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Renin-angiotensin system activation and imbalance of matrix metalloproteinase-9/tissue inhibitor of matrix metalloproteinase-1 in cold-induced stroke

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ABSTRACT

Aims: In the present study, we investigated the roles of renin-angiotensin system (RAS) activation and imbalance of matrix metalloproteinase-9 (MMP-9)/tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in cold-induced stroke during chronic hypertension, as well as the protective effects of captopril and recombinant human TIMP-1 (rhTIMP-1).

Main methods: Rats were randomly assigned to sham; 2-kidney, 2-clip (2K-2C); 2K-2C + captopril, and 2K-2C + rhTIMP-1 groups. After blood pressure values had stabilized, each group was randomly divided into an acute cold exposure (ACE) group (12-h light at 22 °C/12-h dark at 4 °C) and a non-acute cold exposure (NACE) group (12-h light/12-h dark at 22 °C), each of which underwent three cycles of exposure. Captopril treatment was administered via gavage (50 mg/kg/d), while rhTIMP-1 treatment was administered via the tail vein (60 μg/kg/36 h).

Key findings: In the 2K-2C group, angiotensin II (AngII) and MMP-9 levels increased in both the plasma and cortex, while no such changes in TIMP-1 expression were observed. Cold exposure further upregulated AngII and MMP-9 levels and increased stroke incidence. Captopril and rhTIMP-1 treatment inhibited MMP-9 expression and activation and decreased stroke incidence in response to cold exposure.

Significance: The present study is the first to demonstrate that cold exposure exacerbates imbalance between MMP-9 and TIMP-1 by activating the RAS, which may be critical in the initiation of stroke during chronic hypertension. In addition, our results suggest that captopril and rhTIMP-1 exert protective effects against cold-induced stroke by ameliorating MMP-9/TIMP-1 imbalance.

1. Introduction

Stroke is the second leading cause of death and the third leading cause of disability worldwide, and the global burden of stroke continues to increase [1,2]. More and more Epidemiological and observational data has indicated that the cold weather is associated with an increased occurrence of acute stroke events [3,4], and is a risk factor for stroke mortality [5,6]. However, the mechanisms underlying cold-induced stroke remain to be elucidated as a result of experimental data regarding the effects of low ambient temperature on stroke remain insufficient.

Matrix metalloproteinases (MMPs) are a proteinase family of degrading extracellular matrix (ECM). Among them, MMP-9 has been shown to play a key role in cerebrovascular diseases [7]. Tissue inhibitors of matrix metalloproteinases (TIMPs) are specific endogenous inhibitors of MMPs, consisting of four known members (TIMP-1-4). Under physiological conditions, a balance is maintained between the expression of MMPs and TIMPs. As a specific endogenous inhibitor with the greatest affinity for MMP-9, TIMP-1 helps to maintain normal vascular function in the ECM [8]. Under various pathological conditions, relative increases in MMP-9 expression and activity disrupt the balance between MMP-9 and TIMP-1, resulting in over-degradation of

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the vascular matrix—a process that has been implicated in cerebral ischemia [9] and intracerebral hemorrhage [10]. After acute ischemic stroke, the level of MMP-9 increased and associated with increased risk of mortality and poor prognosis in patients [11]. There are adequate evidences showed that plasma MMP-9 concentration increased have closely associated with the spontaneous hemorrhagic transformation [12] and tissue plasminogen activator-induced hemorrhagic transformation in patients with ischemic stroke [13,14].

The RAS can be divided into circulating and local components. Angiotensin-converting enzyme transforms angiotensin I into AngII. AngII is the primary component and effector of the RAS, serving as a crucial inflammatory factor. Accumulating evidence has indicated that MMPs are vital target genes for AngII, which helps to promote MMP-9 expression and activation [15]. Moreover, previous studies have reported that exposure to cold increases RAS activity, thereby enhancing the expression of AngII [16]. Our previous study has suggested that cold-induced upregulation of MMP-9 plays a pivotal role in initiating cold-induced stroke during hypertension [17]. More recent evidence has demonstrated that MMP-9 inhibition and genetic knockdown of MMP-9 may represent novel therapeutic targets for cerebrovascular diseases [18–20].

Therefore, in the current study, we aimed to verify the hypothesis that cold exposure aggravates imbalance between MMP-9 and TIMP-1 by increasing MMP-9 expression and activation, thereby leading to acute stroke events via RAS activation. We also aimed to determine whether inhibition of AngII using captopril or elevation of TIMP-1 levels via rhTIMP-1 would exert protective effects for cold-induced stroke.

2. Material and methods

2.1. Ethics statement

All experimental and animal care procedures/protocols were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No.8023,revised 1978). The protocols (Numbers L2017014 and L2017187) were approved by the Ethical Committee of Animal Experiments at Southern Medical University. All efforts were made to minimize the number of animals used and their suffering.

2.2. Subjects and study design

A total of 170 healthy male Sprague–Dawley rats (weighted 80–120 g and aged one month) were obtained from the Animal Center of Southern Medical University in Guangzhou, China (License No. 00166709 and No. 00184201). All animals were housed in a specific pathogen free environment with free access to a standard pellet diet and water under a 12-h light (8:00 am–8:00 pm)/12-h dark (8:00 pm–8:00 am) cycle throughout the study. All animals were adapted to the specific pathogen free environment for at least 3 days before the experimental operation. All rats were initially assigned to a sham group ($n = 20$) or a 2K-2C renovascular hypertension group ($n = 150$), following which the animals in the 2K-2C renovascular hypertension group were randomly assigned to one of the following three groups: a 2K-2C group, a 2K-2C + captopril group, and a 2K-2C + rhTIMP-1 group. Each of these groups included 50 rats and was further divided into two groups: an ACE group and a NACE group. The 2K-2C renovascular hypertensive operation was performed as previously described [21]. Briefly, the rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (45 mg/kg), and the bilateral renal arteries were constricted using ring-shaped silver clips with an inner diameter of 0.20 mm to induce hypertension. Sham rats underwent the same surgical procedure without clip placement. SBP and body weight were measured before surgery and every four weeks for 12 weeks. SBP was measured via tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) three times in a blinded manner, and the average values were used for statistical analyses. Neurological examinations were performed at least two times per day. Rats highly suspected of having stroke, other complications, and those that had died were replaced. According to the China Meteorological Administration, a cold wave is defined as a rapid drop in temperature of > 10 °C within 24 h, with a minimum temperature lower than 5 °C. Artificial cold exposure was introduced using an intelligent artificial climate box (RXZ-328 A; Ningbo Jiangnan Instrument Factory, Ningbo, China). In the ACE group, the temperature was set at 22 °C for 12 h (8:00 am to 8:00 pm) and 4 °C for 12 h (8:00 pm to 8:00 am) for three consecutive days. In the NACE group, the temperature was maintained at 22 °C for the same duration. The relative humidity was maintained at 65% as previously described. During this period, captopril was administered intragastrically to rats in the 2K-2C + captopril group once per day (50 mg/kg/d; Cat. no. C7510, Solarbio, Beijing, China). In the 2K-2C + rhTIMP-1 group, rhTIMP-1 was administered via the tail vein (60 μg/kg/36 h; Cat. no. PHC8023; Invitrogen, Carlsbad, CA, USA). Rats in the sham and 2K-2C groups were administered equal volumes of normal saline via gavage. Following three cycles of cold exposure or when seizures, hemiplegia, consciousness disturbance, or death were observed, rats were deeply anesthetized and sacrificed. Blood samples and brain tissue were then collected. Stroke lesions were detected via hematoxylin and eosin (HE) staining, and the plasma and brains of rats without stroke were used for detection experiments.

2.3. Incidence of stroke

During the artificial cold exposure period, neurologic deficits were evaluated three times per day as previously described [22]. Scores were assigned as follows: 0: no neurological impairment; 1: unable to extend the contralateral forelimb; 2: mild circling to the contralateral side; 3: falling to the contralateral side; 4: failing to walk spontaneously, loss of consciousness. Magnetic resonance imaging was used to examine a sample of rats with suspected stroke following cold exposure. Following removal, the brain was sectioned into five pieces, and stroke lesions were assessed via visual observation and HE staining.

2.4. Enzyme-linked immunosorbent assay

Blood was collected before and after exposure, following which the samples were centrifuged to separate plasma (1000 *g* for 15 min at 4 °C) and stored at −80 °C until use. Plasma concentrations of AngII, MMP-9, and TIMP-1 were measured in a blinded manner using ELISA kits (Cusabio, cat. no. CSB-E04494r; CSB-E08005r; CSB-E08008r; Wuhan, China), in accordance with the manufacturer's instructions.

2.5. Western blot analysis

Cerebral cortex tissues were homogenized in ice-cold lysis buffer, following which the extracts were centrifuged at 12,000 *g* for 15 min at 4 °C. Protein concentrations were normalized, and the extracts were boiled for 5 min. Equal amounts of protein (30 μg) were separated via 10% SDS-PAGE, following which they were transferred to PVDF membranes (Millipore, Boston, MA, USA). Membranes were blocked with 5% BSA in TBST for 90 min at 25 °C, following which they were incubated with primary antibody mouse anti-AngII polyclonal antibody (1:750; cat. no. NB100-62346; Novus Biologicals), rabbit anti-MMP-9 polyclonal antibody (1:1000; cat. no. ab38898; Abcam), rabbit anti-TIMP1 polyclonal antibody (1:600; cat. no. ab61224; Abcam), or rabbit anti-β-actin monoclonal antibody (1:8000; cat. no. AB0033; Abway) overnight at 4 °C. The membranes were washed and further incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobin G or goat anti-mouse immunoglobin G for 60 min at 25 °C. The membranes were washed, and immunoreactive bands were detected using the Immobilon Western Chemilum HRP Substrate

(Millipore; cat. no. WBKLS0100; Boston; MA; USA). Densitometric analyses of protein bands were performed using Image J (version 1.8.0 National Institutes of Health, Bethesda, MD, USA).

2.6. Immunohistochemistry

Animals were killed and immediately infused with normal saline for exsanguination. The brains were carefully collected, and brain sections were fixed in 4% PFA, following which they were embedded in paraffin. The tissue sections were cut into 4-μm sections and blocked with 10% normal goat serum for 40 min at 25 °C, following which they were incubated in dark humidified chambers with primary antibodies for AngII (1:750; cat. no. NB100-62346; Novus Biologicals), MMP-9 (1:1000; cat. no. ab38898; Abcam), and TIMP-1(1:600; cat. no. ab61224; Abcam) overnight at 4 °C. The sections were then rewarmed for 30 min at 25 °C, washed, and treated with an anti-rabbit or anti-mouse HRP-conjugated secondary antibody for 60 min at 25 °C. Positive staining was assessed by adding 3,3′-diaminobenzidine buffer to the sections, which were viewed and imaged using a light microscope (Olympus BX41; Olympus Corp, Tokyo, Japan). Positive staining intensity was measured using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.7. In situ zymography

In situ gelatinolytic activity in the cerebral cortex was measured using fluorescein isothiocyanate-labeled DQ-gelatin (EnzCheck Gelatinase Assay Kit; cat. no. E12055; Invitrogen). Briefly, 10-μm frozen unfixed brain tissues were incubated with DQ-gelatin (40 μg/ml in 1× reaction buffer) in dark humidified chambers for 2 h at 37 °C. Some sections were incubated in the absence of the substrate or the presence of the metalloproteinase inhibitor 1,10-Phenanthroline monohydrate (10 mmol/L). Sections were examined and imaged using a fluorescence microscope (Leica Imaging Systems Ltd., Cambridge, England). Gelatinolytic activity was detected as bright green fluorescence, which indicates DQ-gelatin breakdown. The fluorescence intensity was evaluated using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.8. Statistical analysis

All results are expressed as the mean ± standard deviation, and all statistical analyses were performed using SPSS version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data for stroke incidence were analyzed using chi-square and Fisher's exact tests. Pre- and post-exposure ELISA values were compared between the ACE and corresponding NACE groups using paired *t*-tests or Wilcoxon signed-rank tests. *P* values of < 0.05 were considered significant.

3. Results

3.1. Baseline systolic blood pressure (SBP) and body weight

Baseline SBP was similar in all groups. Stable hypertension attacks were observed in 100% of 2K-2C rats (150/150), while severe hypertension (SBP ≥ 220 mm Hg after the 2K-2C operation) was observed in 45 of 150 (30%) rats. However, SBP increased significantly 4 weeks after induction ($P < 0.00$), steadily rising and stabilizing until the 12-week mark. Baseline body weight was also similar in all four groups, although significant differences in body weight were observed between the sham group and the remaining groups 8 weeks following the operation ($P \leq 0.001$) (see Supplementary Material). Moreover, prior to cold exposure, there were no significant differences in SBP or body weight between ACE and NACE rats in each of the four groups.

Table 1
Stroke incidence in each group following exposure.

Group	Sham	2K-2C	2K-2C + captopril	2K-2C + rhTIMP-1	*P* value
NACE	0/10(0%)	5/25(20%)	4/25(16%)	3/25(12%)	0.471
ACE	0/10(0%)	12/25(48%)[#, &]	7/25(28%)	5/25(20%)[*]	0.021

P values are based on chi-square/Fisher's exact tests. 2K-2C, 2Kidney, 2 clip; ACE, acute cold exposure; NACE, nonacute cold exposure; rhTIMP-1, recombinant human tissue inhibitor of matrix metalloproteinase-1.
[#] $P < 0.001$ versus sham group.
[*] $P < 0.05$ versus 2K-2C ACE group.
[&] $P < 0.05$ versus 2K-2C NACE group.

3.2. Incidence of stroke

Table 1 shows the incidence of stroke after cold exposure in all groups. A few stroke rats exhibited obvious neurological deficiencies without severe weakness. Ten stroke rats experienced hemiplegia, while two has died. The stroke subtypes included intracerebral hemorrhage, cerebral infarction, and mixed stroke. Magnetic resonance imaging (MRI) revealed that stroke lesions were widely distributed throughout the cerebral cortex, although lesions were observed in the cerebral medulla in two rats (Fig. 1).

3.3. Detection of plasma AngII, MMP-9, and TIMP-1 levels

Changes in plasma concentrations of AngII, MMP-9, and TIMP-1 before and after exposure were determined via enzyme-linked immunosorbent assay (Tables 2 and 3). AngII and MMP-9 levels were significantly higher in the 2K-2C group than in the sham group, although there was no significant difference in TIMP-1 levels between these two groups. Cold exposure further increased levels of AngII and MMP-9 but had no effect on TIMP-1 levels in the 2K-2C ACE group, relative to levels observed in the 2K-2C NACE group. Administration of captopril (50 mg/kg/d) attenuated increases in AngII and MMP-9 expression following cold exposure. However, intravenous injection of rhTIMP-1 (60 μg/kg/36 h) had no significant effect on AngII or MMP-9 levels. Neither treatment significantly altered TIMP-1 levels.

3.4. Expression of AngII, MMP-9, and TIMP-1 in the cerebral cortex

AngII, MMP-9, and TIMP-1 expression in the cerebral cortex was determined via Western blotting and immunohistochemistry. AngII and MMP-9 levels were significantly higher in the 2K-2C group than in the sham group, while no significant differences in TIMP-1 expression were observed between the two groups. Cold exposure further increased the expression of AngII and MMP-9 relative to levels observed in the 2K-2C NACE group. However, no significant changes in TIMP-1 expression were observed. Treatment with captopril attenuated both AngII and MMP-9 expression. Treatment with rhTIMP-1 also attenuated MMP-9 expression. However, captopril did not obviously affect TIMP-1 expression, although slight decreases in TIMP-1 expression were observed following treatment with rhTIMP-1 (Figs. 2, 3 and 4).

3.5. Gelatinolytic activity in the cerebral cortex

Our analyses of in situ gelatinolytic activity revealed that levels of active MMPs were higher in rats with chronic hypertension than in sham rats. Relative to levels observed in the 2K-2C NACE group, cold exposure further increased MMP activity in the 2K-2C ACE group. However, cold-induced activation of MMP-9 was significantly attenuated following treatment with captopril and rhTIMP-1, the latter of which was more effective (Fig. 5).

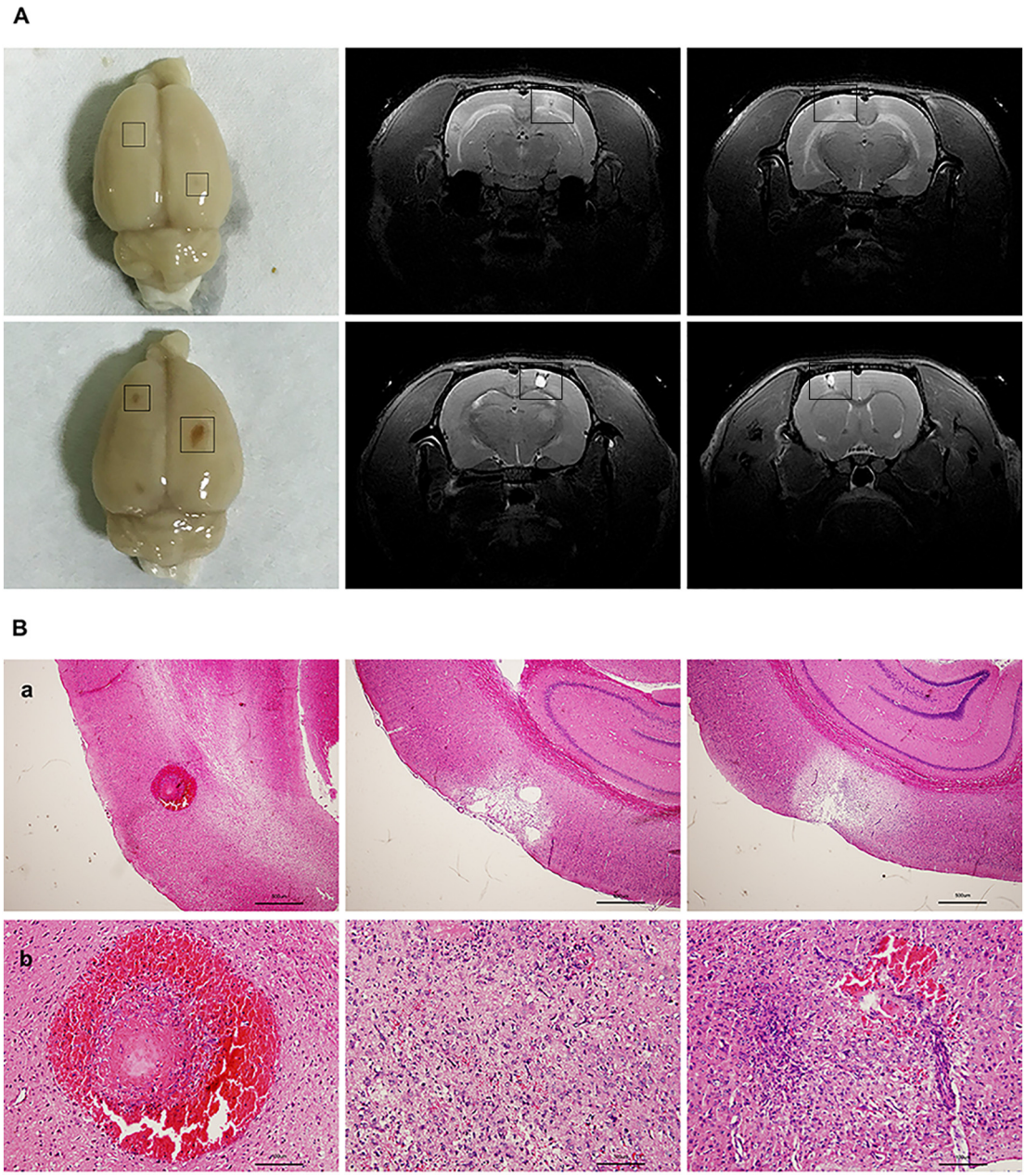

Fig. 1. A. Brains of stroke rats and the corresponding MR images. B. HE staining of stroke brains. (a and b) Infarcted and hemorrhagic lesions in the cerebral cortex (a: ×40, scale bar: 500 μm; b: ×200, scale bar: 100 μm).

Table 2
Plasma concentrations of AngII, MMP-9, and TIMP-1 in each ACE group before and after cold exposure.

Groups		AngII(pg/ml)	MMP-9(ng/ml)	TIMP-1(ng/ml)
Sham(n = 10)	Before	2.62 ± 1.10	40.65 ± 8.22	4.22 ± 0.86
	After	3.09 ± 0.94	43.78 ± 10.28	4.74 ± 2.38
2K-2C(n = 10)	Before	14.37 ± 2.69	117.18 ± 27.01	5.30 ± 1.40
	After	23.22 ± 6.54*	153.45 ± 32.35**	5.88 ± 1.72
2K-2C+ captopril(n = 10)	Before	13.55 ± 2.32	111.45 ± 26.06	5.01 ± 1.46
	After	11.24 ± 2.02	118.31 ± 36.22	6.03 ± 2.91
2K-2C+ rhTIMP-1(n = 10)	Before	13.91 ± 2.03	114.80 ± 30.94	5.95 ± 1.82
	After	19.47 ± 4.16*	113.28 ± 47.57	6.02 ± 2.36

P values are based on paired *t*-tests/Wilcoxon paired tests. 2K-2C, 2Kidney, 2 clip; ACE, acute cold exposure; NACE, non-acute cold exposure; rhTIMP-1, recombinant human tissue inhibitor of matrix metalloproteinase-1; AngII, angiotensin II, MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

* $P < 0.05$ versus respective before group.

** $P < 0.01$ versus 2K-2C before group.

Table 3
Plasma concentrations of AngII, MMP-9, and TIMP-1 in each NACE group before and after exposure.

Groups		AngII (pg/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)
Sham(n = 10)	Before	2.51 ± 1.28	38.97 ± 8.10	4.24 ± 1.23
	After	2.52 ± 1.18	38.86 ± 7.20	4.72 ± 1.96
2K-2C(n = 10)	Before	14.31 ± 4.09	119.08 ± 39.42	4.27 ± 0.98
	After	12.69 ± 1.75	119.51 ± 21.83	5.34 ± 2.32
2K-2C + captopril(n = 10)	Before	13.87 ± 3.03	110.22 ± 35.43	5.42 ± 1.50
	After	10.14 ± 0.95*	84.85 ± 39.63	6.31 ± 1.81
2K-2C + rhTIMP-1(n = 10)	Before	13.34 ± 3.39	111.76 ± 40.69	5.28 ± 1.70
	After	12.58 ± 1.76	93.35 ± 27.46	5.64 ± 2.01

* $P < 0.05$ versus 2K-2C + captopril before group; P values are based on paired *t*-tests/Wilcoxon paired tests. 2K-2C, 2Kidney, 2 clip; ACE, acute cold exposure; NACE, non-acute cold exposure; rhTIMP-1, recombinant human tissue inhibitor of matrix metalloproteinase-1, AngII, angiotensin II; MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

4. Discussion

Numerous epidemiological studies have indicated that cold temperatures are associated with increases in the occurrence of stroke in those at risk. Our results demonstrated that cold exposure aggravates MMP-9/TIMP-1 imbalance in hypertensive rats via RAS activation, making rats with chronic hypertension more susceptible to stroke. Increases in MMP-9 expression and activation due to chronic hypertension contributed to MMP-9/TIMP-1 imbalances in the plasma and cortex. Moreover, cold exposure further increased MMP-9 expression and activation in parallel with levels of AngII. Inhibition of AngII expression due to captopril treatment or supplementation with exogenous rhTIMP-1 effectively attenuated increases in MMP-9 expression and activation, and such changes were accompanied by decreases in the incidence of cold-induced stroke. These results suggest that cold exposure induces RAS activation, thereby aggravating MMP-9/TIMP-1 imbalance during chronic hypertension, which are closely related to the initiation of stroke. Thus, direct decreases in AngII or direct inhibition of MMP-9 may exert protective effects against cold-induced stroke by attenuating MMP-9/TIMP-1 imbalance.

It is widely accepted that hypertension is among the most critical risk factors for stroke [23,24]. Recent research has indicated that patients with hypertension are more susceptible to cold-associated stroke than those without [4]. Previous study has observed the stroke lesions in 2K-2C renovascular hypertensive rats are similar to those observed in patients with hypertension [21]. So, in the present study, we used 2K-2C renovascular hypertensive rats model to investigate stroke outcomes and prevention efforts following artificial cold exposure.

MMPs are well recognized enzymes that act to degrade the ECM. MMPs are inhibited by TIMPs to maintain normal ECM metabolism in the vascular wall. Numerous studies have demonstrated that upregulation of MMPs alters the balance between MMPs and TIMPs, leading to over-degradation of the ECM, which has been closely associated with hypertensive vascular remodeling and dysfunction [25]; the destabilization and rupture of atherosclerotic plaques [26,27]; the formation, progression, and rupture of cerebral aneurysms [28]; and BBB dysfunction [29]. AngII is upregulated in patients and experimental animals with hypertension via RAS activation. AngII is the most potent inflammatory factor inducing MMP expression, acting via the NF-κB [30], P38MAPK [31], JAK-STAT [32], and MEK/ERK [33] signaling and oxidative stress pathways [15]. In addition, AngII-mediated intracellular Ca^{2+} overload may represent the mechanism by which the balance between MMP-9 and TIMP-1 is altered [34]. In the central nervous system, MMP-9 expression is significantly increased in the cortex in rats with chronic hypertension [35]. These findings suggest that hypertension more strongly stimulates changes in MMP-9 expression than changes in TIMP-1 expression. Moreover, AngII is involved in MMP-9 activation [36]. In the present study, AngII and MMP-9 expression significantly increased in the plasma and cerebral cortex, and levels of active MMPs increased in 2K-2C chronic hypertensive rats. However, we observed no significant differences in TIMP-1 levels. These findings suggest that chronic hypertension upregulates AngII and MMP-9 expression in the cortex. Furthermore, plasma and cortical imbalances in MMP-9/TIMP-1 expression associated with chronic hypertension may explain the occurrence of spontaneous stroke in 2K-2C rats.

Cold exposure activates the sympathetic nervous system, thereby stimulating the RAS to increase AngII levels in both animals and humans [16,37]. In the present study, cold exposure further increased AngII levels in the plasma and cortex, and these changes were accompanied by increases in MMP-9 in rats with chronic hypertension. These results suggest that cold exposure contributes to AngII-mediated increases in MMP-9 expression. Previous studies have indicated that cold exposure promotes the infiltration of inflammatory cells [38], and that AngII is involved in the recruitment of inflammatory cells in models of vascular disease [39]. Neutrophils act as a pivotal cellular source of MMP-9 [40,41], promoting the degradation of collagen IV in the basal lamina and the BBB via MMP-9 [42]. Meanwhile, other studies have indicated that AngII may stimulate MMP-9 expression in other inflammatory cells, such as eosinophils [43], macrophages [44], and monocytes [45]. Furthermore, the RAS exhibits a strong correlation with atherosclerosis during chronic hypertension [46]. The fibrous caps of atherosclerotic plaques are composed of ECM, helping to maintain plaque stability. Excessive MMP-9 expression results in over-degradation of the fibrous plaque, causing plaque instability and rupture [47]. In our study, MMP-9 expression and levels of enzymatically active MMPs further increased following cold exposure. Therefore, the aforementioned pathological processes may also be closely related to hemorrhagic stroke and cerebral infarction following cold exposure.

Because angiotensin converting enzyme inhibitors (ACEIs) block the conversion of angiotensin I into AngII, they are commonly used in the treatment of hypertension and in stroke prevention [48]. MMPs are structurally organized into three domains: an N-terminal propeptide domain, an internal catalytic domain, and a C-terminal hemopexin-like domain [49]. Several studies have reported that ACEIs can inhibit MMPs activation via indirect mechanisms as a result of MMPs and Angiotensin-converting enzyme both are zinc-dependent endopeptidases and catalytic domains are similar [50]. Thus, ACEI simultaneously exerts an inhibitory effect on MMPs [51]. Captopril is a common anti-hypertensive drug in the ACEI class. Previous studies have demonstrated that captopril normalizes the MMP-9/TIMP-1 balance by decreasing MMP-9 levels and increasing TIMP-1 levels [34]. In addition, captopril inhibits MMP-9 activity by directly binding to its active center [52]. In our study, intragastric administration of captopril reduced circulating and cortical levels of AngII expression and decreased MMP-9 expression and activity. Moreover, such changes were accompanied by decreases in the incidence of stroke. However, captopril had no obvious effect on TIMP-1 expression in our study. This difference may be associated with short-term administration of the drug, or it may indicated that captopril primarily acts to inhibit excessive increases in

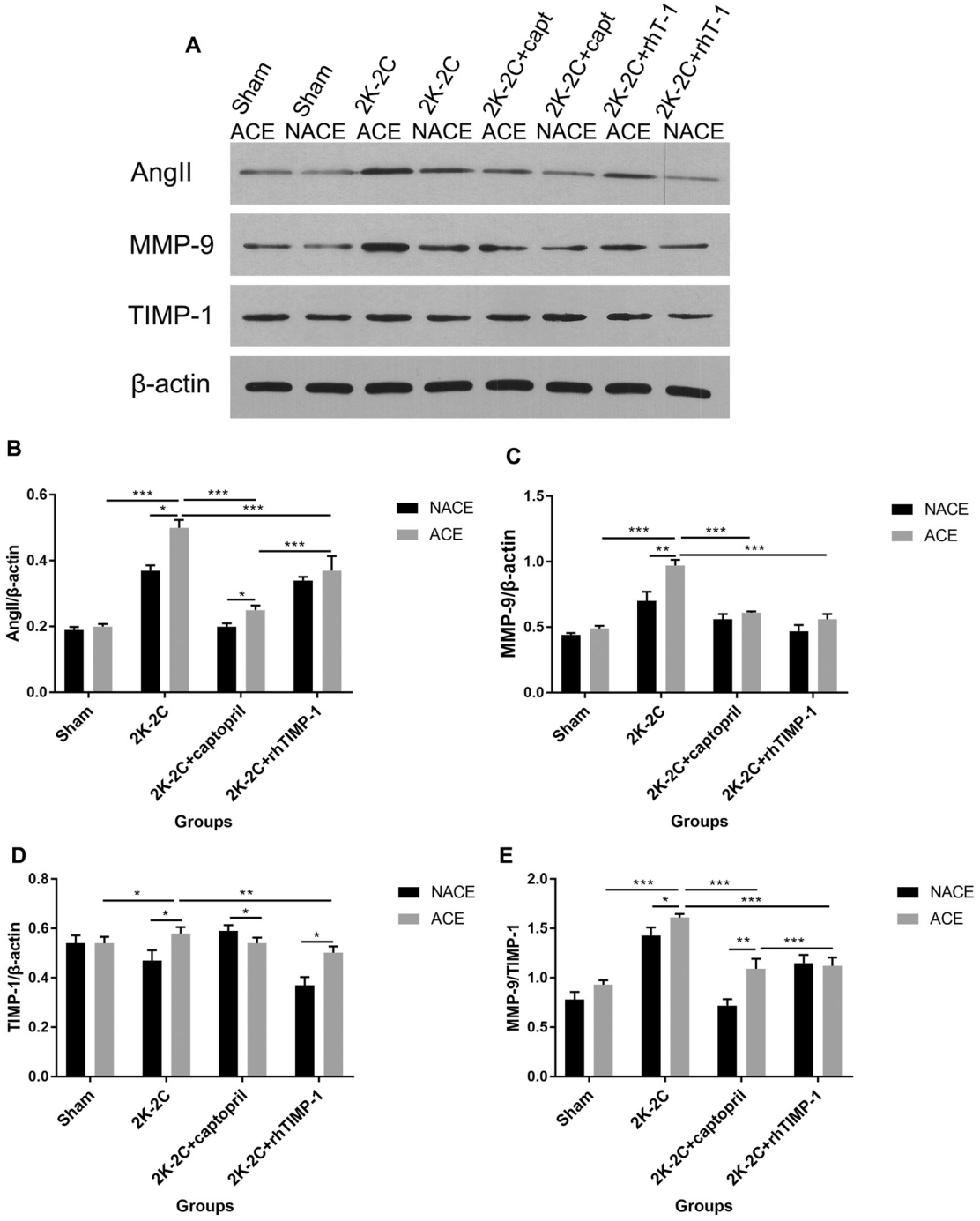

Fig. 2. A. Protein expression of AngII, MMP-9, and TIMP-1 in the cerebral cortex determined via Western blotting. β-actin served as the internal control for each experiment. Densitometric analysis of AngII (B), MMP-9 (C), TIMP-1 (D), and MMP-9/TIMP-1 (E). $N = 4$ in each group. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate ACE group versus the respective NACE group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.00$ indicate 2K-2C group versus the other three groups, and 2K-2C + captopril group versus 2K-2C + rhTIMP-1 group in ACE groups. AngII, angiotensin II; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; rhTIMP-1, recombinant human tissue inhibitor of metalloproteinase-1; NACE, non-acute cold exposure; ACE, acute cold exposure; 2K-2C, 2Kidney, 2 clip.

MMP-9 during cold exposure. Our findings verify that AngII impairs the balance between MMP-9 and TIMP-1, which may trigger stroke onset following cold exposure. Captopril indirectly inhibits MMP-9 expression and activation by inhibiting AngII to alleviate the imbalance between MMP-9 and TIMP-1, thereby exerting protective effects against cold-induced stroke.

The activity of MMP-9 is strongly inhibited by endogenous TIMP-1, which has both N-terminal and C-terminal domains. The N-terminal domain primarily forms high-affinity complexes with MMP-9, which non-covalently bind to the catalytic domain of MMP-9 in a 1:1 stoichiometric to inhibit active MMP-9, whereas the C-terminal domain forms a complex with proMMP-9 to inhibit proMMP-9 activation [53]. Previous studies have indicated that intravenously injected TIMP-1 nanoparticles [54] and intracerebroventricular injection of TIMP-1 cDNA plasmids [55] increase TIMP-1 levels within the central nervous system, helping to maintain the integrity of the BBB. One recent study reported a long blood half-life (42.2 h) for rhTIMP-1 (60 μg/kg) following intravenous injection in rats with stroke. Moreover, the authors

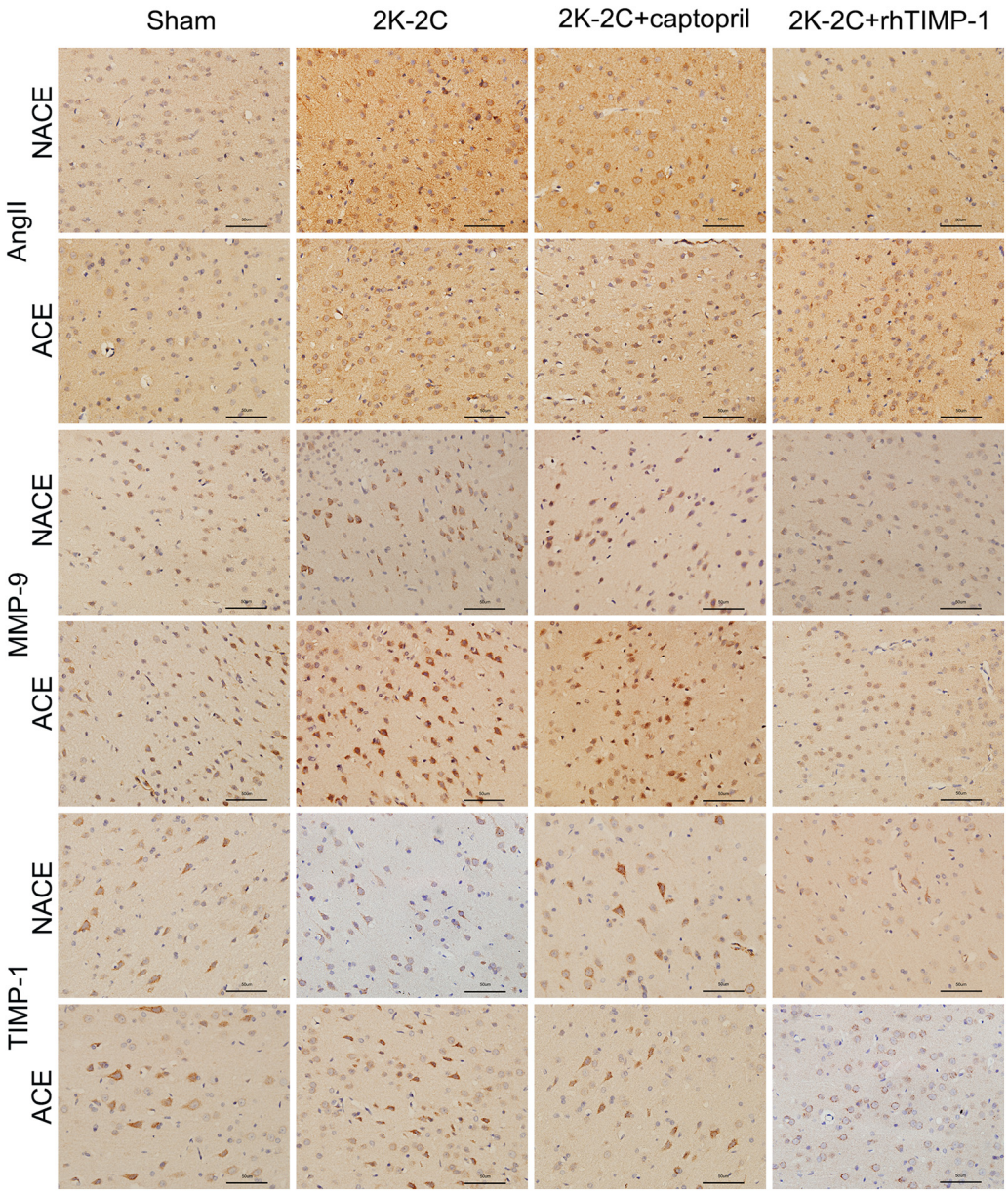

Fig. 3. Protein expression of AngII, MMP-9, and TIMP-1 in the cerebral cortex as measured via immunohistochemical staining. Representative photographs of the immunohistochemical localization of AngII, MMP-9, and TIMP-1 in the cerebral cortex (×400, scale bar: 50 μm). AngII, angiotensin II; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; rhTIMP-1, recombinant human tissue inhibitor of metalloproteinase-1; ACE, acute cold exposure; NACE, non-acute cold exposure; 2K-2C, 2Kidney, 2 clip.

reported that rhTIMP-1 can cross the BBB and remain in the brain for a long time [56]. Based on these findings, we administered exogenous TIMP-1 via the tail vein (rhTIMP-1, 60 μg/kg/36 h) during the study period. Such treatment decreased the expression and activation of MMP-9 in the cortex, and these changes were accompanied by a decrease in the incidence of stroke. These findings indicate that supplementation with rhTIMP-1 may compensate for decreases in endogenous TIMP-1 expression, which may inhibit excessive increases in MMP-9 expression and activation. Interestingly, we observed that supplementation with exogenous rhTIMP-1 was associated with a slight negative feedback mechanism for TIMP-1 expression in the cortex. This finding supports the notion that cold exposure exacerbates MMP-9/TIMP-1 imbalance. Our findings also indicated that direct inhibition of MMP-9 activation via exogenous rhTIMP-1 exerts a neuroprotective effect against cold-induced stroke by alleviating the imbalance between MMP-9 and TIMP-1.

Future studies should aim to determine whether plasma MMP-9 and TIMP-1 levels and the MMP-9/TIMP-1 ratio can be used as a promising biomarker to predict the risk of cold-induced stroke, and whether circulating MMP-9 and TIMP-1 levels also reflect tissue levels. Moreover, further studies are required to examine changes in the structure of microvessels due to changes in MMP-9/TIMP-1, as well as the roles of other MMPs and TIMPs.

5. Conclusion

Overall, the present study is the first to demonstrate that cold exposure exacerbates MMP-9/TIMP-1 imbalance during chronic hypertension via RAS activation, and that such changes are strongly associated with the incidence of cold-induced stroke. Furthermore, our results suggest that captopril and rhTIMP-1 exert protective effects against cold-induced stroke by ameliorating MMP-9/TIMP-1 imbalance.

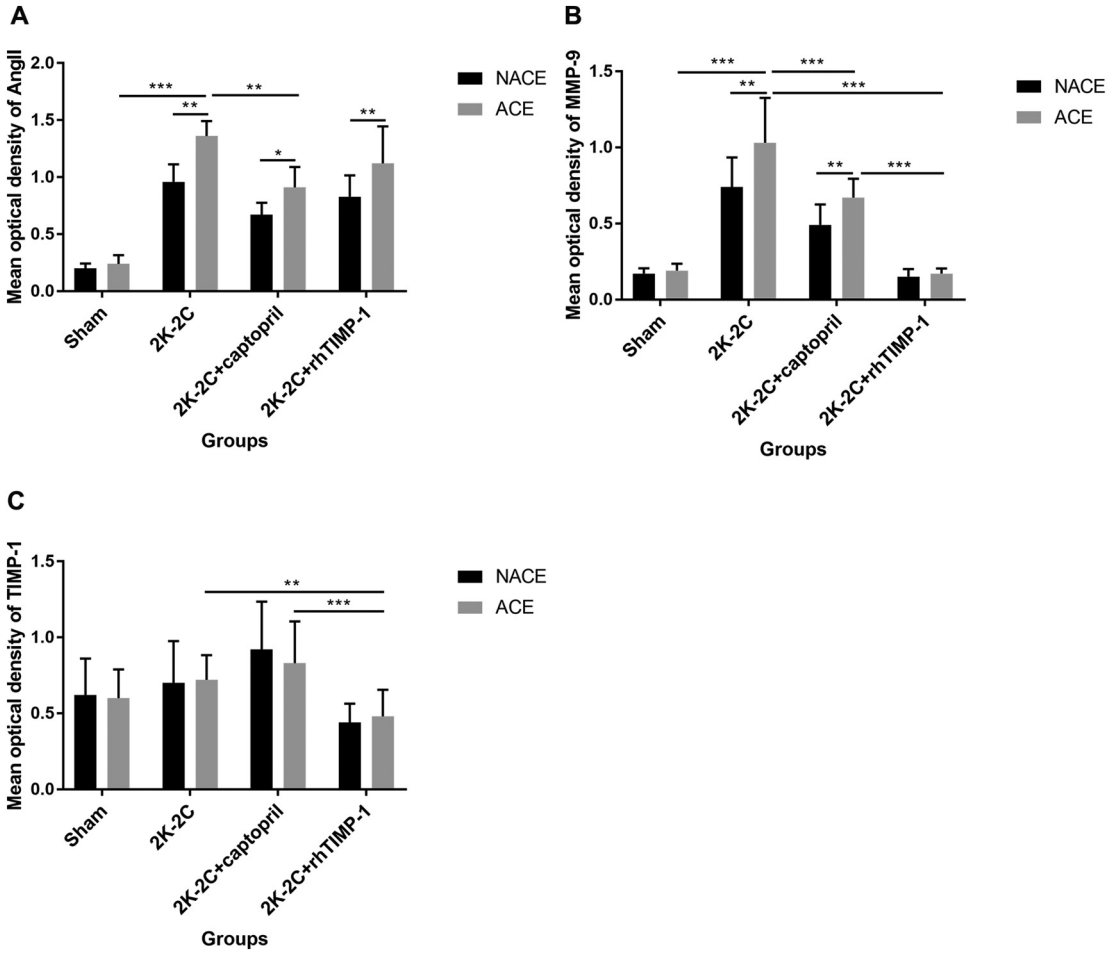

Fig. 4. (A, B, and C) Densitometric analyses of AngII, MMP-9, and TIMP-1 during immunohistochemical staining. $N = 5$ in each group. Data are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate ACE group versus the respective NACE group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.00$ indicate 2K-2C group versus the other three groups, and 2K-2C + captopril group versus 2K-2C + rhTIMP-1 group in ACE groups. AngII, angiotensin II; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; rhTIMP-1, recombinant human tissue inhibitor of metalloproteinase-1; ACE, acute cold exposure; NACE, non-acute cold exposure; 2K-2C, 2Kidney, 2 clip.

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Abbreviations

RAS	renin-angiotensin system
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of matrix metalloproteinase
rhTIMP	recombinant human tissue inhibitor of matrix metalloproteinase
2K-2C	2Kidney, 2 clip
AngII	angiotensin II
ECM	extracellular matrix
BBB	blood-brain barrier
ACE	acute cold exposure
NACE	non-acute cold exposure
ACEI	angiotensin converting enzyme inhibitor
SBP	systolic blood pressure
MRI	magnetic resonance imaging
HE	hematoxylin and eosin
HRP	horseradish peroxidase

Author contributions

Yu-ying Su and Huan-min Li contributed to designing and performing the experiments; to the acquisition, analysis, and interpretation of data; and to the writing of the manuscript.

Zhen-xing Yan, Ming-chun Li, and Ji-peng Wei contributed to designing the experiments, to drafting and guiding the work, and to revising the manuscript for critical intellectual content.

Wen-xia Zheng, Si-qin liu, and Yi-ting Deng contributed to designing the experiments, to analyzing the data, and to the writing of the manuscript.

Hui-fang Xie and Chun-guang Li contributed to designing the experiments, are accountable for all aspects of the experiments, and approved the final version of the manuscript to be published.

All authors have approved the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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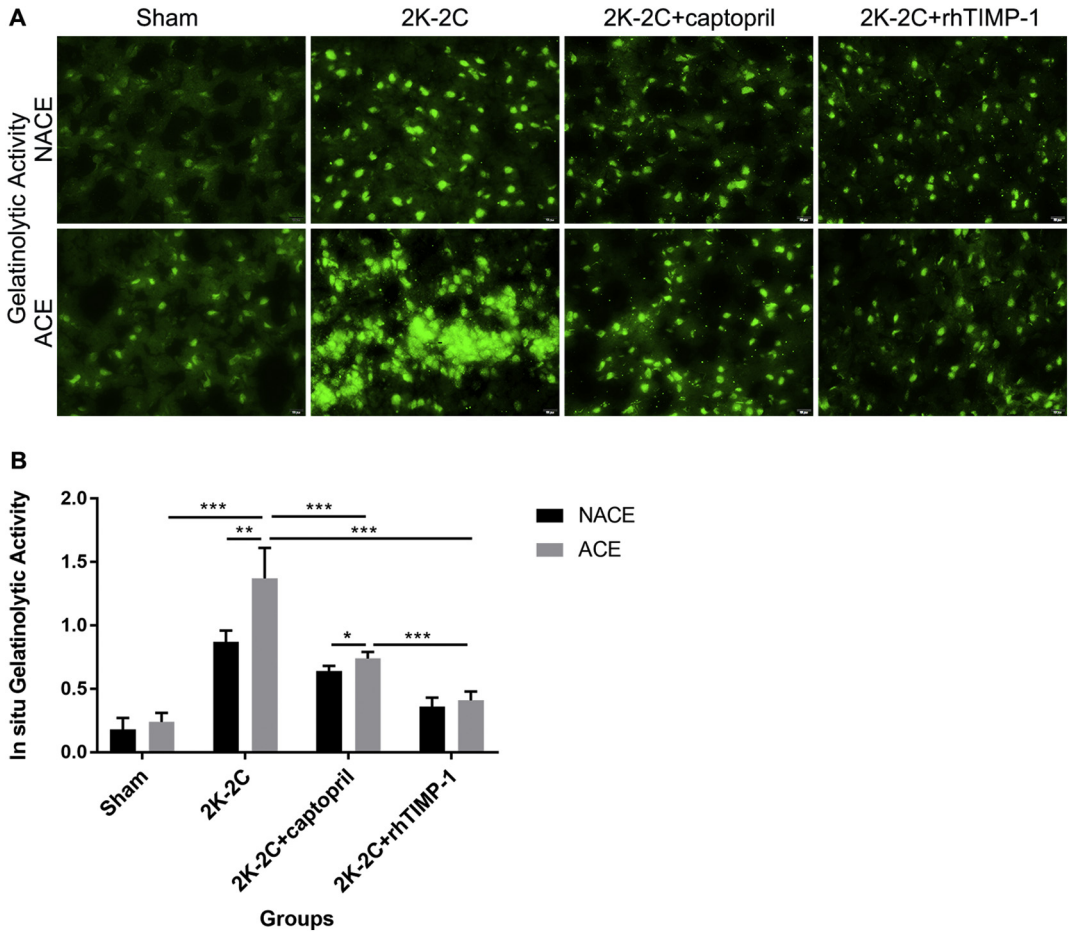

Fig. 5. Gelatinase activity in frozen cerebral cortex samples was determined via in situ zymography (×400, scale bar: 20 μm). A. Representative photographs of gelatinase activity in the cerebral cortex (bright green color). Bar graphs show quantified values of gelatinolytic activity (B). N = 5 in each group. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$ indicate ACE group versus the respective NACE group, $*P < 0.05$, $**P < 0.01$, $***P < 0.00$ indicate 2K-2C group versus the other three groups, and 2K-2C + captopril group versus 2K-2C + rhTIMP-1 group in ACE groups. MMPs, matrix metalloproteinases; ACE, acute cold exposure; NACE, non-acute cold exposure; rhTIMP-1, recombinant human tissue inhibitor of metalloproteinase-1; 2K-2C, 2Kidney, 2 clip. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Data Availability statement

The SPSS statistics data used to support the findings of this study are available from the corresponding author upon request.

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