

Rosiglitazone Protects Blood-Brain Barrier Integrity Following Ischemic Stroke by Reducing MMP-9 Expression Through a Caveolin-1-Dependent Pathway.

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Research

Keywords: Rosiglitazone, Ischemic stroke, Caveolin-1, Blood-brain barrier, MMP-9

Posted Date: June 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34911/v1>

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Abstract

Background: Rosiglitazone (RSG) is a widely used antidiabetic drug which activates peroxisome proliferator-activated receptor- γ . Recent work have shown that RSG can up-regulate caveolin-1 levels and ameliorate both chronic and acute brain injury. However, whether rosiglitazone can ameliorate ischemic injury in the brain via a caveolin-1-dependent pathway remains unknown.

Methods: Adult male sprague-dawley rats were randomly divided into sham operation group, model group, rosiglitazone group and rosiglitazone+daidzein group. The rat models of middle cerebral artery occlusion (MCAO) was established using the suture method, with ischemia for 2 hours and reperfusion for 22 hours. Neurological deficits were evaluated by the methods of Longa's standard scoring. Cerebral infarction volume was observed by staining with 2, 3, 5-triphenyltetrazolium chloride. Evans blue content reflects BBB permeability. The expressions of caveolin-1, matrix metalloproteinase-9(MMP-9) and occludin were detected by immunofluorescent staining and western blot.

Results: In this study, we found that the expression of caveolin-1 was increased in a rat model of stroke, and treatment with RSG significantly increased the levels of caveolin-1, reduced the release of MMP-9, and increased the expression of occludin. On the other hand, a caveolin-1 inhibitor daidzein canceled the protective roles of RSG in the MCAO model.

Conclusions: These data unveil that RSG might protect blood-brain barrier integrity by down-regulating the levels of MMP-9 via a caveolin-1-dependent pathway.

Introduction

Acute ischemic stroke (AIS) is a major cause of mortality and morbidity in the adult population worldwide[1]. Intensive basic and clinical studies have revealed multiple risk factors for stroke and clarified many mechanisms underlying acute ischemic injury. However, current treatment for AIS remains largely dependent on recombinant tissue plasminogen activator (rtPA)-mediated thrombolytic intervention or mechanical thrombectomy in appropriate patients. During and after AIS, blood-brain barrier (BBB) damage facilitates injury progression and increases the risk of haemorrhagic transformation, limiting the use of rtPA[2]. As for mechanical thrombectomy, just only a few percentages of patients meet the eligibility criteria[3]. Therefore, there is a continuous urgent need to explore potential therapeutic targets to improve neurological function following AIS.

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a member of the nuclear receptor superfamily, acting as a ligand-activated transcription factor[4]. Administration of PPAR- γ agonists has been shown to protect against cerebral ischemia through multiple mechanisms, such as by inhibition of post-ischemic oxidative stress[5, 6], apoptosis[5], and ischemia-induced inflammation[6–9]. Recent in vitro and vivo research, Rosiglitazone (RSG), a PPAR γ agonist, was shown to increase caveolin-1 expression and protect against traumatic brain injury[10, 11]. Caveolin-1 is a principal marker of caveolae which are invaginations of the plasma membrane[12]. Caveolin-1 has been shown to protect the integrity of BBB by

decreasing matrix metalloproteases (MMPs), which degrade tight junctions during cerebral ischemia[13]. So, in this study, we investigated whether RSG can protect against AIS by modifying the expression of matrix metalloproteinase-9 (MMP-9) via a caveolin-1-dependent pathway.

In this study, we investigated the role played by RSG in the expression of caveolin-1. We also evaluated the effects of RSG on the levels of MMP-9 and occludin. Expression of caveolin-1 was downregulated by intraperitoneally injected with a caveolin-1 inhibitor daidzein. BBB permeability and the expression of MMP-9 were monitored. The results showed that PPAR γ ligand RSG protected the integrity of BBB by reducing the level of MMP-9 via its ability to up-regulate caveolin-1.

Methods

Ethics

All experiments were performed in accordance with Guidelines for the Care and Use of Laboratory Animals formulated by the National Institutes of Health. The Second Xiangya Hospital Animal Care Committee of Central South University approved this study. Rats were housed in standard cages with ambient temperature of 22 ± 1 °C, humidity of 40–50%, a 12-hour dark/light cycle, and free access to food and tap water.

Animals and Groups

Adult male Sprague-Dawley (SD) rats weighing 220 to 260 g were provided by the HUNAN SJA LABORATORY ANIMAL CO., LTD [license no. SCXK (Xiang) 2019-0004]. 96 SD rats were divided randomly into four groups: Sham group(24 rats), MCAO group (24 rats), RSG group(24 rats), and RSG + daidzein group (24 rats). RSG (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 2 mg/mL. In the RSG group, rats received RSG immediately after MCAO at a dose of 10 mg/kg, q12 h. In the RSG + daidzein group, intraperitoneal administration of Daidzein (Sigma-Aldrich) was given along with RSG immediately after the onset of stroke at a dose of 0.6 mg/kg.

Animal Model of MCAO

Animal model of MCAO was established in SD rats as Longa et al. described[14]. Briefly, after the rats were injected with 10% (w/v) chloral hydrate (300 mg/kg), a silicone-coated nylon monofilament with a heatblunted tip was passed through the bifurcation of the common carotid artery to the internal carotid artery until the tip reached the origin of the right middle cerebral artery. After 120 minutes of occlusion, the monofilament was withdrawn slowly to allow for 22 hours of reperfusion. The body temperature of rats should be maintained at 37 ± 0.5 °C until recovery. Rats in the sham operation group were subject to the same surgery except for the filament insertion.

Neurological deficits score

The neurological defects were assessed by two independent examiners who were blind to each experiment treatment before the killing[14]. 0: no symptoms of neurological deficits; 1: left forepaws cannot be fully extended ; 2: circling or rotating to the contralateral side; 3: falling to the contralateral side; 4: No spontaneous activity with depressed levels of consciousness. Those with a score of 1–3 were included in the experiment, and those who were not satisfied were excluded. Other excluded conditions included subarachnoid hemorrhage, and rats died within 24 hours.

Triphenyltetrazolium chloride staining

To assess the infarction areas, after the rats were fully anesthetized, they were sacrificed by rapid cervical dislocation. Then, the brain tissue were quickly harvested and placed in a refrigerator at -20 °C for 20 minutes. Then we removed the olfactory bulb and cerebellum, and cut the brain tissue into 5 continuous coronal sections with a thickness of 2 mm. The sections were incubated with 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) in the dark at 37 °C for 30 minutes and were flipped every 10 minutes. Then the brain slices were fixed in 4% (w/v) paraformaldehyde solution at 4 °C overnight. Pictures were taken and infarct volume was calculated with Image J software (ImageJ, MD, USA).

Evans Blue extravasation

Evans blue (EB; Sigma) was dissolved in saline and rats were injected with 2% EB (4 ml/kg) via the tail vein. After 2 hours, rats were perfused with 0.9% (w/v) sodium chloride to flush away the intravascular dye from the blood vessels. Then the nonischemic and ischemic brain tissues were quickly harvested, weighed, and homogenized and proteins were precipitated with formamide solution at 60°C for 24 hours. After centrifuging at 5000 g for 5 minutes, the absorbance at 620 nm was measured with a multifunctional microplate reader (Eppendorf, Germany) in the supernatant. The extravasation of EB was evaluated with a standard curve and expressed as microgram per gram of brain tissue.

Immunofluorescence staining

At 24 hours after MCAO, the brains were removed after perfusion with 0.9% sodium chloride and 4% paraformaldehyde. Then the brain tissue were immersed in 4% paraformaldehyde solution for post fixation, dehydrated in gradient sucrose solutions of 15% and 30% at 4 °C, embedded in optimal cutting temperature compound and cut into 20 µm thick serial coronal sections. Sliced tissues were then blocked with buffer containing 1% goat serum, and 0.2% Triton in PBS and incubated with the following primary antibodies: rabbit polyclonal anti-MMP-9 (1: 500, Abcam), rabbit polyclonal anti-caveolin-1 (1: 500, Abcam), and rabbit monoclonal anti-occludin antibody (1:100, Abcam). After overnight incubation at 4 °C, sections were washed four times, secondary fluorescent antibodies were added and incubated for 1 hour

in the dark at room temperature, and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The sections were observed using a fluorescence microscope (Olympus, Japan).

Western Blot

After 22 hours of reperfusion, rats in each group were intraperitoneally injected with 10% chloral hydrate. After deep anesthesia, the ischemic brain tissues were quickly removed and were homogenized with RIPA lysis buffer. The mixtures were incubated on ice for 30 minutes and then centrifuged for 15 min at 4 °C. After collecting the supernatant and denaturing it, equal amounts of protein samples were electrophoretically separated in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and then transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were placed in an incubation box containing 5% BSA and blocked for 2 h at room temperature on a shaker. Then the membranes were incubated with primary antibodies against MMP-9 (1:5000, Abcam), caveolin-1 (1:5000, Abcam), and occludin (1:1000, Abcam) at 4 °C overnight. After washing the membranes with tris-buffered saline and 0.1% Tween-20 (TBS-T) for 7 min × 4 times, they were incubated with the corresponding secondary antibodies (1:3000, Santa Cruz) at room temperature for 2 hours. Then membranes were washed four times with TBS-T for 7 minutes each, and were developed with the enhanced chemiluminescence (ECL) (Servicebio, China). The membranes incubated with GADPH primary antibodies (1:4000, Sigma) were regarded as the loading control. The bands were visualized using the Gel image analysis system (Bio-Rad, Italy) and quantified using the Image J software. The final results were standardized and expressed as the ratios of target proteins to GADPH.

Statistical analysis

Data were presented as mean ± SD. Statistical analysis was performed with GraphPad Prism software (version 8.3) and data were analyzed with one-way ANOVA or two-way ANOVA followed by Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results

TTC staining

The cerebral infarct volume in each group was evaluated using TTC staining. No infarction was found in the sham-operated rats. The RSG group ($P < 0.05$) showed a significant reduction in the infarct volumes compared with the MCAO group. Compared with the RSG group, the RSG + daidzein group ($P < 0.05$) showed significant increases (Figure.1).

Neurological deficit score

Neurological defects were evaluated and scored at 24 h following ischemia. Compared with the sham operation group, the MCAO model group had significantly higher neurological deficit scores; Compared with MCAO group, the rats treated with RSG showed significant reduced neurological deficit scores. In comparison to RSG intervention group, rats in RSG + daidzein group showed higher long scores (Table.1).

Table 1. Neurological deficit score of each group (n=6, $\bar{x} \pm S$)

Group	Grade
Sham group	0
MCAO group	2.75 \pm 0.43*
RSG group	1.29 \pm 0.54*#
RSG+daidzein group	2.38 \pm 0.48*#▲
*Compared with the sham group, P < 0.05; # Compared with the MCAO group, P < 0.05; ▲Compared with the RSG group, P < 0.05	

BBB permeability

To assess BBB permeability after ischemia, the content of Evans blue in brain tissue was measured at 24 h after ischemic stroke. In the sham operation group, rat brain tissues were almost free of Evans blue, while the model group showed more Evans blue in the ischemic hemisphere. The extravasation of Evans blue in contralateral hemisphere was significantly lower than that in the ischemic hemisphere of rats with cerebral ischemia. Compared with the MCAO group, RSG-treated group showed significantly reduced Evans blue content. The protective effect of RSG was abolished by downregulation of caveolin-1 (Figure.2).

The expression of caveolin-1, MMP-9 and occludin in each group detected by Western blot

We used western blot to quantify the protein levels of caveolin-1, MMP-9 and occludin. The expression of caveolin-1 and MMP-9 proteins was increased in ischemic brains, while the expression of occludin was down-regulated after cerebral ischemia. RSG intervention significantly reduced the MMP-9 protein levels, but further increased the expression of caveolin-1 and occludin. To confirm that the protective effect of RSG on BBB damage was achieved by upregulating caveolin-1, we administered the caveolin-1 antagonist daidzein (0.6 mg/kg) along with RSG immediately after the onset of stroke through intraperitoneal injection. We found that daidzein treatment together with RSG reversed their expression.

These results suggest that caveolin-1 upregulation by RSG may contribute to amelioration of the BBB disruption in stroke rats. (Figure.3)

The changes of caveolin-1, MMP-9 and occludin in each group detected by immunofluorescence assay

We further performed immunofluorescence to detect the expression of caveolin-1, MMP-9 and occludin in each group. Compared with the sham group, occludin in the MCAO model group changed from a continuous linear structure to a discontinuous or even a spot-like structure, indicating that the tight junctions were damaged after ischemia-reperfusion injury. Compared with the model group, the RSG-treated group had better continuity of occludin. Compared with the sham group, the fluorescence intensity of MMP-9 and caveolin-1 were significantly increased in the model group. After treatment with RSG, the fluorescence intensity of caveolin-1 was further increased, while that of MMP-9 was significantly decreased. Daidzein could cancel the effect of RSG on caveolin-1, MMP-9, and occludin. (Figure.4)

Discussion

This study showed reduced Evans blue extravasation in RSG-treated rats compared with untreated rats suffering from ischemic brain injury, suggesting that prevention of BBB disruption is one of the protective mechanisms of RSG following cerebral ischemia. BBB is composed of endothelial cells and their linking tight junctions, pericytes, astrocytic endfeet, and extracellular matrix. It is crucial in the maintenance of the homeostatic microenvironment for normal neuronal function, pointing to the BBB as an important target for the attenuation of brain damage in stroke. Tight junction proteins have been generally believed to play an important role in regulating BBB permeability and function[15, 16], and the decreased tight junction proteins have correlation with the compromised BBB integrity. Tight junction proteins comprise different proteins such as occludin, claudin-5, and ZO-1. In the present study, we demonstrated that RSG significantly increased the tight junction proteins expression after ischemic stroke, indicating that RSG could effectively protect BBB integrity via promoting tight junction expression. The relationship between MMP-9 and tight junction proteins has been clearly shown. Cerebral ischemic injury can lead to damage of BBB by degrading tight junction proteins through the up-regulation of MMP-9[17]. Reports have shown several agents that prevent the up-regulation of MMP-9 and improve the outcome of cerebral ischemia. In this study, we also showed the decrease in the expression of MMP-9 by RSG, suggesting that RSG can prevent ischemic brain damage by stabilizing the disrupted BBB via decreasing MMP-9 and increasing tight junction proteins. Our data are in agreement with a previous study which showed that RSG decreased the levels of MMP-9 after experimental stroke[5].

Caveolin-1 is essential for the formation of caveolae which generally distributed in endothelial cells, smooth muscle cells, skeletal myoblasts, adipocytes, and fibroblasts[18]. Caveolin-1 modulates multiple cellular functions such as cell proliferation, cellular senescence, lipid metabolism, cholesterol tracking, and signal transduction[19, 20]. In the brain, caveolin-1 is primarily expressed in endothelial cells,

pericytes, and astroglial cells[21, 22], and caveolin-1 can regulate neuronal signaling and promote dendritic growth[23]. Changes in caveolin-1 can cause a series of changes in BBB permeability, neuroinflammation, oxidative stress, apoptosis, neurogenesis and angiogenesis[12]. Previous reports have suggested that caveolin-1 can regulate MMP-9. Compared with wild-type mice, caveolin-1(-/-) mice exhibited higher proteolytic activity and disruption of tight junction proteins, while re-expression of caveolin-1 showed the opposite effect[13]. RSG is a widely used antidiabetic drug which activates the PPAR- γ . RSG treatment can prevent ischemic stroke through a variety of mechanisms, such as inhibiting inflammation, apoptosis and oxidative stress[24–26]. Studies have shown that RSG can increase the expression of caveolin-1 in cancer cells and macrophages[27, 28]. Recently, Zhao et al. provided evidence that RSG can protect brain tissue and BBB by increasing the level of caveolin-1 in diffuse axonal injury[10, 11]. However, whether RSG can play a protective role on BBB in animal models of cerebral ischemia/reperfusion injury via a caveolin-1-dependent pathway remains unknown, so we conducted this experiment.

In this study, we found that the level of caveolin-1 was increased in ischemic brains, and RSG further increased caveolin-1 expression. There have been some studies investigating the expression and potential role of caveolin-1 after cerebral ischemic stroke, but the results are inconsistent. Gu et al showed that caveolin-1 was reduced in cerebral ischemia/reperfusion injury[29], and the decrease in caveolin-1 was associated with increased MMP-9 activity and increased BBB permeability. Their previous studies found that the down-regulation of caveolin-1 may be related to the production of nitric oxide (NO) after ischemia-reperfusion injury[30], and a non-selective nitric oxide synthase inhibitor N (G) -nitro -L-arginine methyl ester (L-NAME) reduced the production of NO, retained the expression of caveolin-1, inhibited the activity of MMPs and reduced the permeability of BBB[29], suggesting that caveolin-1 may play a protective role. In addition, experimental studies have found that caveolin-1 can regulate nitric oxide synthase to inhibit the production of nitric oxide products[31, 32]. However, Zhang et al found that caveolin-1 was increased after cerebral ischemia, and the neuroprotection effect of green tea polyphenols may be related to the down-regulation of caveolin-1[33]. Huang et al also showed that Cerebralcare Granule ameliorated BBB disruption caused by cerebral ischemia, which may be related to the inhibition of caveolin-1[34]. The discrepancy is probably related to different stroke models or detection condition.

To investigate whether the protective effect of RSG during cerebral ischemia was mediated by its caveolin-1 up-regulating effect, we administered both RSG and the caveolin-1 antagonist daidzein in the RSG + daidzein group. We found that the therapeutic effect of RSG on BBB was canceled in the presence of daidzein. Compared with the RSG group, the rats in the RSG + daidzein group showed increased neurological function score, increased cerebral infarction volume, and increased Evans blue content in ischemic hemisphere, indicating that caveolin-1 plays a key role in the RSG-afforded protection in AIS. Moreover, after the administration of daidzein, the level of caveolin-1 decreased, while the level of MMP-9 increased, suggesting that caveolin-1 might play a protective role by inhibiting the expression of MMP-9. The results of our study are similar to that of a recent research conducted by Yang et al[35], they showed that the expression of caveolin-1 was up-regulated in MCAO-injured mice, and pretreatment with cystatin

C could further increased caveolin-1 and decreased the enzymatic activity of MMP-9. The up-regulation of caveolin-1 during ischemia may be a protective compensatory response to prevent brain damage.

Conclusions

In summary, RSG might protect the integrity of BBB in ischemia reperfusion injury by reducing the levels of MMP-9 via its ability to up-regulate caveolin-1.

Abbreviations

rosiglitazone (RSG); middle cerebral artery occlusion (MCAO); matrix metalloproteinase-9(MMP-9); acute ischemic stroke (AIS); recombinant tissue plasminogen activator (rtPA); Evans blue (EB) blood-brain barrier (BBB); peroxisome proliferator-activated receptor- γ (PPAR γ); sprague-dawley (SD); 2,3,5-triphenyltetrazolium chloride (TTC);

Declarations

Funding

This work was supported by the National Natural Science Foundation of China (Grant no.81271298)

Competing interests

The authors declare no conflict of interest.

Ethics approval

The Second Xiangya Hospital Animal Care Committee of Central South University approved this study.

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Authors' contributions

Qianyi Huang participated in the design of the study, implemented animal experiments, drafted and revised the manuscript. Wei Zhong was responsible for animal experiments and statistical processing. Xia Yi provided technical support. Zhen Qin performed the statistical analysis and revised the manuscript. Xiangqi Tang supervised the study and revised the manuscript. All authors read and approved the final manuscript.

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Figures

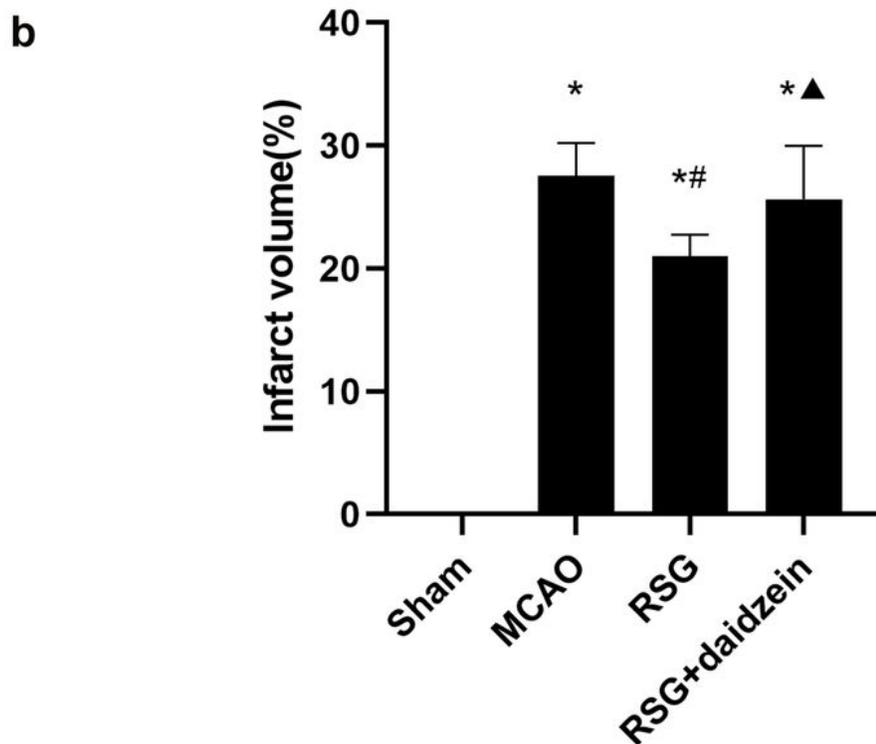
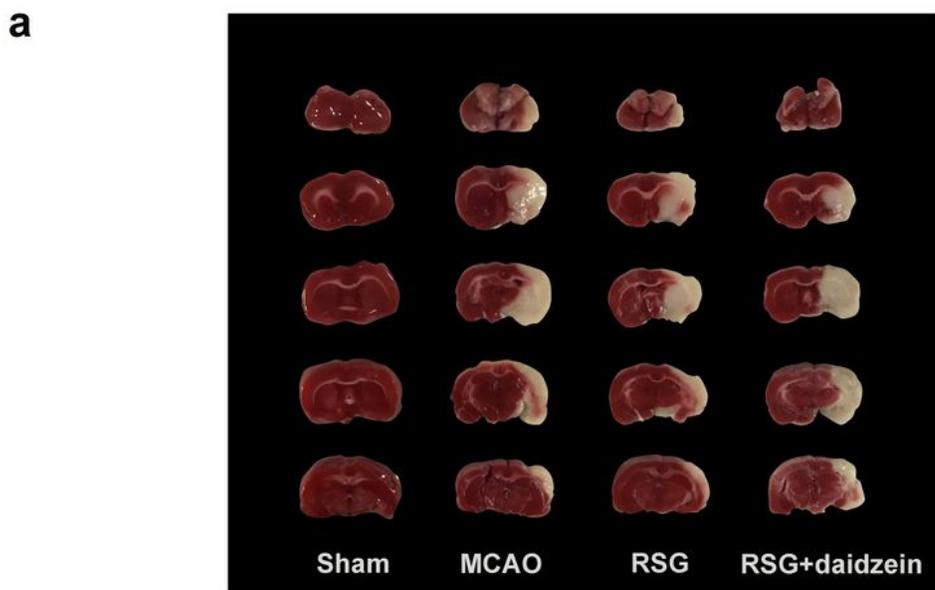


Figure 1

Effects of RSG and RSG + daidzin (caveolin-1 antagonists) on the infarct volume of rats. a TTC staining. b Relative infarct volume. (*P < 0.05, versus the sham group; #P < 0.05, versus the MCAO group; ▲P < 0.05, versus the RSG group;). Data are presented as the mean ± SD.

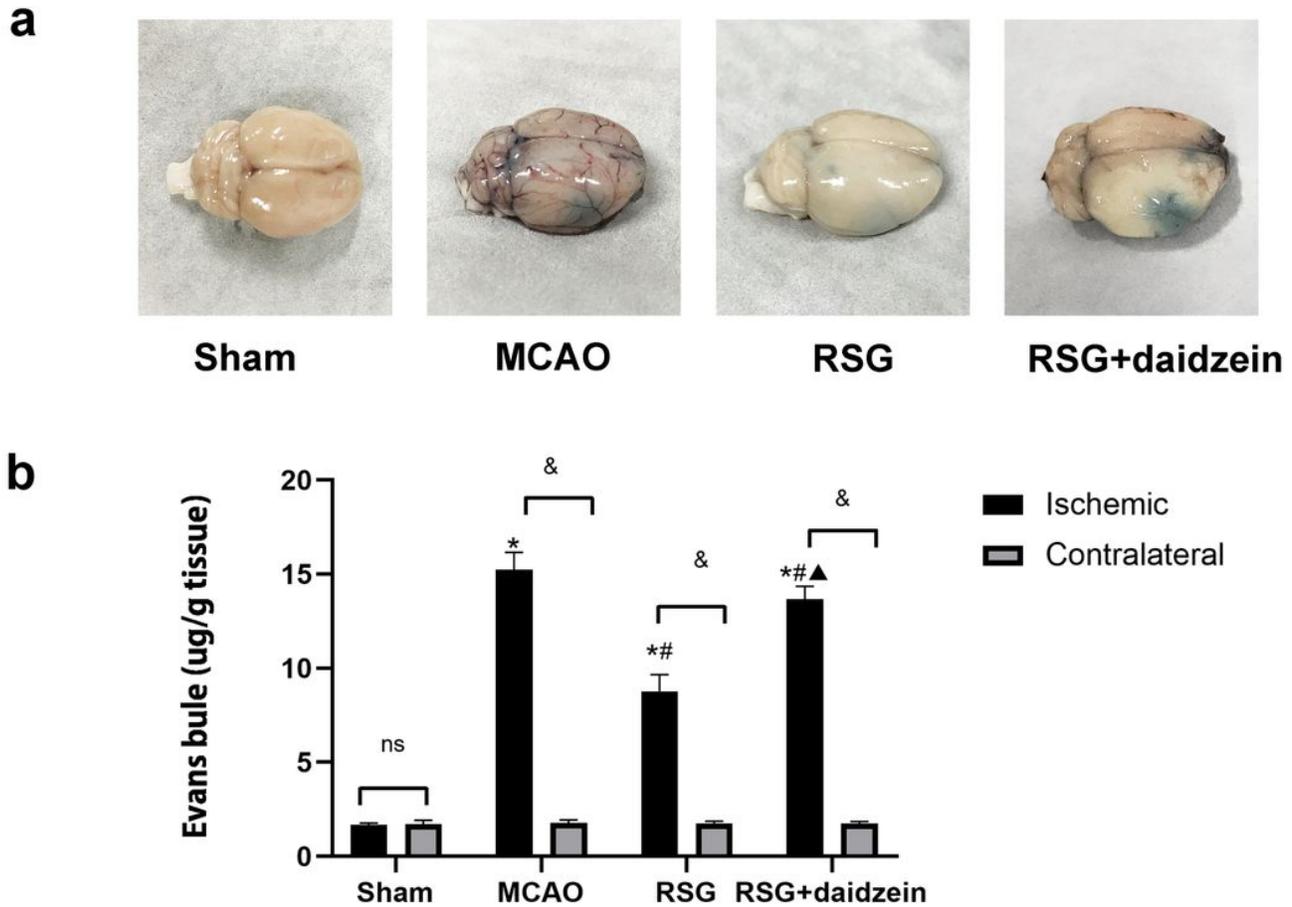


Figure 2

Effects of RSG and RSG + daidzin (caveolin-1 antagonists) on the permeability of the BBB following cerebral ischemia. a Photos representing Evans blue leakage in different groups. b The statistical analysis of the Evans blue diffusion (*P < 0.05, versus the sham group; #P < 0.05, versus the MCAO group; ▲P < 0.05, versus the RSG group; &P < 0.05, versus the contralateral brain). Data are presented as the mean ± SD.

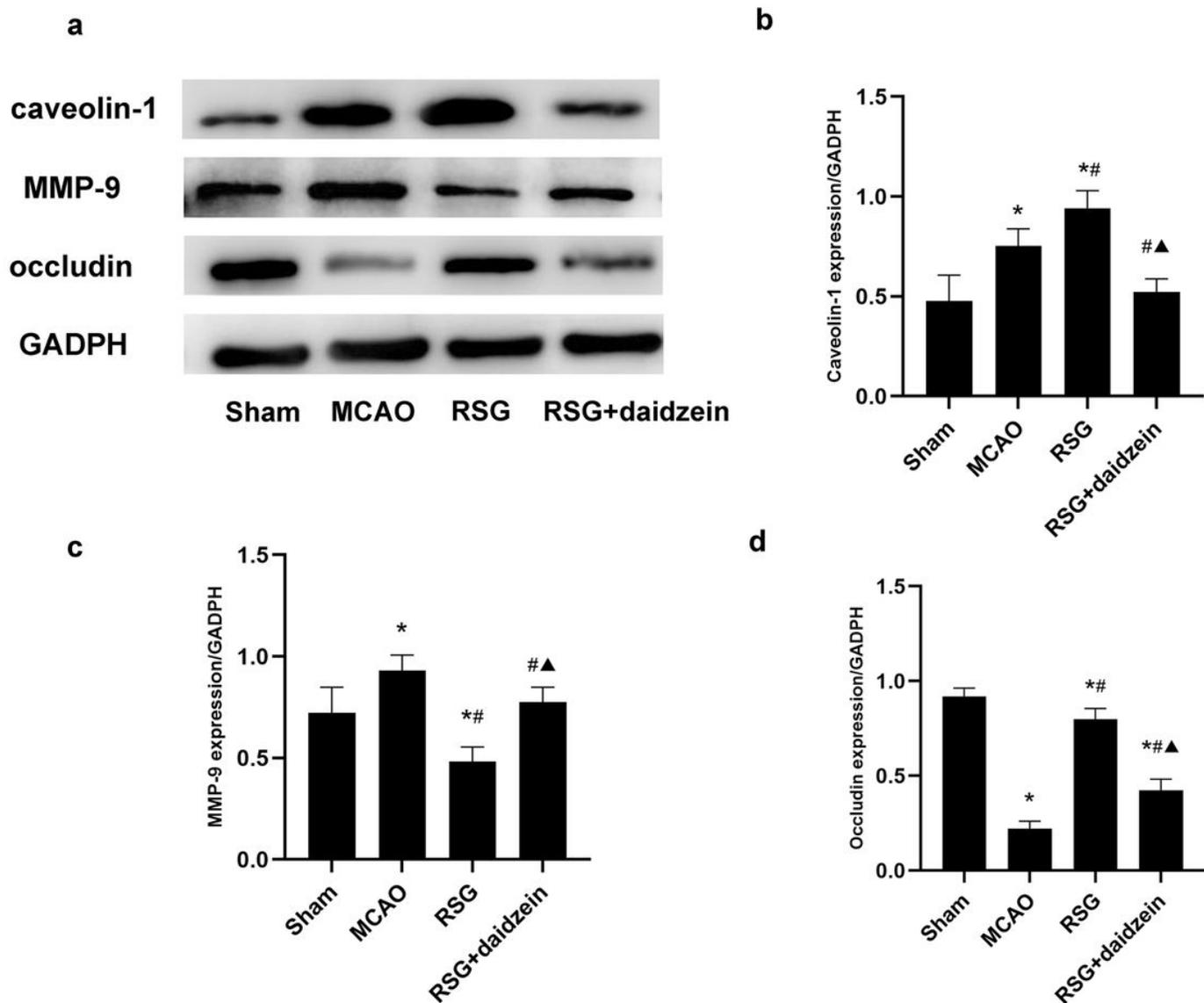


Figure 3

The levels of expression of MMP-9, caveolin-1, and occludin determined by Western blot. GADPH was used as an internal control. The bar graphs show the statistical analysis of the expression levels of MMP-9, caveolin-1, and occludin in the different experimental groups. All data are presented as the mean \pm SD (n = 6; *P < 0.05, versus the sham group; #P < 0.05, versus the MCAO group; ▲P < 0.05, versus the RSG group).

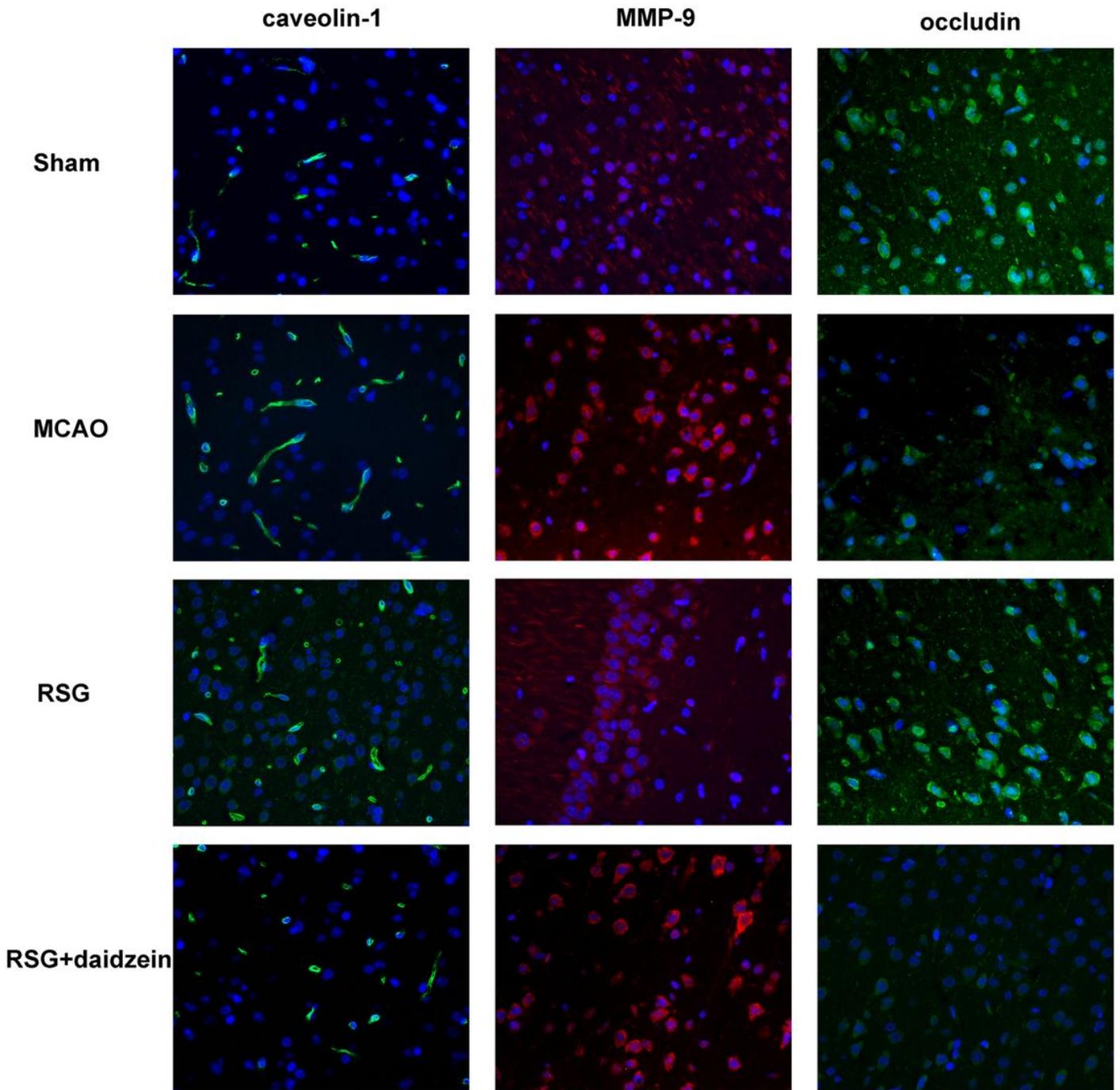


Figure 4

The expression levels of MMP-9, caveolin-1, and occludin assessed by immunofluorescence staining ($\times 400$ magnification, $n = 6$).