significantly attenuated infarction volume and reduced apoptotic cell death in MCAO rat model of cerebral ischemia–reperfusion injury [37]. Current study aimed to further explore the potentials of using PDC as an adjunct therapy with t-PA to prevent HT and promote therapeutic outcome. Practically, we should further explore the dose-dependent responses to the PDC treatment in protecting blood–brain barrier and attenuating HT during thrombolytic treatment for saving ischemic brain tissues as a potential therapeutic drug in future.

Taken together, we conclude that PDC could prevent hemorrhagic transformation and improve neurological outcome during cerebral ischemia–reperfusion injury with delayed t-PA treatment via inhibiting peroxynitrite-mediated MMP activation. Targeting peroxynitrite formation could be a potential adjuvant therapeutic strategy to reduce t-PA-mediated hemorrhage complication and potentially extend the current narrow treatment time window.

Acknowledgments

This work was supported by Hong Kong General Research Fund (GRF No. 777611M and No. 776512M), Research Grant Council, Hong Kong SAR; and Grant from National Natural Science Foundation of China (No. 31270902).

Conflict of Interest

The authors declare no conflict of interest.

References

- Embersson J, Lees KR, Lyden P, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. *Lancet* 2014;**384**:1929–1935.
- Lansberg MG, Bluhmki E, Thijs VN. Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a metaanalysis. *Stroke* 2009;**40**:2438–2441.
- Jauch EC, Saver JL, Adams HP Jr, et al. Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the

- American Heart Association/American Stroke Association. *Stroke* 2013;**44**:870–947.
4. Xu AD, Wang YJ, Wang DZ, Chinese Stroke Therapy Expert Panel for Intravenous Recombinant Tissue Plasminogen A. Consensus statement on the use of intravenous recombinant tissue plasminogen activator to treat acute ischemic stroke by the Chinese Stroke Therapy Expert Panel. *CNS Neurosci Ther* 2013;**19**:543–548.
5. Clark WM, Wissman S, Albers GW, Jhamandas JH, Madden KP, Hamilton S. Recombinant tissue-type plasminogen activator (alteplase) for ischemic stroke 3 to 5 hours after symptom onset. The ATLANTIS Study: a randomized controlled trial. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke. *JAMA* 1999;**282**:2019–2026.
6. group I, Sandercock P, Wardlaw JM, et al. The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the third international stroke trial [IST-3]): a randomised controlled trial. *Lancet* 2012;**379**:2352–2363.
7. Lapchak PA. Hemorrhagic transformation following ischemic stroke: significance, causes, and relationship to therapy and treatment. *Curr Neurol Neurosci Rep* 2002;**2**:38–43.
8. Wardlaw JM, Murray V, Berge E, et al. Recombinant tissue plasminogen activator for acute ischaemic stroke: an updated systematic review and meta-analysis. *Lancet* 2012;**379**:2364–2372.
9. Strbian D, Sairanen T, Meretoja A, et al. Patient outcomes from symptomatic intracerebral hemorrhage after stroke thrombolysis. *Neurology* 2011;**77**:341–348.
10. Kleindorfer D, Lindsell CJ, Brass L, Koroshetz W, Broderick JP. National US estimates of recombinant tissue plasminogen activator use ICD-9 codes substantially underestimate. *Stroke* 2008;**39**:924–928.
11. Jickling GC, Liu D, Stamova B, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab* 2014;**34**:185–199.
12. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J Cereb Blood Flow Metab* 2007;**27**:697–709.
13. Park KP, Rosell A, Foerch C, et al. Plasma and brain matrix metalloproteinase-9 after acute focal cerebral ischemia in rats. *Stroke* 2009;**40**:2836–2842.
14. Tsuji K, Aoki T, Tejima E, et al. Tissue plasminogen activator promotes matrix metalloproteinase-9 upregulation after focal cerebral ischemia. *Stroke* 2005;**36**:1954–1959.
15. Cuadrado E, Ortega L, Hernandez-Guillamon M, et al. Tissue plasminogen activator (t-PA) promotes neutrophil degranulation and MMP-9 release. *J Leukoc Biol* 2008;**84**:207–214.
16. Lapchak PA, Chapman DF, Zivin JA. Metalloproteinase inhibition reduces thrombolytic (tissue plasminogen activator)-induced hemorrhage after thromboembolic stroke. *Stroke* 2000;**31**:3034–3040.
17. Sumii T, Lo EH. Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal ischemia in rats. *Stroke* 2002;**33**:831–836.
18. Castellanos M, Leira R, Serena J, et al. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. *Stroke* 2003;**34**:40–46.
19. Montaner J, Molina CA, Monasterio J, et al. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation* 2003;**107**:598–603.
20. Ramos-Fernandez M, Bellolio MF, Stead LG. Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. *J Stroke Cerebrovasc Dis* 2011;**20**:47–54.
21. Jin X, Liu J, Yang Y, Liu KJ, Yang Y, Liu W. Spatiotemporal evolution of blood brain barrier damage and tissue infarction within the first 3 h after ischemia onset. *Neurobiol Dis* 2012;**48**:309–316.
22. Liu J, Jin X, Liu KJ, Liu W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. *J Neurosci* 2012;**32**:3044–3057.
23. Machado LS, Sazonova IV, Kozak A, et al. Minocycline and tissue-type plasminogen activator for stroke: assessment of interaction potential. *Stroke* 2009;**40**:3028–3033.
24. Lu A, Suofu Y, Guan F, Broderick JP, Wagner KR, Clark JF. Matrix metalloproteinase-2 deletions protect against hemorrhagic transformation after 1 h of cerebral ischemia and 23 h of reperfusion. *Neuroscience* 2013;**253**:361–367.
25. Suzuki M, Tabuchi M, Ikeda M, Tomita T. Concurrent formation of peroxynitrite with the expression of inducible nitric oxide synthase in the brain during middle cerebral artery occlusion and reperfusion in rats. *Brain Res* 2002;**951**:113–120.
26. Virag L, Szabo E, Gergely P, Szabo C. Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicol Lett* 2003;**140–141**:113–124.
27. Gursoy-Ozdemir Y, Can A, Dalkara T. Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia. *Stroke* 2004;**35**:1449–1453.
28. Gu Y, Zheng G, Xu M, et al. Caveolin-1 regulates nitric oxide-mediated matrix metalloproteinases activity and blood-brain barrier permeability in focal cerebral ischemia and reperfusion injury. *J Neurochem* 2012;**120**:147–156.
29. Chen XM, Chen HS, Xu MJ, Shen JG. Targeting reactive nitrogen species: a promising therapeutic strategy for cerebral ischemia-reperfusion injury. *Acta Pharmacol Sin* 2013;**34**:67–77.
30. Cuzzocrea S, Mazzon E, Costantino G, et al. Beneficial effects of n-acetylcysteine on ischaemic brain injury. *Br J Pharmacol* 2000;**130**:1219–1226.
31. Dhar A, Kaundal RK, Sharma SS. Neuroprotective effects of FeTMPyP: a peroxynitrite decomposition catalyst in global cerebral ischemia model in gerbils. *Pharmacol Res* 2006;**54**:311–316.
32. Suofu Y, Clark J, Broderick J, et al. Peroxynitrite decomposition catalyst prevents matrix metalloproteinase activation and neurovascular injury after prolonged cerebral ischemia in rats. *J Neurochem* 2010;**115**:1266–1276.
33. Thiagarajan M, Kaul CL, Sharma SS. Neuroprotective efficacy and therapeutic time window of peroxynitrite decomposition catalysts in focal cerebral ischemia in rats. *Br J Pharmacol* 2004;**142**:899–911.
34. Al-Nimer MS, Al-Mahdawi AM, Sakeni RA. Assessment of nitrosative oxidative stress in patients with middle cerebral artery occlusion. *Neurosciences* 2007;**12**:31–34.
35. Shen J, Ma S, Chan P, et al. Nitric oxide down-regulates caveolin-1 expression in rat brains during focal cerebral ischemia and reperfusion injury. *J Neurochem* 2006;**96**:1078–1089.
36. Yamashita T, Kamiya T, Deguchi K, et al. Dissociation and protection of the neurovascular unit after thrombolysis and reperfusion in ischemic rat brain. *J Cereb Blood Flow Metab* 2009;**29**:715–725.
37. Xu M, Chen X, Gu Y, et al. Baicalin can scavenge peroxynitrite and ameliorate endogenous peroxynitrite-mediated neurotoxicity in cerebral ischemia-reperfusion injury. *J Ethnopharmacol* 2013;**150**:116–124.
38. Liu W, Hendren J, Qin XJ, Shen J, Liu KJ. Normobaric hyperoxia attenuates early blood-brain barrier disruption by inhibiting MMP-9-mediated occludin degradation in focal cerebral ischemia. *J Neurochem* 2009;**108**:811–820.
39. Chen J, Sanberg PR, Li Y, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 2001;**32**:2682–2688.
40. Shen J, Gu Y. Insights into mechanisms of blood-brain barrier permeability-roles of free radicals, matrix metalloproteinases, and caveolin-1. In: Laher I, editor. *Systems biology of free radicals and antioxidants*. Berlin, Heidelberg: Springer, 2014:2049–2067.
41. Lakhan SE, Kirchgessner A, Tepper D, Leonard A. Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. *Front Neurol* 2013;**4**:32.
42. Fagan SC, Nagaraja TN, Fenstermacher JD, Zheng J, Johnson M, Knight RA. Hemorrhagic transformation is related to the duration of occlusion and treatment with tissue plasminogen activator in a nonembolic stroke model. *Neurol Res* 2003;**25**:377–382.
43. Fagan SC, Garcia JH. Hemorrhagic transformation in focal cerebral ischemia: influence of time to artery reopening and tissue plasminogen activator. *Pharmacotherapy* 1999;**19**:139–142.
44. Hacke W, Donnan G, Fieschi C, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. *Lancet* 2004;**363**:768–774.