Original Article
Knockdown of tissue inhibitor of metalloproteinase 1 suppresses cell apoptosis in cerebral infarction rat model

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Abstract: Cerebral infarction is a kind of ischemic stroke that severely threatens patient's health and life. Tissue inhibitor of metalloproteinase 1 (TIMP1) is reported to be promoted in the brain of middle cerebral artery occlusion (MCAo)-induced rabbit model, but its roles in regulating cerebral infarction are not fully understood. This study aimed at revealing the expression and function of TIMP1 in cerebral infarction. MCAo-induced cerebral infarction rat model was used to analyze TIMP1 expression in the brain. TIMP1-specific small interfering RNA was intracerebroventricularly injected before model construction. Brain histological observation was performed to analyze infarction area by TTC staining and cell apoptosis by TUNEL assay. Results showed that both mRNA and protein levels of TIMP1 were up-regulated in brain of cerebral infarction rat model compared to sham operation group ($P < 0.001$). Knockdown of TIMP1 reduced infarction area ($P < 0.05$) and suppressed cell apoptosis in brain cortex and hippocampus ($P < 0.01$), which was also confirmed by the expression changes of cell apoptosis indicators B-cell lymphoma extra-large (Bcl-XL) and caspase 3 (CASP3). These results suggest that knockdown of TIMP1 can suppress cerebral infarction progression and cerebral cell apoptosis in rat model, implying the pro-apoptotic roles of TIMP1 in cerebral infarction. So TIMP1 is a potential target for treating cerebral infarction, but further mechanism research is still necessary.

Keywords: Cerebral infarction, tissue inhibitor of metalloproteinase 1 (TIMP1), cell apoptosis, middle cerebral artery occlusion (MCAo)

Introduction

Cerebral infarction is a kind of ischemic stroke caused by insufficient blood supply to the brain and the consequent ischemic necrosis of brain tissues. It has become a common risk of death worldwide, inflicting great life threats and living burdens on patients. Hypertension, smoking, diabetes mellitus and other risk factors contribute to the high mortality of cerebral infarction patients, and they differ in young and old patients [1-3]. Cerebral infarction also occurred after traumatic brain injury, resulting in serious outcome and high mortality, despite the relatively low incidence [4]. With the help of computed tomography magnetic resonance imaging, it is much easier to detect the infarction area in the brain. Treatment strategies rely on the use of thrombolytic drugs [5] and surgery methods like thrombectomy [6], carotid endarterectomy [7] and stent angioplasty [8].

Progression of cerebral infarction is associated with accelerated cell apoptosis and promoted inflammatory responses. Existed studies indicate the recruitment of leukocytes to the ischemic brain induced by matrix metalloproteinase 9 (MMP9) contributes to the blood-brain barrier (BBB) breakdown and the ultimate neuronal injury [9, 10]. It is reported that activation of STAT3 attenuates neuronal apoptosis and inflammation during cerebral ischemia/reperfusion via regulating nuclear factor κB (NF-κB), tumor necrosis factor α (TNF-α) and members of B-cell lymphoma (BCL) family [11]. BCL family members and Caspase proteins, such as BCL extra-large (Bcl-XL) and caspase 3 (CASP3) are frequently used as indicators of cell apoptosis [12, 13]. With the development of molecular therapy research, the mechanism involved in cerebral infarction has been investigated and more key factors are being discovered, such as interleukins and several microRNAs [14, 15].

Tissue inhibitor of metalloproteinases 1 (TIM-P1) is produced by connective tissue cells and macrophages under the induction of various cytokines. It binds to MMPs to control their activation states, and thus is an endogenous inhibitor of metalloproteinases [16]. Previous studies have shown the up-regulated level of TIMP1 in the brain of middle cerebral artery occlusion (MCAo)-induced cerebral ischemia/reperfusion rabbit model [17], whereas the specific role of TIMP1 during cerebral infarction is unknown. So the aim of this study is to investigate the expression and functions of TIMP1 in cerebral infarction. The cerebral infarction rat model was induced with the MCAo method, and knockdown of TIMP1 was achieved by small interfering RNA (siRNA) injection to the rat brain. The brain samples were collected to analyzed TIMP1 expression, infarction area and cell apoptosis changes. These results would help to uncover the role of TIMP1 in cerebral infarction and help to understand the development and regulation of this disease.

Materials and methods

Animal model

Thirty male Sprague-Dawley rats (250-300 g, SPF degree, Vital River Laboratories, Beijing, China) were randomly divided into five groups, namely Control, Sham, Model, Scramble and TIMP1 siRNA group, to ensure that five individuals could be detected in each group. Rats in the Control group did not receive siRNA injection or operation. During the construction of cerebral infarction model, rats in the Sham group underwent the sham operation. The other rats received siRNA injection and then the whole operation procedure. The animal experiments were approved by a local ethics committee and performed according to the instruction of our institute.

The cerebral infarction model was induced by MACo method. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg). A cervical midline incision was made to expose the common carotid artery (CCA), external carotid artery (ECA) and pterygopalatine artery. A nylon cord (d = 0.26 mm) coated with paraffin was inserted into the right CCA. Then the skin was sutured and the cord end was fixed on the skin. After 2 h of ischemia, the cord was removed carefully for reperfusion. The Sham group received all the procedures except

the insertion of cord. After the operation, the rats were kept warm to a body temperature of about 37°C. The model was successfully constructed if the rats woke up with left hemiparesis or standing instability. At 24 h after the injection, rats of the five groups were sacrificed for brain tissue sampling for the following histological observation, RNA and protein extraction.

siRNA injection

At 2 d before the model construction, rats of the Scramble and TIMP1 siRNA groups were anesthetized and received siRNA injection intracerebroventricularly (0.8 mm posterior to the bregma, 1.5 mm left/right to the midline, and 4.5 mm ventral to the bregma). The TIMP1-specific siRNA and the scramble siRNA expression vectors (5 µg, GenePharma, Shanghai, China) were injected into rats of the TIMP1 siRNA and Scramble group, respectively, at the rate of 1 µL/min. For the Model group, blank vectors were injected.

TTC staining

The cerebral infarction area of rats was analyzed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Briefly, the whole brain tissues were sampled and immediately stored at -20°C for 20 min. Then they were cut into 2 mm-thick slabs, immersed in 2% TTC solution, and incubated at 37°C for 30 min. After the incubation, the slabs were fixed in 10% formaldehyde solution for 4 h and photographed. The infarction area (white) compared to the total area was analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

TUNEL assay

The apoptotic cells in the cortex and hippocampus of rat brain were detected by TUNEL assay using In Situ Cell Death Detection Kit, POD (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the tissue samples were embedded in paraffin and cut into 6 µm-thick slices. After deparaffinage and rehydration, the sections were digested in Proteinase K at 37°C for 10 min, incubated in 50 µL of TUNEL reaction solution in dark at 37°C for 1 h, and then in 50 µL of converter-POD for 30 min. The sections were washed with phosphate buffered saline (PBS) for three times between steps. After incubation in diaminoben-
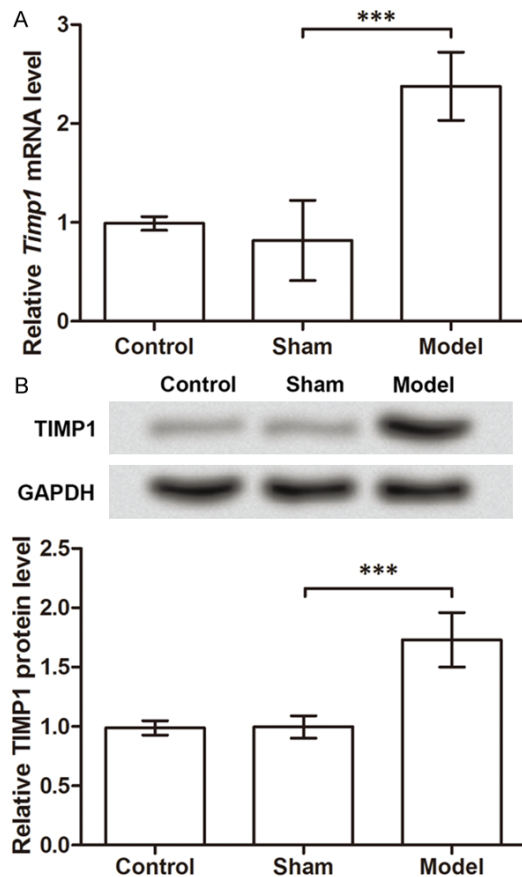
Figure 1. Up-regulation of TIMP1 in the brain of cerebral infarction rat model. Control, rats without any treatment. Sham, rats received sham operation during model construction. Model, cerebral infarction rats induced with MACo operation. A. qPCR shows that *Timp1* mRNA expression is up-regulated in the brain of cerebral infarction model (n = 5). B. Western blot shows that TIMP1 protein expression is up-regulated in the brain of cerebral infarction model. The histogram shows the relative intensity of protein bands compared to GAPDH (n = 5). ***P < 0.001. TIMP1, tissue inhibitor of metalloproteinases 1.

zidine for 10 min, the apoptotic cell number was counted under an optical microscope (Leica Microsystems, Wetzlar, Germany).

Real-time quantitative PCR (qPCR)

Total RNAs of the brain were extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNase I (Invitrogen) was used to remove any DNA contamination. The extracted RNA samples were quantified by NanoDrop 2000 (Thermo Fisher Scientific, Carlsbad, CA) and examined by agarose gel electrophoresis. First-strand complementary DNAs were synthesized using 1 µg RNAs by PrimeScript 1st Strand cDNA Synthesis Kit (Ta-

KaRa, Dalian, China). qPCR was performed with 20 ng cDNA templates and the gene-specific primers for rat *Timp1* (Fw: 5'-ATC GCG GGC CGT TTA AGG A-3' and Rv: 5'-CAA GGG ATG GCT GAA CAG GA-3') on LightCyler 480 platform (Roche, Basel, Switzerland). Rat *Gapdh* (Fw: 5'-CGC ATT GCC AGA CAT ATC AGC-3' and Rv: 5'-AGG TGA AGC AGG CTC AAT CAA-3') was used as an internal control. Experiments including the blank control of qPCR were repeated for five times. qPCR data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein of the brain samples were extracted using the protein lysis buffer (Beyotime, Shanghai, China) according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate 20 µg of total protein. The protein bands on the gel were transferred to a polyvinylidene fluoride membrane and then the membrane was blocked in 5% skim milk at room temperature for 2 h, after which it was incubated in the specific primary antibodies for TIMP1, Bcl-XL and CASP3 (ab61224, ab178844 and ab179517, Abcam, Cambridge, UK) at 4°C overnight. Anti-GAPDH (ab181602) was used as an internal control. After washed in PBS for five times, the membrane was incubated in the horse reddish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Signals were developed using EasyBlot ECL Kit (Sangon Biotech, Shanghai, China) and analyzed by ImageJ.

Statistical analysis

All experiments were repeated for five times. Results were represented as the mean ± standard deviation, and data were analyzed using *t* test and one-way ANOVA in SPSS 20 (IBM, New York, USA). Differences were significant if *P* < 0.05.

Results

TIMP1 is up-regulated in cerebral infarction model

To begin with, qPCR and western blot were performed to investigate whether TIMP1 was aberrantly expressed in the brain of cerebral infarction rat model. qPCR results showed that *Timp1* mRNA was significantly up-regulated in the rat brain after induction of cerebral infarction (*P* < 0.001, **Figure 1A**), and consistently, TIMP1 pro-
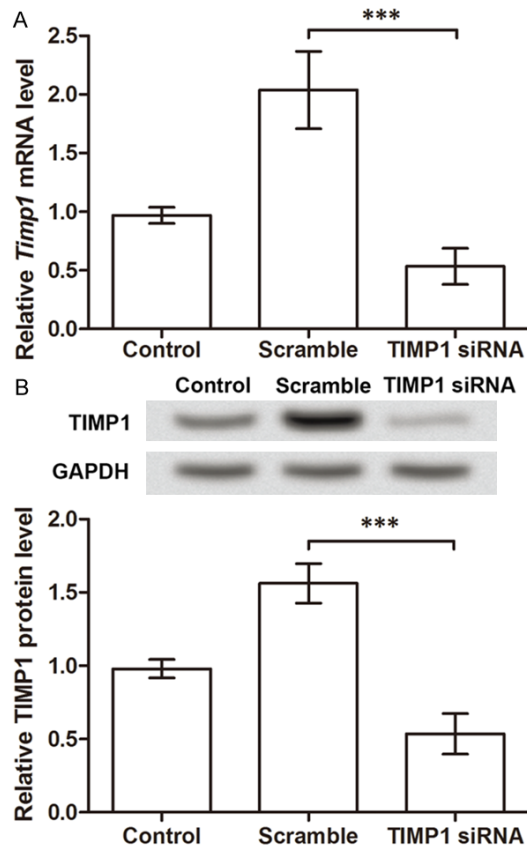
Figure 2. TIMP1 is inhibited by its specific siRNA. Control, rats without any treatment. Scramble, rats injected with scramble siRNA vectors as control and then induced with MACo operation. TIMP1 siRNA, rats injected with TIMP1-specific siRNA vector and then induced with MACo operation. A. qPCR shows *Timp1* mRNA level is inhibited by siRNA injection (n = 5). B. Western blot shows TIMP1 protein level is inhibited by siRNA injection. The histogram shows the relative intensity of protein bands compared to GAPDH (n = 5). ***P < 0.001. TIMP1, tissue inhibitor of metalloproteinases 1.

tein level was also promoted in the Model group compared to the Sham group (P < 0.001, **Figure 1B**). These results indicated the aberrant expression of TIMP1 in both mRNA and protein levels, implying the potential relationship of TIMP1 and cerebral infarction progression. So the following experiments of this study focused on the functions of TIMP1 in cerebral infarction and cerebral cells.

Knockdown of TIMP1 reduces cerebral infarction area

In order to reveal the role of TIMP1 in cerebral infarction, we performed knockdown of TIMP1 using its specific siRNA. Before analyzing the infarction area change, we detected the levels
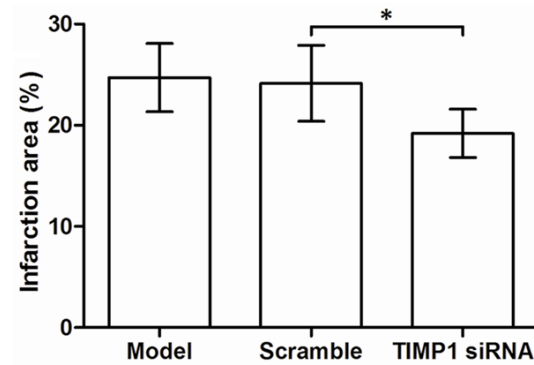

Figure 3. Percent of infarction area in the brain of cerebral infarction rat model is reduced by knockdown of TIMP1. Model, cerebral infarction rats induced with MACo operation. Scramble, rats injected with scramble siRNA vectors as control and then induced with MACo operation. TIMP1 siRNA, rats injected with TIMP1-specific siRNA vector and then induced with MACo operation. *P < 0.05. TIMP1, tissue inhibitor of metalloproteinases 1.

of TIMP1 to test if siRNA injection could successfully inhibit TIMP1 expression. qPCR results showed that TIMP1-specific siRNA obviously inhibited *Timp1* mRNA expression in the brain of rat model compared to the Scramble group (P < 0.001, **Figure 2A**). Similarly, TIMP1 protein expression was also inhibited by its siRNA (P < 0.001, **Figure 2B**). So the siRNA used in this study was effective in TIMP1 knockdown, and the injected rat groups were used to investigate functions of TIMP1.

Then the infarction area of the brain was detected by TTC method. Results showed that the percent of infarction area in cerebral infarction rat model was about 25%, while knockdown of TIMP1 decreased the infarction area to about 20% (P < 0.05, **Figure 3**). This result indicated histologically that knockdown of TIMP1 could help to alleviate cerebral infarction in the induced rat model, which implied that TIMP1 played roles of promoting the progression of cerebral infarction.

Knockdown of TIMP1 inhibits apoptosis of cerebral cells

TIMP1 has been reported to regulate cell proliferation and apoptosis in some diseases [18], thus in this study, its effects on cerebral cell apoptosis were investigate. TUNEL assay was performed in brain tissue sections of the Control groups and the siRNA injection groups, and results showed that the cerebral cell apoptosis was accelerated in cerebral infarction rat
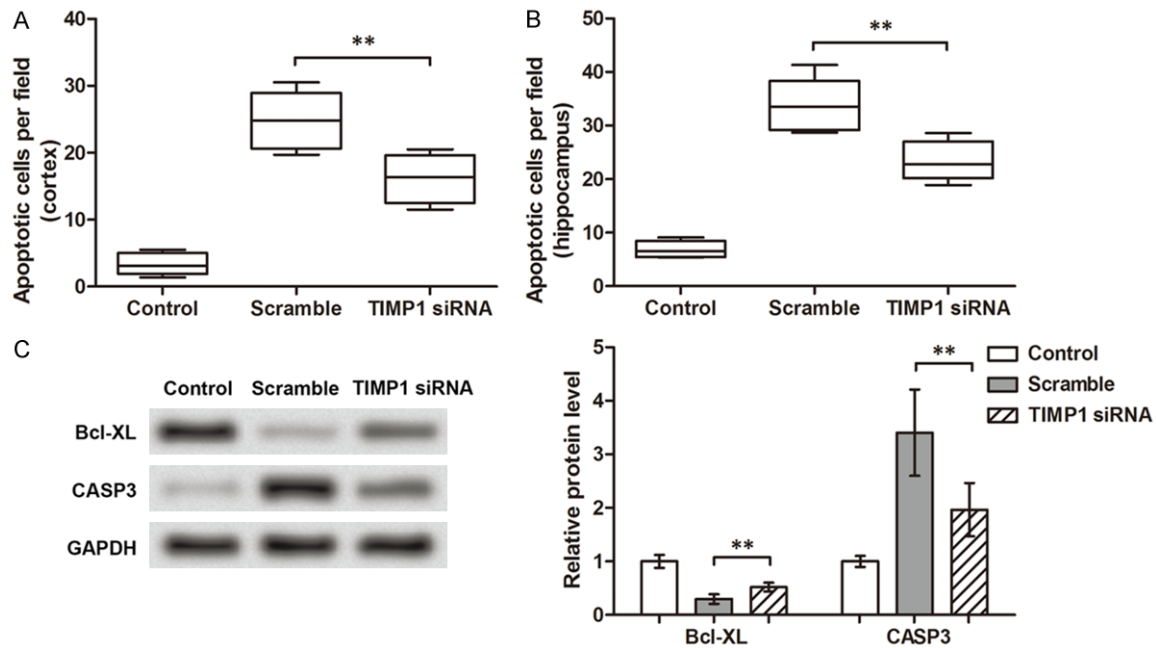
Figure 4. Knockdown of TIMP1 inhibits cell apoptosis in the brain of cerebral infarction rat model. Control, rats without any treatment. Scramble, rats injected with scramble siRNA vectors as control and then induced with MACo operation. TIMP1 siRNA, rats injected with TIMP1-specific siRNA vector and then induced with MACo operation. A. TUNEL assay shows that knockdown of TIMP1 inhibits cerebral cell apoptosis in cortex (n = 5). B. TUNEL assay shows that knockdown of TIMP1 inhibits cerebral cell apoptosis in hippocampus (n = 5). C. Western blot shows the promoted Bcl-XL level and the inhibited CASP3 level when TIMP1 is knocked down. Histogram shows the relative intensity of protein bands compared to GAPDH (n = 5). $**P < 0.01$. TIMP1, tissue inhibitor of metalloproteinases 1. Bcl-XL, B-cell lymphoma extra-large. CASP3, caspase 3.

model, whereas inhibiting TIMP1 led to the suppression of cerebra cell apoptosis both in brain cortex and hippocampus compared to the Scramble group ($P < 0.01$, **Figure 4A** and **4B**). It seemed that knockdown of TIMP1 could significantly inhibit cerebral cell apoptosis. Then the expression of apoptotic factors was examined to verify the changes in cell apoptosis. The anti-apoptotic factor Bcl-XL was down-regulated in the rat model, and promoted with the knockdown of TIMP1 ($P < 0.01$); the pro-apoptotic factor CASP3 showed the opposite changes pattern (**Figure 4C**), which was in agreement with the TUNEL assay results. So the knockdown of TIMP1 caused the suppressed cerebral cell apoptosis in the rat model, implying that TIMP1 might facilitate apoptosis during cerebral infarction.

Discussion

This study reveals the role of TIMP1 in altering infarction area and cerebral cell apoptosis in a MCAo-induced cerebral infarction rat model with knockdown of TIMP1. TIMP1 level is increased in the brain of model rats. The following experiments show that knockdown of TIMP1 reduces infarction area and inhibits cerebral cell apoptosis, indicating a pro-apoptotic function of TIMP1 during cerebral infarction.

This study detected an up-regulated TIMP1 mRNA and protein levels in the brain of cerebral infarction rats, which implied the potential association of TIMP1 and cerebral infarction progression. TIMP1 has been reported to be a promising marker to predict myocardial infarction for its high expression in the plasma of myocardial infarction patients [19]. In acute cerebral infarction, its excessive concentration in the plasma is also detected, together with the promoted MMP9 level that may be generated from the alteration of BBB integrity [20, 21]. Higher MMP9 and TIMP1 levels are likely to indicate a worse prognosis of cerebral infarction [22]. Based on the previous findings, the up-regulated level of TIMP1 shown in this study is not a disparity, and implies the possible involvement of TIMP1 in cerebral infarction.

The effects of TIMP1 on changing cerebral infarction area and cell apoptosis were ana-

lyzed by histological observation after TTC staining and TUNEL assay. Knockdown of TI-MP1 reduced infarction area in the rat model brain, and inhibited cerebral cell apoptosis as indicated by TUNEL assay and apoptotic factor changes. The direct association of TIMP1 with cerebral cell apoptosis has not been reported before, though it is confirmed to suppress tumor growth and angiogenesis in a murine xenotransplant model [23]. TIMP2, but not TIMP1, can suppress atherosclerotic plaque progression via inhibiting the migration and apoptosis of macrophages [24]. So it seems that the role of TIMP1 in cell apoptosis is not quite conserved among difference cell types. The results of this study confirm the pro-apoptotic role of TIMP1 in the brain of cerebral infarction rat model, suggesting that TIMP1 may be an alternative target for alleviating cerebral infarction.

In MCAo-induced cerebral ischemia/reperfusion rodent model, ginkgolide B, rosuvastatin and glycyrrhizin are able to attenuate NF-κB, TNF-α and interleukins-induced inflammatory responses to suppress cell apoptosis [25-27], suggesting the modulation of cell apoptosis in cerebral infarction may be related to control of inflammatory responses. The mechanisms of TIMP1 in modulating cerebral cell apoptosis may involve the regulation of apoptotic factors such as Bcl-XL and CASP3, as was shown in this study. But the detail pathways like the inflammatory signaling are to be investigated in future research.

Numerous studies of TIMP1 focus on its relationship with MMPs, since TIMP1 is an endogenous inhibitor of MMPs and plays crucial roles in the modulation of ECM degradation. Studies in MCAo-induced ischemia/reperfusion rat models confirm that inhibiting MMP9 allows effective control of cerebral infarction area [28, 29], which appears to conflict with the roles of TIMP1 in inhibiting MMPs and promoting cerebral cell apoptosis. A relative reasonable explanation may be that it is the ratio of MMP/TIMP1, rather than the TIMP1 concentration, that is close related to the modulation of diseases including cerebral infarction. For example, MMP3 is elevated in inflammatory bowel disease, while the TIMP1 level remains stable [30]. In rat model of brain edema following acute cerebral infarction, the ratio of MMP9/TIMP1 is positively correlated with brain water

content (BWC), but no correlation is found between TIMP1 and BWC [31]. So it is necessary to associate TIMP1 with MMPs when investigating the mechanism of cerebral cell apoptosis in the following studies.

To sum up, this study reveals the anabatic expression of TIMP1 in the brain of cerebral infarction rat model, which may facilitate cerebral infarction and cell apoptosis. Inhibiting TIMP1 is promising to attenuate cerebral infarction and suppress cell apoptosis. This study implies the potential of using TIMP1 as a target for treating cerebral infarction, and further mechanism research is essential to understand the modulation of cerebral infarction.

Disclosure of conflict of interest

None.

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