Resveratrol attenuates ischemic brain damage in the delayed phase after stroke and induces messenger RNA and protein express for angiogenic factors

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Background: **It has been reported recently that resveratrol preconditioning can protect the brain from ischemia–reperfusion injury. However, it was unclear whether resveratrol administration after stroke was beneficial to the delayed phases after focal cerebral ischemia injury. This study investigated the effects and possible protective mechanism of resveratrol on the delayed phase after focal cerebral ischemia injury in mice.**

Methods: **Mice were randomly assigned to five groups according to the time of administration of resveratrol. Control group mice received a corresponding volume of saline solution (0.9% NaCl) containing 20% hydroxypropyl h-cyclodextrin by gavage and were exposed to middle cerebral artery (MCA) occlusion and reperfusion injury. The treatment groups received resveratrol (50 mg/kg/d, gavage) until day 7. Ischemia group mice received their first dose 5 minutes before MCA ischemia, reperfusion group mice received their first dose 5 minutes before MCA reperfusion, first-day, group mice received their first dose 24 hours after MCA reperfusion, and third-day group mice received their first dose at 72 hours after MCA reperfusion. Brain injury was evaluated by triphenyltetrazolium chloride staining and neurologic examination 7 days after reperfusion. The microvascular cell number was examined with immunohistochemistry staining. Effect of resveratrol on matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) gene expression was investigated with reverse transcriptase-polymerase chain reaction and Western blot.**

Results: **The mean neurologic scores and infarct volumes of the ischemia and reperfusion groups were lower than that of the control group at 7 days after MCA reperfusion ($P < .05$). Immunohistochemistry staining showed significantly less reduction in the number of microvessels in the cortical area of mice of the ischemia and reperfusion groups compared with controls. The ischemic hemispheres of the ischemia and reperfusion groups showed significantly ($P < .05$) elevated levels of protein of MMP-2 and VEGF.**

Conclusions: **Resveratrol administration by gavage provided an important neuroprotective effect on focal cerebral ischemic injury in the delayed phase. The elevated MMP-2 and VEGF levels might be important in the neuroprotective effect of resveratrol administration by inducing angiogenesis. (J Vasc Surg 2008;48:709-14.)**

Clinical Relevance: **Strokes can induce infarction size or neurologic disability and cause brain injury in millions of people world wide each year. However, there is no approved therapy currently, and so it is necessary to develop new treatments in the field of primary and secondary stroke to improve the prognosis. This study identified the benefits of early administration of resveratrol by gavage in the delayed phases after focal cerebral ischemic injury and further supports the possible use of resveratrol as a therapeutic agent to ameliorate ischemic infarction. Resveratrol may thus be considered as a potential candidate in the armamentarium of drugs for the early treatment in patients who sustain a stroke.**

Stroke causes 5.5 million deaths and the loss of 49 million disability-adjusted life-years worldwide each year.[1,2] In the Western world, ischemic stroke is a leading cause of death and disability. Although the incidence and mortality of stroke have been decreasing regularly over time, development of new treatments during the acute phase in the field of primary and secondary stroke prevention is still necessary to improve prognosis.[3] The focus of current research is, therefore, to find novel drugs to be used in the therapy for ischemic stroke.

Resveratrol (3,4′,5-trihydroxy-trans-stilbene), a natural polyphenolic compound, is found in a few edible materials.[4] In addition to inhibiting carcinogenesis at multiple stages in a wide variety of cancer models,[5,6] decreasing platelet aggregation,[7] enhancing stress resistance, and extending the lifespan of various organisms from yeast[8] to vertebrates,[9] it is also confirmed that resveratrol reduces infarction volume and decreases brain water content at an extremely low dosage in a variety of in vitro and in vivo models for cerebral ischemic stroke.[10-13]

Huang et al[13] first showed that a low dosage of resveratrol was able to effectively reduce the infarct volume in Long-Evans rats subjected to focal cerebral ischemia, with-

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Competition of interest: none.

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out any side effects. Similar results were observed in a study of male Wistar rats[14] that reported that resveratrol preconditioning prevented motor impairment, increased the levels of malondialdehyde, reduced glutathione, and decreased the volume of infarct after middle cerebral artery occlusion at the dose of 20 mg/kg intraperitoneally for 21 days.

In rat hippocampal neurons, resveratrol inhibited voltage-activated K^+ currents, suggesting that it may be useful for treating ischemic brain injury.[15] In addition, resveratrol has been found to ameliorate cell death through phosphoinositide3-kinase pathway in organotypic hippocampal cultures exposed to oxygen-glucose deprivation.[16] Resveratrol also decreased delayed neuronal cell death and glial cell activation in the hippocampus in Mongolian gerbils after stroke induced by the occlusion of common carotid arteries.[17]

Previous studies about the role of resveratrol in ischemic–reperfusion injury have concentrated on resveratrol preconditioning.[10-17] If we can confirm that resveratrol treatment after ischemia was beneficial for brain protection and neurologic function recovery during the delayed phase, perhaps we can find a novel therapy for promoting recovery of ischemic–reperfusion injury.

In our study we examined the effect of resveratrol in the mouse model of focal cerebral ischemia. The neuroprotection was evaluated by infarct volume measurements and neurologic examination. Meanwhile, stroke recovery is closely associated with neuronal plasticity, neurogenesis, and angiogenesis. Matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) are crucial for this process because they can remodel components involved in extracellular matrix and blood vessel formation. So the effects of resveratrol on MMP-2 and VEGF gene expression in the delayed phase after middle cerebral artery (MCA) occlusion in mice were investigated to provide a mechanistic basis for the protective effect of resveratrol on cerebral ischemia–reperfusion.

MATERIAL AND METHODS

Animals. Cerebral focal ischemia surgery was performed in adult male Balb/c mice, weighing 28 to 32 g, obtained from the animal center of the Fourth Military Medical University. Mice were allowed free access to water and chow until surgery. Resveratrol (Sigma, St Louis, Mo) was prepared at a concentration of 6 mg/mL in saline solution (0.9% NaCl) containing 20% hydroxypropyl h-cyclodextrin (American Maize-Products Co, Hammond, Ind). Mice were weighed, and resveratrol was administrated orally (50 mg/kg/d) to intact mice by gavage after surgery. Mice were euthanized with an overdose of pentobarbital 7 days after MCA reperfusion.

Experimental groups. All mice were randomly assigned to five groups according to the time of administration of resveratrol. Control group (CG) mice received a corresponding volume of saline solution (0.9% NaCl) containing 20% hydroxypropyl h-cyclodextrin by gavage and were exposed to MCA occlusion and reperfusion injury. All mice in the treatment groups were treated with resveratrol (50 mg/kg/d, gavage) until day 7. Ischemia group (IG) mice and reperfusion group (RG) mice received their first dose 5 minutes before MCA ischemia and reperfusion, respectively. First-day group (OG) mice received their first dose 24 hours after MCA reperfusion, and third-day group (TG) mice received their first dose 72 hours after MCA reperfusion.

Induction of focal cerebral ischemia. Mice were anesthetized with 2.5% halothane for induction and maintained with 1.0% to 1.5% halothane in 70% nitrous oxide and 30% oxygen with a face mask. Sufficiency of occlusion and reperfusion of the MCA were monitored by laser Doppler flowmetry. MCA occlusion was induced with an 8-0 nylon monofilament coated with a silicone hardener mixture through the internal carotid artery, as described.[18] Anesthesia did not exceed 15 minutes. After 2 hours of ischemia, the animals were reanesthetized for 2 minutes and reperfusion was induced by removing the monofilament. Body temperature was maintained at 37°C until recovery from anesthesia.

Neurologic examination. Neurologic examination was performed before euthanasia. A neurologic grading system with a 5-point scale (0 to 4), described by Menzies et al,[19] was used: 0, no apparent deficits; 1, left forelimb flexion; 2, decreased grip of the left forelimb while the tail is pulled; 3, spontaneous movement in all directions; left circling only if pulled by the tail; 4, spontaneous left circling.

Infarct volume measurement. Seven days after reperfusion, animals (n = 8 for each group) were euthanized with an overdose of pentobarbital anesthesia, and the brains were removed. For 2,3,5-triphenyltetrazolium chloride (TTC)-stained sections, brains were sectioned coronally into four 2-mm slices in a mouse brain matrix (RBM-2000C; Activational Systems, Ann Arbor, Mich). Slices were stained with 2.5% TTC (Sigma) in phoshate-buffered saline (PBS), followed by 10% formalin overnight. The infarct area of each TTC-stained section was measured using an image analysis system on the posterior surface of each section.

Because larger infarcts were associated with significant edema, areas in each section were corrected for edema as follows. The relative size of the cortical infarct was expressed as a percentage: [100% × (contralateral cortex × [total ipsilateral cortex − cortical infarct])/ipsilateral cortex]. The relative size of each striatal infarct was similarly corrected. Corresponding volumes were then calculated for the total set of slices. All measurements were done by an investigator blinded to treatment assignment.

Immunohistochemistry. To examine the microvascular cell number, a rabbit polyclonal anti-CD34 antibody (Boster, China) against human and mouse was used to stain the vascular endothelial cells.

Staining protocol. The pieces were fixed for 6 hours in 10% neutral phosphate-buffered formalin. Tissues were dehydrated using graded alcohols, cleared with xylene, and embedded in paraffin wax. To minimize antigen denaturalization, tissues were not exposed to temperatures >60°C

during processing. For increased tissue section adhesion, 2-μm sections were collected on poly-L coated slides. The tissue sections were deparaffinized in xylene (10 minutes, twice) and rehydrated in a graded ethanol series up to distilled water.

For heat-induced antigen retrieval, the samples were treated for 2 minutes in an autoclave at 121°C containing 10% citrate buffer. After washing twice with PBS (pH 7.4) for 3 minutes, the sections were treated with 0.3% hydrogen peroxide in the methanol for 30 minutes at room temperature to inhibit endogenous peroxidase activity. After washing three times with PBS for 30 minutes, these sections were blocked with 0.1M PBS plus 3% skimmed milk and 3% goat serum for 2 hours at room temperature, and washed twice in distilled water and PBS for 3 minutes. The anti-CD34 antibody diluted in 0.1M PBS (1:200) was applied onto the sections, and these sections were maintained 48 hours at 4°C in a humidified chamber, and afterwards washed twice in PBS for 3 minutes. The tissue sections was covered with a peroxidase-based secondary antibody for 30 minutes and then washed in PBS for 5 minutes.

To confirm the presence of immunocomplexes, 3,3′-diaminobenzidine (DAB) was used as a chromogene. The samples were washed twice in distilled water, contrasted with Mayer's hematoxylin for 1 minute, rinsed gently with tap water, dehydrated in a graded series of ethanol, cleared in xylene, and cover slipped with 1,3, -diethyl-8-phenylxanthine. Digital microscope images were captured by means of an Olympus CH 30 microscope (Olympus, Tokyo, Japan). Negative controls were made by omission of the primary antibody.

Quantification of microvessels. Microscopic analysis was performed by one independent pathologist. The number of CD34-tained microvessels in every section was determined on the ischemic side and the nonischemic side. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells, were considered for microvascular assessment. The number of microvessels detected in the basal ganglia and cortex on the ischemic side were compared with the number of microvessels in the nonischemic hemisphere.

RNA isolation and reverse transcriptase-polymerase chain reaction. On day 7, the level of messenger RNA (mRNA) for MMP-2 and VEGF was examined (n = 5, per group) by using reverse transcriptase-polymerase chain reaction (RT-PCR). Isolation of total RNA was performed using the Trizol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. For this, 5 μg of total RNA was incubated with 100 U of SuperscriptTM II reverse transcriptase (Invitrogen), deoxyribonucleotide triphosphate (175μM), oligo(dT) (200nM), dithiothreitol (1μM), and reaction buffer in a final volume of 20 μL at 37°C for 60 min. After a final denaturation at 94°C for 5 minutes, 1 μL of complimentary DNA was subjected to polymerase chain reaction (PCR).

PCR amplifications used the following program: step 1, 94°C for 30 seconds; step 2, 53°C for 30 seconds; step 3, 72°C for 30 seconds. Thirty cycles were performed for the amplification of MMP-2 and VEGF.

PCR amplifications of β-actin used the following program: step 1, 94°C for 30 seconds; step 2, 56°C for 30 seconds; step 3, 72°C for 30 seconds; 30 cycles for β-actin. The last cycle was ended by 10 minutes of elongation at 72°C.

Sequences for mouse MMP-2, VEGF, and β-actin were obtained from GenBank (Los Alamos, NM). The following sequences of primers were used:

1. MMP-2: 5′GCT GCC ACG AGG AAT AGG 3′, 5′TCA ACG GTC GGG AAT ACA 3′;
2. VEGF: 5′AAG ATG AGG AAG GGT AAG C 3′, 5′GAG ACA ATG GGA TGA AAG G 3′;
3. β-actin: 5′AAC CCT AAG GCC AAC CGT GAA AAG 3′, 5′TCA TGA GGT AGT CTG TCA GGT 3′.

The lengths of the MMP-2, VEGF, and β-actin amplicons were 490 bp, 556 bp and 242 bp, respectively. PCR products were visualized on 1.5% agarose gels stained by ethidium bromide and UV transillumination. Semiquantitative analysis was conducted using a computerized densitometric imager.

Preparation of tissue extracts. On day 7 after reperfusion, four mice per group were euthanized with an overdose of pentobarbital and then transcardially perfused with ice-cold PBS (pH 7.4). The brains were removed quickly and divided into ipsilateral and contralateral hemispheres. Ischemic tissue and matching tissue from the contralateral hemisphere was dissected, frozen immediately in liquid nitrogen, and stored at −80°C. Brain samples were homogenized in lysis buffer (50mM Tris-HCl, pH 7.6; 150mM NaCl; 0.1% polyoxyethylene 23 lauryl ether, 0.1% deoxycholic acid, 10 μg/mL leupetin, 2 μg/mL aprotinin, 1mM phenylmethylsulfonyl fluoride) on ice. After centrifugation, the supernatant was collected, and total protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, Calif).

Western blot analysis. To investigate protein expression patterns in ischemic brains, equal amounts (40 μg) of total protein extracts were prepared. After mixing with 2× sample buffer, each sample was separated by Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a reducing condition for MMP-2, VEGF (anti-mouse MMP-2 and VEGF, Santa Cruz Biotechnology), and β-actin (antimouse β-actin, Sigma). Proteins were transferred after separation onto a polyvinylidene diflouride membrane using a commercial semi-dry blotting apparatus (Bio-Rad). All blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; pH 7.4) containing 0.1% Tween 20 (TBS-T) at 4°C overnight.

Next, the filters were incubated with the primary antibodies and diluted in blocking buffer for 2 hours at room temperature. The respective dilution rates of the primary antibodies were 1:400, 1:400, and 1:5000 for MMP-2, VEGF, and β-actin. The membrane was washed with PBS-T and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for

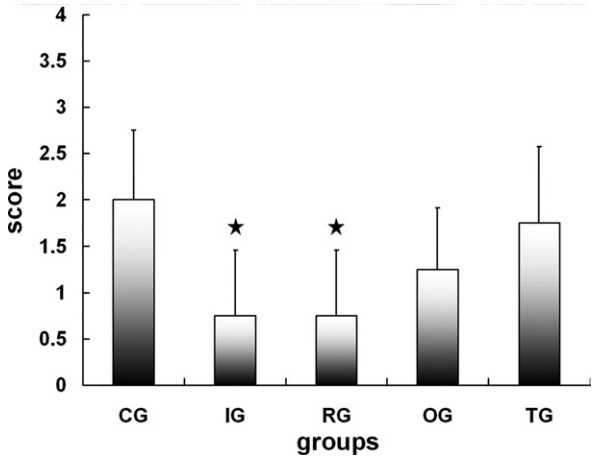

Fig 1. Neurologic scores of mice 7 days after middle cerebral artery reperfusion. *0,* no apparent deficits; *1,* left forelimb flexion; *2,* decreased grip of the left forelimb while the tail is pulled; *3,* spontaneous movement in all directions; left circling only if pulled by the tail; *4,* spontaneous left circling. Scores for mice in the ischemia group *(IG)* and reperfusion group *(RG)* are significantly different from the control group *(CG)* scores (*$P < .05$). Data are given as means ± standard deviation (n = 8, per group). *OG,* First-day group; *TG,* third-day group.

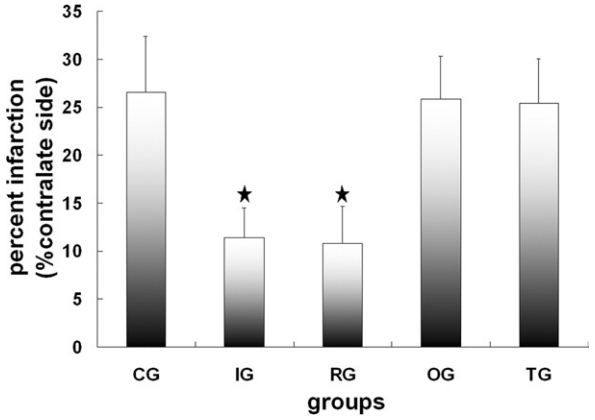

Fig 2. Infarct volumes of mice in 7 days after middle cerebral artery reperfusion. Volumes for mice in the ischemia group *(IG)* and reperfusion group *(RG)* are significantly different (*$P < .05$) from the control group *(CG)*. Data are given as means ± standard deviation (n = 8, per group). *OG,* First-day group; *TG,* third-day group.

1 hour. Finally, antigen was detected by using the standard chemical luminescence method (Santa Cruz Biotechnology, Inc).

Statistical analysis. All values are given as mean ± standard deviation. Differences between groups were compared by using analysis of variance with the Bonferroni correction for multiple comparisons. Values of $P < .05$ were considered to indicate statistical significance.

RESULTS

Neurologic examination. The scores of OG and TG mice were not different from those of CG mice on day 7 after MCA reperfusion ($P > .05$). However, the scores of the IG and RG mice were lower than that of the CG mice ($P < .05$; Fig 1).

TTC staining: infarct size. On day 7 after MCA reperfusion, the infarct sizes of the OG and TG mice were not different from those of the CG mice ($P > .05$). However, the infarct sizes of IG and RG mice were lower than those of CG mice ($P < .05$; Fig 2).

Vascular density. The Table reports the reduction of the number of the microvessels in the basal ganglia region and the cortex for each study group compared with the nonischemic side. In the cortical area of mice, the number of the microvessels in IG and RG mice significantly exceeded the number of the microvessels in CG mice ($P < .05$).

MMP-2 and VEGF mRNA expression. To determine whether indeed resveratrol was involved in the regulation of MMP-2 and VEGF at the mRNA level, we did a semiquantitative RT-PCR. Although there was no statistical difference among the five groups ($P > .05$), we noted a slight increase of MMP-2 and VEGF mRNAs level in IG and RG mice, whereas β-actin mRNA levels remained unchanged (Fig 3).

Table. Reduction of CD34-stained microvessels in the cortex and the basal ganglia on the ischemic side compared with the nonischemic side[a]

Groups (n = 5)	*Cortex, %*	*Basal ganglia, %*
Control group	53 ± 8	61 ± 7
Ischemia group	93 ± 5[b]	63 ± 7
Reperfusion group	82 ± 7[b]	65 ± 8
First-day group	64 ± 7	60 ± 6
Third-day group	54 ± 6	63 ± 7

[a]Data are given as means ± standard deviation.
[b]The ischemia group and reperfusion group are significantly different ($P < .05$) from the control group in the cortex on the ischemic side compared with the nonischemic side.

MMP-2 and VEGF protein expression. To determine the effect of resveratrol on protein expression of MMP-2 and VEGF, tissue extracts were analyzed by Western blot. In Western blot analysis, tissue extracts from IG and RG mice showed more strong immunoreactive bands on Western blots of MMP-2 and VEGF ($P < .05$). Extracts from OG mice did not show the significant up-regulation of MMP-2 and VEGF. However, there was a slight decrease of VEGF protein expression in TG mice, but β-actin protein expression remained unchanged (Fig 4).

DISCUSSION

This current study showed two major findings. First, resveratrol administration after stroke is beneficial to reduce infarct volume and recover neurologic function in the delayed phase after stroke. This notion is partly supported by the recent research that treatment with resveratrol (0.1

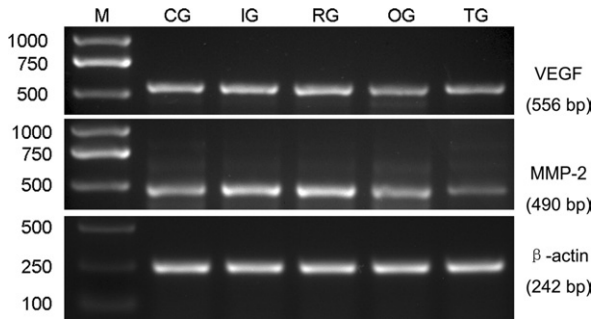

Fig 3. Results of reverse transcriptase-polymerase chain reaction assay for matrix metalloproteinase-2 *(MMP-2)* and vascular endothelial growth factor *(VEGF)* in the delayed phases show a slight increase occurred in MMP-2 and VEGF messenger RNAs level in the ischemia group *(IG)* and reperfusion group *(RG)*. Mouse β-actin was used as the internal standard. The sizes of MMP-2, VEGF, and β-actin were 490 bp, 556 bp, and 242 bp, respectively. DNA markers *(M)* are as indicated on the left (n = 5, per group). *CG,* Control group; *OG,* first-day group; *TG,* third-day group.

and 1 μg/kg) intravenously after 1 hour of MCA occlusion decreased the lactate dehydrogenase in plasma and malondialdehyde in focal cerebral ischemic injury brain tissue, whereas the level of NO in plasma was increased.[20]

Second, the effect of resveratrol is more prominent in the ischemia group and reperfusion group compared with other groups on day 7 after MCA occlusion. This means that the early resveratrol administration during the ischemia–reperfusion injury might be more beneficial to reduce infarct volume and recover neurologic function. In addition, previous studies on the role of resveratrol on ischemia–reperfusion injury concentrated on resveratrol preconditioning.[10-17] This study confirmed that early treatment with resveratrol after ischemia was also beneficial for brain protection and recovery of neurologic function.

However, the mechanism of the protection of resveratrol after stroke remains to be studied. It was confirmed that resveratrol could enhance myocardial angiogenesis both in vivo and in vitro by inducing VEGF, and pretreatment with resveratrol markedly reduced infarct size 24 hours after myocardial infarction and increased capillary density in the peri-infarct myocardium.[21] Fukuda et al[22] demonstrated that resveratrol significantly up-regulated the protein expression profiles of VEGF and increased capillary density and also that pharmacologic preconditioning with resveratrol 3 weeks after myocardial infarction improved left ventricular function.

In addition, resveratrol can markedly raise the proliferation, migration, and adhesion of endothelial progenitor cells in vitro as well as endothelial progenitor cells mobilization in a low dosage.[23] These effects appear to be related with angiogenesis. Our study also identified that angiogenesis induced by resveratrol might play an important role in the delayed phase after stroke, and resveratrol significantly up-regulated the expression of VEGF and MMP-2, which could induce angiogenesis.

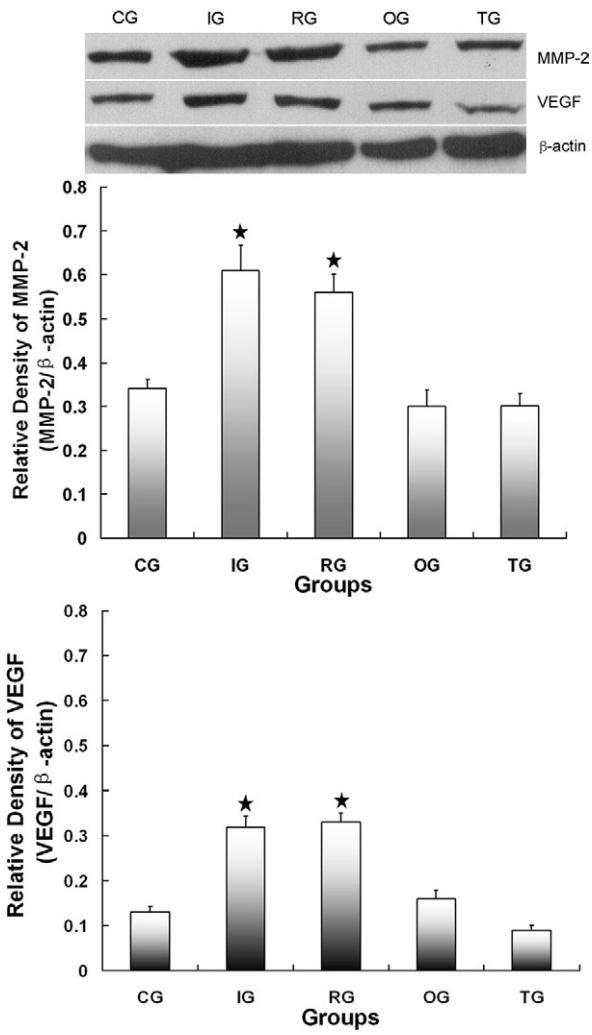

Fig 4. Top, Western blot analysis of stroke-induced levels of matrix metalloproteinase-2 *(MMP-2)* and vascular endothelial growth factor *(VEGF)* in ischemic mouse brain tissue extracts in the delayed phases. Data are representative examples. Results showed that **(Middle)** MMP-2 and **(Bottom)** VEGF proteins in the ischemia group *(IG)* and reperfusion group *(RG)* were expressed at a high level. Extracts from the first-day group *(OG)* and third-day group *(TG)* did not show MMP-2 and VEGF significant up-regulation. Mouse β-actin was used as the internal standard. Data are given as means ± standard deviation (n = 4, per group; $*P < 0.05$).

Angiogenesis is a very important factor in stroke recovery. MMP-2 and VEGF may be crucial for this process because they can remodel all components of extracellular matrix and blood vessel formation. Our study is consistent with these general principles. Given all these considerations, we suggest that angiogenesis induced by resveratrol might play important role in the delayed phase after stroke. Together with the free radical scavenging, antiplatelet aggregation, and anti-inflammatory effects of resveratrol in preconditioning in cerebral ischemia–reperfusion injury,

resveratrol should be a potential target for developing anti-ischemic drugs.

CONCLUSION

Our finding confirmed that resveratrol administration after stroke is beneficial to reduce infarct volume and in the recovery of neurologic function in the delayed phase after stroke. Studies are underway to more completely define the role of resveratrol in stroke and to determine how resveratrol may affect this process after stroke.

AUTHOR CONTRIBUTIONS

Conception and design: WD
Analysis and interpretation: DG, WD
Data collection: HZ, NL
Writing the article: WD, FL
Critical revision of the article: XZ, WD
Final approval of the article: WD, DG, HZ, XZ, NL, FL
Statistical analysis: XZ, NL
Obtained funding: XZ
Overall responsibility: XZ, WD
WD and NL contributed equally to the article

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