

A new nitronyl nitroxide radical with salicylic acid framework attenuates blood–brain barrier disruption and oxidative stress in a rat model of middle cerebral artery occlusion

Lei Chen^{a,b}, Shanbo Ma^b, Min Shi^a, Qiaofeng Wang^a and Yi Miao^c

Objectives A new nitronyl nitroxide radical with a salicylic acid framework (SANR) has been demonstrated to exert antioxidant effects in the previous study by our team. The current study has assessed the protective effect of SANR on cerebral ischemia and reperfusion (I/R) in rat models.

Methods Sprague–Dawley rats were randomly divided into four groups: sham, I/R, 10, and 20 mg/kg SANR + I/R groups. A total of 120 min of middle cerebral artery occlusion (MCAO) caused cerebral ischemia. Survival rates were calculated, and neurological deficits were evaluated by a blinded experimenter. Cerebral infarct area, apoptosis cells, and blood–brain barrier (BBB) leakage were measured by 2,3,5-triphenyltetrazolium chloride staining, terminal deoxynucleotidyl transferase dUTP nick-end labeling, and Evans blue assay, respectively. Reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and 8-hydroxy-2-deoxyguanosine (8-OHdG) also were detected to assess oxidation damage caused by cerebral I/R.

Results Treatment with SANR significantly promoted survival of rats with cerebral I/R injury. SANR meliorated

neurologic deficit and infarct area, improved BBB permeability, and reduced neuronal apoptosis. SANR also reduced ROS levels and the content of MDA and increased SOD and GSH-Px activity in a dose-dependent manner. Furthermore, SANR could inhibit the expression of 8-OHdG.

Conclusion Our results suggested that SANR has a neuroprotective effect against cerebral I/R injury, and its effect mechanism is related to the antioxidant function. *NeuroReport* 33: 129–136 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

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^aSchool of Medicine, Xi'an Peihua University, ^bDepartment of Pharmacy, Xijing Hospital, Fourth Military Medical University and ^cDepartment of Anesthesia and Perioperative Medicine, Affiliated Children Hospital, Xi'an Jiaotong University, Xi'an, China

Correspondence to Yi Miao, MM, Department of Anesthesia and Perioperative Medicine, Affiliated Children Hospital, Xi'an Jiaotong University, Xi'an 710003, China
Tel:+8602987692119; e-mail: miaoyi8@163.com

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Introduction

Cerebral artery embolism or atherosclerotic thrombotic occlusion is described as ischemic stroke, accounting for 80% of all stroke categories [1]. Recombinant tissue plasminogen activator (rt-PA) is the sole drug approved by the FDA for the treatment of acute ischemic stroke. However, because of the narrow treatment window, only 2% of stroke patients received rt-PA treatment, and 50% of stroke patients successfully achieved reperfusion [2]. Moreover, rt-PA treatment can elevate the risk of a brain hemorrhage. Therefore, the clinical application of rt-PA is limited.

Oxidative damage is the essential pathogenesis of neuronal loss and memory impairment after ischemia and reperfusion (I/R) injury [3,4]. The brain is highly susceptible to oxidative damage induced by ischemia due to the

extreme speed of oxidative metabolism and insufficient nerve recovery ability. Once an ischemic stroke occurs, the accumulation of reactive oxygen species (ROS) and reactive nitrogen species exceed the endogenous antioxidant defense mechanism, resulting in dynamic balance changes of energy consumption and mitochondrial function [5]. These so-called ischemic cascade events lead to neuronal death through apoptosis, necrosis, and autophagy [6,7]. In addition, the consequent outbreak of ROS cascades after rapid recovery of cerebral blood flow caused by thrombolysis is also the cause of reperfusion injury. Thus, the development of a protective strategy against oxidative stress may be an effective way to process patients with ischemic stroke [8,9].

In recent years, nitroxyl radicals have been considered a virtual cluster of antioxidant substances protecting against I/R injury and neurodegenerative ailments [10,11]. Unlike natural antioxidants that act in a sacrificial manner, nitroxyl radicals are self-replenishing. In our previous study, a new α -nitronyl (NIT) nitroxide radical with salicylic acid framework (SANR) was shown to

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exert beneficial effects of antioxidants [12]. However, it remains unclear whether SANR protects against ischemia-induced neuronal injury, and further investigation is required. Thus, the present study was designed to assess the protective effects of SANR on cerebral I/R injury through rat models.

Materials and methods

Reagents

SANR (293.3 g/mol, >99% purity; Fig. 1) was synthesized by our laboratory and the detailed synthetic procedures were described in the previous study [12]. ROS-dihydroethidium (DHE) assay kit was purchased from KeyGen Corporation (Nanjing, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Apoptosis Kit was obtained from Servicebio company (Wuhan, China). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) detection kits were provided from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The antibody of 8-hydroxy-2-deoxyguanosine (8-OHdG) was purchased from the Japan Institute for the Control of Aging (JaICA, Shizuoka, Japan).

Experimental protocol

Rats were divided randomly into the following four groups: (a) sham-operated group (Sham), (b) I/R group, (c) I/R + 10 mg/kg SANR group, and (d) I/R + 20 mg/kg SANR group. SANR was diluted in 0.5% dimethyl sulfoxide for injection. Rats were intraperitoneally injected with solvent or SANR at the onset of reperfusion, and then the same doses were administered every 24 h for seven continuous days.

Animals and focal cerebral ischemia

Sprague-Dawley rats (male, 230–250 g) were supplied from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). Rats were fed and maintained in a specific sterile environment and cared for under ARRIVE guidelines. The study protocol was approved and supervised by the Institutional Animal Care and Use Committee. Transient focal cerebral ischemia was caused by intraluminal MCAO surgery following the previous method [13]. Briefly, rats were anesthetized with 3% isoflurane and maintained under anesthesia with 1.5%

isoflurane during surgery. Rats were placed on a hot plate, and the temperature was controlled at 37 ± 1 °C. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed and isolated. A nylon thread was inserted from ECA into ICA to occlude the right middle cerebral artery. After 120 min of ischemia, the nylon thread was carefully pulled out to restore blood circulation and achieve reperfusion. The sham-operated rats underwent the same surgical process except that ICA was not inserted with a nylon thread. A laser doppler flow meter was used to monitor the changes of regional cerebral blood flow (rCBF) before ischemia (baseline) and during maintenance I/R, respectively. Rats were excluded from the study if their rCBF failed to fall below 40% of baseline.

Survival and neurological deficit evaluation

Survival rate was measured within 7 days after MCAO by the ratio of the number of survived rats to the total number of rats. On the 1st, 3rd, 5th, and 7th day after MCAO, neurological deficits were assessed by a blinded experimenter according to the modified neurologic severity score (mNSS) [14]. The scoring system consists of three experiments, including motor test, sensory test, and beam balance test, with 0 indicating no defects and 18 indicating maximum defects.

Infarct area measurement

After the final evaluation of neurological deficit, the rat was sacrificed, and the brain was carefully removed into ice saline to rinse the blood. Brain blocks were cut into 2-mm thick coronal sections using a rodent brain matrix. Brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37 °C for 7 min and fixed with 4% paraformaldehyde. Noninfarcted tissue is stained dark red and infarcts are white. The stained slices were photographed and infarct areas were measured by ImageJ software. To exclude possible confounding effects of brain edema, the corrected infarct area was calculated according to the previously described formula [15].

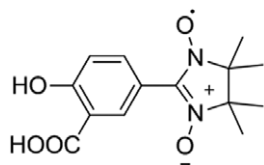
Blood-brain barrier permeability assay

The extravasation of Evans blue (EB) assessed blood-brain barrier (BBB) permeability. Briefly, the EB dye was injected intravenously through a tail vein on the 7th day after MCAO. Cortical tissues of the ischemic hemisphere were weighed and homogenates were centrifuged at 10 000g for 10 min. The supernatant was collected, and EB content was determined by a spectrophotometer at 610 nm.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining for neuronal apoptosis

The rat was anesthetized on the 7th day after MCAO and then perfused with 0.1 M PBS for blood rinsing, followed by 4% paraformaldehyde to preserve the sample.

Fig. 1



The chemical structure of SANR. SANR, nitronyl nitroxide radical with a salicylic acid framework.

Brain blocks (3.3–3.8 mm regions behind the bregma) were embedded in paraffin and subsequently cut into 10 μm coronal sections. Neuronal apoptosis was detected by the double immunofluorescence staining of neuron nucleus (NeuN) and TUNEL. Briefly, the coronal section from each group was incubated with the anti-NeuN antibody at 4 °C overnight, followed by fluorescein isothiocyanate-labeled TUNEL staining using a TUNEL Apoptosis Assay Kit according to the manufacturer's instruction. The number of apoptotic neurons and NeuN staining cells from the peri-infarction cortex was counted by an inverted fluorescence (Olympus, Tokyo, Japan). The experiment in each group was conducted in six replicates. The data were expressed as a percentage of TUNEL-positive neurons vs. NeuN-labeled cells.

Measurement of reactive oxygen species generation

ROS levels were measured with the DHE-ROS assay kit as described by the manufacturer. A fluoro-spectrophotometer obtained signal intensities with excitation and emission at 488 and 590 nm, respectively.

Detection of superoxide dismutase, glutathione peroxidase, and malondialdehyde

Cortical tissues of the ischemic hemisphere were homogenized in cold saline, and the homogenates were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was collected to measure the activity of SOD and GSH-Px as well as the content of MDA. The values were expressed as a percent, with the sham group as control.

Immunohistochemistry analysis for 8-hydroxy-2-deoxyguanosine

Immunohistochemistry was carried out using a kit according to the manufacturer's instructions. Briefly, a 10- μm thick coronal section was deparaffinized in xylene and then rehydrated in gradient concentrations of ethanol. The section was incubated with 3% hydrogen peroxide to block endogenous peroxidase, washed with 0.01 M PBS, and preincubated with 5% BSA at 37 °C for 60 min. Then, the section was incubated with the primary antibody at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 90 min at 25 °C. Next, the section was stained with diaminobenzidine for 3–5 min, counterstained with hematoxylin, and differentiated with 1% hydrochloric acid. An Olympus BX51 microscope system (Olympus, Tokyo, Japan) was used to capture images and count immunoreactive cells in the peri-infarction cortex. Two consecutive coronal slices of each brain block were counted and the average value was calculated. Data were obtained from six rats in each group.

Statistical analysis

Data are expressed as mean \pm SD. Multiple groups were evaluated by one-way analysis of variance with Tukey

tests. The survival rate was calculated using the Kaplan-Meier method. GraphPad Prism 8.0 was used for drawing. $P < 0.05$ showed statistical significance.

Results

Effect of SANR on survival rate

Figure 2 shows the survival rate after MCAO. In the I/R group, only 50% of rats survived compared to the sham group (100%). Treatment with 10 and 20 mg/kg SANR could promote the survival rate (75.0 and 91.7%, respectively).

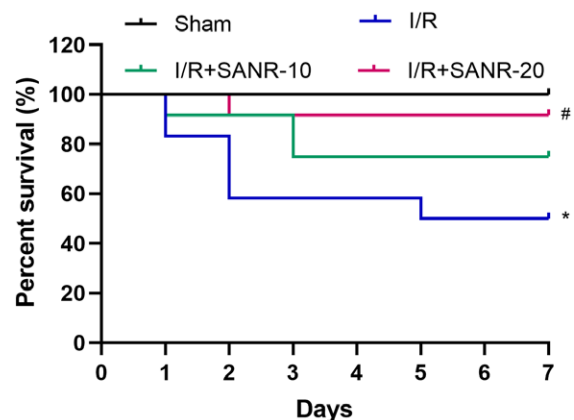
Effect of SANR on neurofunction deficit and infarction area

Neurological deficit evaluation is presented in Fig. 3a. The sham group did not have any neurological deficits, whereas severe neurological deficits on the 3rd, 5th, and 7th day after MCAO were found in the I/R group. Compared to the I/R group, rats that received daily doses of 20 mg/kg SANR markedly alleviated neurofunctional deficits on the 3rd, 5th, and 7th day after MCAO. The 10 mg/kg SANR group ameliorated the neurofunction on the 7th day but had little effect on the neurofunction on the 3rd and 5th day after MCAO. Brain slices were stained by TTC reagent. The sham group showed no infarction. Compared with the sham group, rats in the I/R group had evident cerebral infarction. Treatment with SANR (10 and 20 mg/kg) significantly reduced the infarct area when compared with the I/R group (Fig. 3b and c).

Effect of SANR on cellular apoptosis and blood-brain barrier permeability

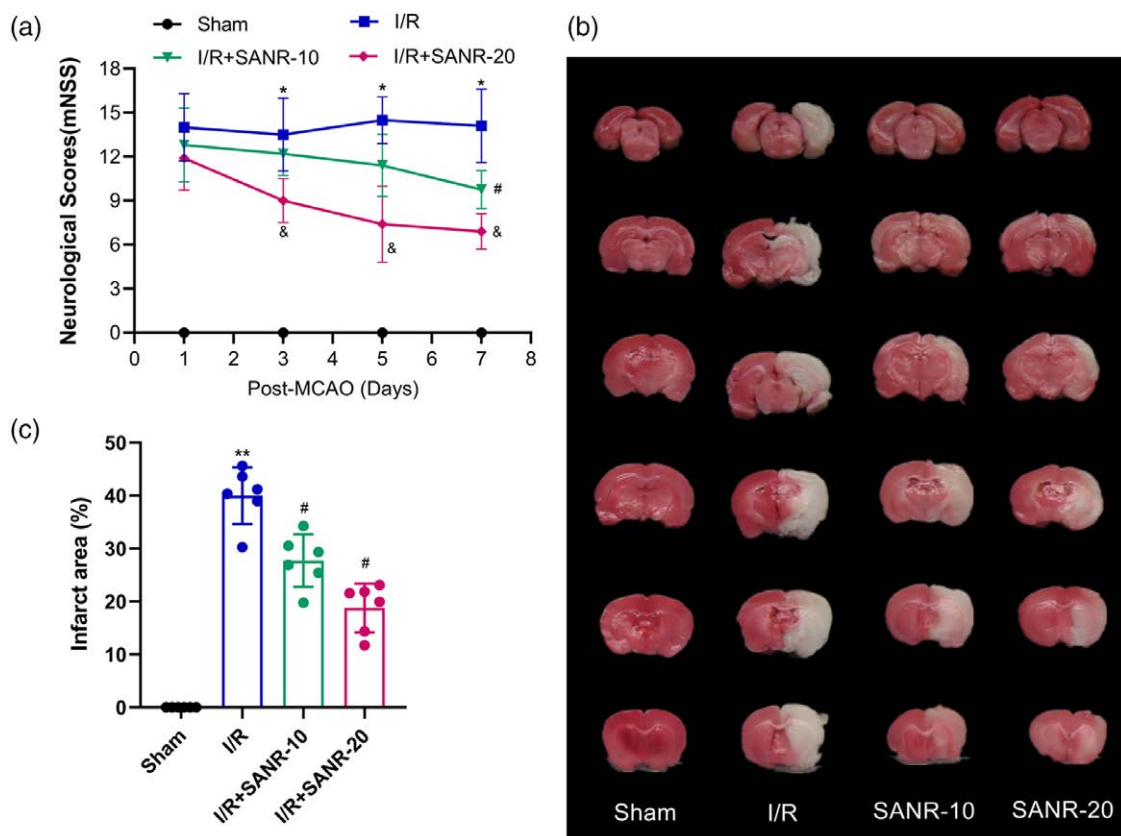
TUNEL staining is presented in Fig. 4a and b; the number of neuronal apoptosis increased obviously in the I/R group compared to the sham group. Compared with the

Fig. 2



Survival rate was evaluated in each group for 7 days after MCAO ($n = 12$). * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the I/R group. MCAO, middle cerebral artery occlusion.

Fig. 3



SANR improved neurologic function and reduced infarct area. Data were expressed as mean \pm SD ($n \geq 5$). (a) The mNSS test was performed on the 1st, 3rd, 5th, and 7th day after MCAO; * $P < 0.05$ vs. the sham group on the 3rd, 5th, and 7th day; # $P < 0.05$ vs. the I/R group on the 7th day after MCAO; & $P < 0.05$ vs. the I/R group on the 3rd, 5th, and 7th day. (b) Representative TTC staining in each group on the 7th day after MCAO. (c) The percentage of the infarct area was measured for each group. ** $P < 0.01$ vs. the sham group; # $P < 0.05$ vs. the I/R group. I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion; mNSS, modified neurologic severity score; SANR, nitronyl nitroxide radical with a salicylic acid framework; TTC, 2,3,5-triphenyltetrazolium chloride.

I/R group, treatment with SANR could dramatically reduce neuronal apoptosis. BBB permeability was measured using EB extravasation on the 7th day after MCAO. Compared with the sham group, EB content was markedly elevated in the I/R group, showing the disruption of BBB. Treatment with 10 and 20 mg/kg SANR showed a reduction in EB content compared with that in the I/R group (Fig. 4c).

Effect of SANR on oxidative stress injury

We evaluated oxidative stress of the peri-infarction cortex by ROS level, the content of MDA, SOD, and GSH-Px activity on the 7th day after MCAO. The results showed that ROS level and MDA content in the I/R group significantly increased compared to the sham group. At the same time, treatment with SANR significantly inhibited the increase of ROS level and MDA content (Fig. 5a and b). On the contrary, the activity of SOD and GSH-Px in the I/R group decreased compared to that in the sham group, and treatment with SANR increased the activity of SOD and GSH-Px (Fig. 4c and d).

Effect of SANR on 8-hydroxy-2-deoxyguanosine expression

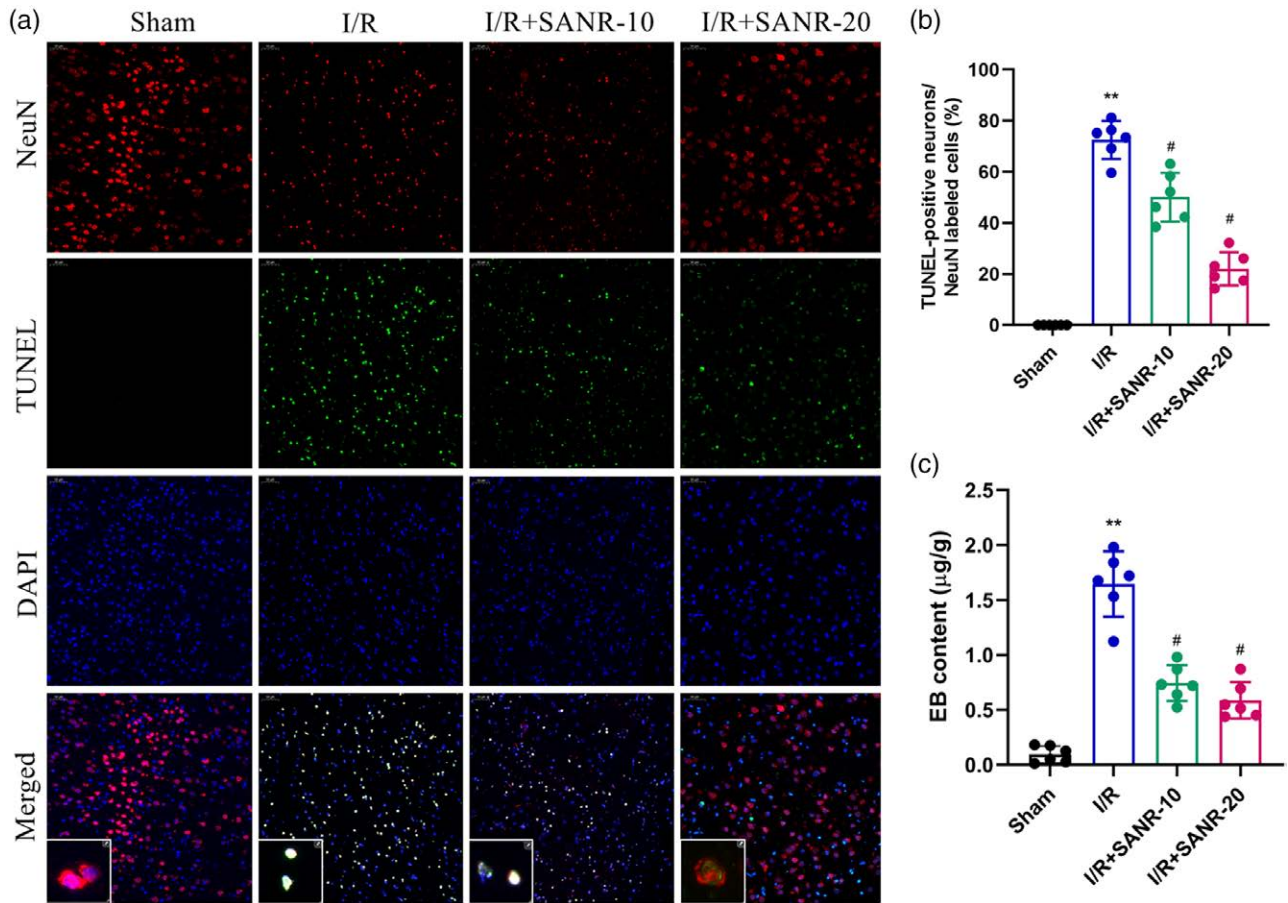
As shown in Fig. 6, a few 8-OHdG-stained cells were observed in the sham group, whereas a significant number of 8-OHdG-stained cells were presented in the I/R group. However, 20 mg/kg SANR could decrease the number of 8-OHdG-stained cells.

Discussion

Ischemic stroke is a leading cause of disability and death in adults [16]. Excessive ROS production is the primary mechanism of cell death after cerebral ischemia [17]. The aim of the current study was to investigate the mechanism of oxidative stress associated with SANR in ischemic stroke. Our study showed that SANR exerts a neuroprotective role in ischemic stroke by reducing oxidative stress injury, strengthening the cellular antioxidant system, and improving BBB disruption.

Free radicals are harmful substances produced in the normal metabolism process of cells, which can damage

Fig. 4



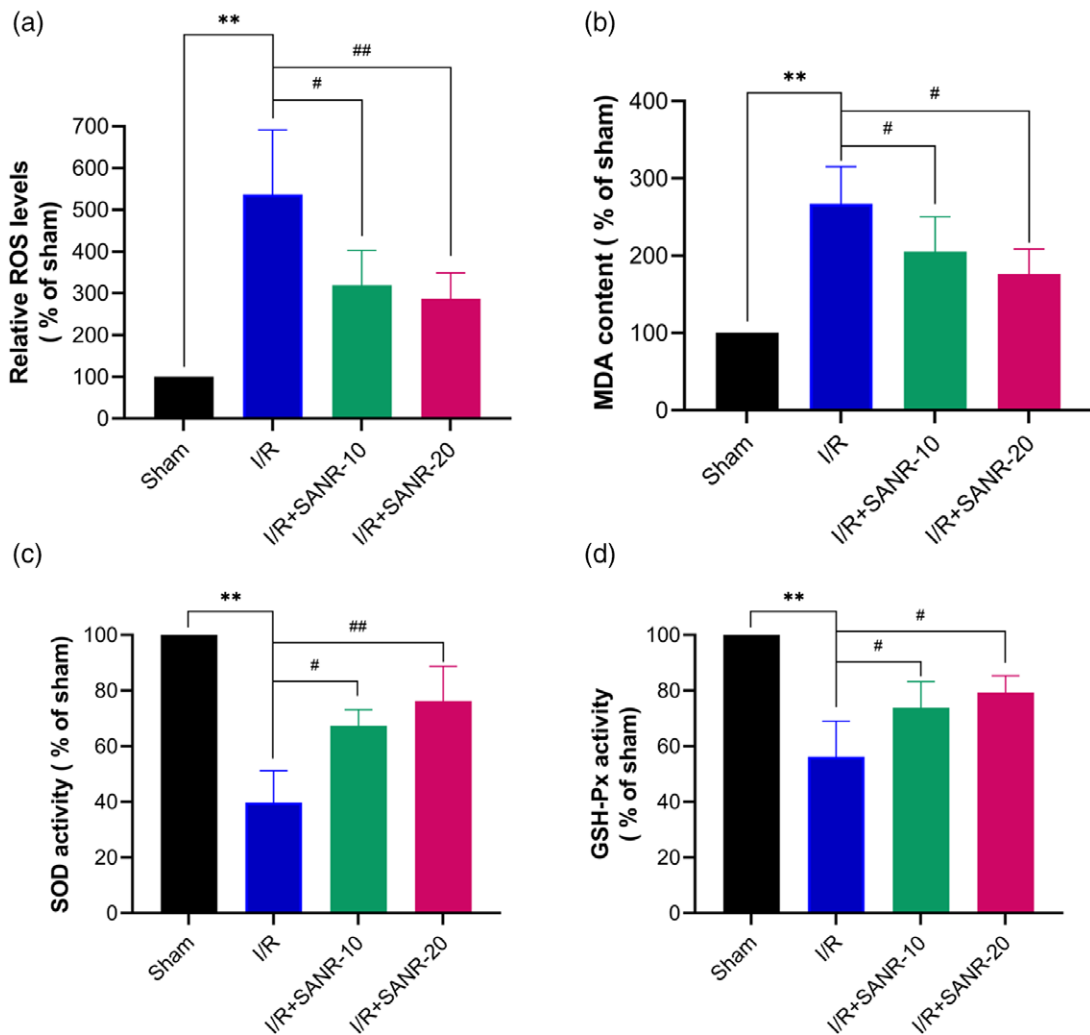
SANR attenuated neuronal apoptosis and improved BBB disruption. (a) The representative figure of TUNEL staining in the peri-infarction cortex. NeuN: red, TUNEL: green, and DAPI: blue. The merged images showed neuronal apoptosis. Scale bar = 50 μm. (b) The percentage of TUNEL-positive neurons vs. NeuN-labeled cells in each group. (c) BBB permeability on the 7th day after MCAO was detected by EB assay. Data were expressed as mean ± SD (n = 6); **P < 0.01 vs. the sham group; #P < 0.05 vs. the I/R group. BBB, blood-brain barrier; DAPI, 4',6-diamidino-2-phenylindole; EB, Evans blue; I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion; NueN, neuron nucleus; SANR, nitronyl nitroxide radical with a salicylic acid framework; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

the body's tissues and cells. The imbalance between the cell's ability to produce free radicals and its ability to resist them is defined as oxidative stress. The brain is the most vulnerable to oxidative stress because of its high rate of oxygen consumption, relatively low antioxidant enzyme levels, and high levels of chemical reactions involving glutamate. After cerebral ischemia injury, numerous ROS are produced, causing significant damage to cells and leading to cell death with lipid peroxidation, protein damage, enzyme inactivation, nucleic acid and DNA damage, cytoskeleton structure destruction, etc [18–20]. Therefore, ROS plays a vital role in ischemic nerve cell death and has become the focus of attention as possible candidates for triggering ischemic cascade and underlying therapeutic targets.

Cellular self-oxidative defense can remove and prevent ROS production. Antioxidant enzymes including SOD, catalase (CAT), GSH-Px, and heme oxygenase (HO-1)

play major roles in cellular defense against ROS. SOD can catalyze the conversion of superoxide anion into H_2O_2 and O_2 and is the primary substance for scavenging free radicals in cells. CAT is an enzyme for scavenging H_2O_2 . GSH-Px can promote the reaction of H_2O_2 and reduced glutathione to generate water and oxidized glutathione. In the animal model [21], overexpression of SOD significantly reduced neuronal apoptosis after cerebral I/R, and GSH-Px decreased infarct area after transient MCAO. On the contrary, decreased SOD activity in animal models intensifies the formation of neuronal damage and brain edema after transient MCAO [22]. ROS can directly induce oxidation of the main and side chains of proteins or indirectly through lipid peroxidation and carbonylation, causing protein main-chain breaking, side-chain β-excision, protein carbonylation, and protein-protein crosslinking, ultimately leading to cell death. ROS damage to biomolecules can produce many specific

Fig. 5



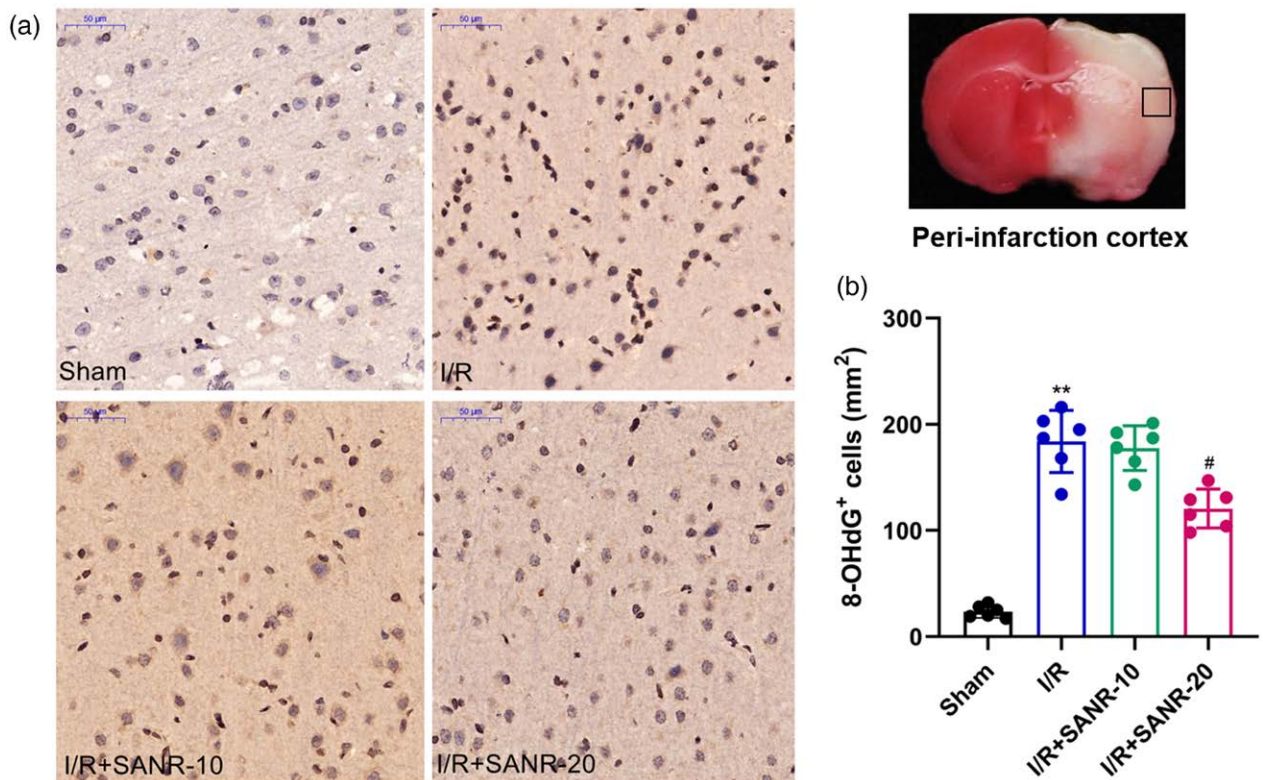
SANR alleviated oxidative damage on the 7th day after MCAO. (a) The level of intracellular ROS was measured indirectly by DCF fluorescence intensity with a fluoro-spectrophotometer. (b–d) The content of MDA and the activity of SOD and GSH-Px were determined by the commercial kits. Data were expressed as mean \pm SD ($n = 6$); ** $P < 0.01$ vs. the sham group; ## $P < 0.01$, # $P < 0.05$ vs. the I/R group. DCF, dichlorofluorescein; GSH-Px, glutathione peroxidase; I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; ROS, reactive oxygen species; SANR, nitronyl nitroxide radical with a salicylic acid framework; SOD, superoxide dismutase.

oxidative metabolites, such as MDA, 4-hydroxynonenal, and 8-OHDG, which are significant indicators of oxidative stress injury [23]. In addition, ROS can also increase the permeability of the BBB, disrupting its normal function. During cerebral ischemia, BBB cells are exposed to the accumulation of free radicals. These free radicals, such as ROS, impair the function of BBB, leading to increased leakage and reduced blood flow. Therefore, reducing the effects of oxidative stress on BBB permeability can improve cerebral I/R injury. Our present study observed the noticeable increase of ROS and MDA and the significant decrease of SOD and GSH-Px in I/R rats. However, treatment with SANR can protect the brain from oxidative stress by scavenging ROS, inhibiting MDA and 8-OHDG, and strengthening the activity of SOD and GSH-Px in

cerebral ischemia. Furthermore, our results also showed that BBB permeability and TUNEL-positive neurons increased in I/R rats, whereas SANR can reduce BBB leakage and TUNEL-positive neurons. All these data support the standpoint that SANR alleviates cerebral I/R injury by decreasing oxidative stress, strengthening the cellular antioxidant system, and improving BBB disruption in the rat MCAO model.

In conclusion, the neuroprotection effect of SANR on I/R-induced oxidative damage was demonstrated in the present study. Furthermore, the effect mechanism of SANR is involved with the antioxidant function. These findings support SANR as a probable candidate for ischemia stroke treatment. Next,

Fig. 6



SANR decreased the number of 8-OHdG-positive cells on the 7th day after MCAO. (a) Representative images of 8-OHdG-positive cells in the peri-infarction cortex. Scale bar = 50 μ M. (b) Quantitative analyses of 8-OHdG-positive cells in different groups. Data were expressed as mean \pm SD ($n = 6$); ** $P < 0.01$ vs. the sham group; # $P < 0.05$ vs. the I/R group. 8-OHdG, 8-hydroxy-2-deoxyguanosine; I/R, ischemia and reperfusion; MCAO, middle cerebral artery occlusion.

antioxidation-related signaling pathways will be investigated to further clarify the neuroprotection of SANR against ischemic stroke.

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L.C. and S.M. designed and performed the biochemical experiments. Y.M. anesthetized and euthanized the animals. M.S. and Q.W. collected and analyzed the data. L.C. wrote the manuscript. Y.M. revised the manuscript. All authors commented on the results and approved the final manuscript.

The datasets used during the present study are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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