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Sestrin2 aggravates oxidative stress of neurons by decreasing the expression of Nrf2

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Abstract. – OBJECTIVE: Oxidative injury is an essential part of the pathological changes of cerebral ischemia-reperfusion. Sestrin2 (Sesn2), a conserved antioxidant protein, is activated under stress to protect cells against oxidative stress. This study mainly explored the function and mechanism of Sesn2 during cerebral ischemia-reperfusion injury in rats.

MATERIALS AND METHODS: Oxygen-glucose deprivation/reoxygenation (OGD/R) model of primary neurons was established. MTS and LDH (lactate dehydrogenase) kit were used to detect cell viability and cell damage by colorimetric method. The superoxide dismutase (SOD) kit and malondialdehyde (MDA) kit were used to access the level of SOD and MDA in neurons. To establish a model of middle cerebral artery occlusion (MCAO) in adult male Sprague-Dawley (SD) rats, the process of cerebral ischemia-reperfusion injury in animals was simulated. Western blot and immunofluorescence were utilized to further examine the relationship between the expression of Sesn2 and Nrf2 (nuclear factor E2 related factor 2).

RESULTS: Sesn2 aggravated neuronal damage and enhanced cell viability in OGD/R model. Intraventricular injection of Sesn2 siRNA lentivirus aggravated nerve function damage in MCAO model, increased cerebral infarction area and water content. Sesn2 overexpression resulted in the increase of total, nuclear levels of Nrf2, as well as the downstream proteins of Nrf2, sulfiredoxin1 (Srx1) and thioredoxin1 (Trx1). On the contrary, after knockdown of Sesn2, we obtained the opposite result. Knockdown of Sesn2 reduced Nrf2, Srx1 and Trx1 levels in rat cerebral cortex in MCAO model.

CONCLUSIONS: Sesn2 promoted the transfer of nuclear Nrf2 to the cytoplasm, it decreased the expressions of Nrf2 and its downstream proteins, Srx1 and Trx1. Meanwhile, it increased the cerebral ischemia-reperfusion injury by changing the distribution of Nrf2.

Key Words:
Sestrin2, Nrf2, Ischemia-reperfusion, Oxygen-glucose deprivation/reoxygenation, Oxidative stress.

Introduction

Stroke is a neurological deficit syndrome caused by acute vascular or blood abnormalities leading to disorders of blood circulation in the brain. It is one of the diseases that severely affects human health and cause deaths worldwide[1-3]. Prevention and treatment of ischemic stroke research have received more and more attention. Cerebral ischemia-reperfusion injury is a very complex pathophysiological process, showing a rapid cascade reaction. The mechanism mainly includes overload of intracellular calcium (Ca), lipid peroxidation, oxygen free radical damage, apoptosis gene activation, excitement (EAA) cytotoxicity, inflammatory cytokine damage, etc.[4-7]. Among them, oxygen free radicals are mainly responsible for cerebral ischemia-reperfusion injury. Therefore, it is urgent to explore the inhibitory method in the production of oxygen free radicals, so as to provide efficient treatment.

Sesn2 (Sestrin2), a highly conserved protein, is the essential member of the Sestrin family. Under stress conditions, such as DNA damage, oxidative stress, hypoxia and other circumstances, Sesn2 is activated[8-10]. Researches[11] have shown that after silencing Sesn2, intracellular reactive oxygen species were significantly upregulated and their sensitivity to ionizing radiation was enhanced. Scholars[12,13] have also shown that Sesn2 had some neuroprotective effects in neurodegenerative diseases; however, the exact mechanism has not been clearly illustrated. So far, cellular function of Sesn2 has been well recognized; however, the effect on cerebrovascular has not been fully elucidated.

As an important endogenous antioxidant factor, NF-E2-related factor 2 (Nrf2) is an essential part of the oxidative stress response[14,15]. In the oxidative stress injury, Nrf2 separated from Keap1 (Kelch-like ECH-associated protein 1) and

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entered into the nucleus[16]. When combined with antioxidant response elements (AREs), the expressions of Phase II detoxification enzymes and antioxidants were regulated by multiple signaling pathways, such as sulfiredoxin1 (Srx1)[17] and thioredoxin1 (Trx1)[18]. Protective effect has been exerted during the pathological process of resisting tumors and oxidation[19]. Studies[20] indicated that Nrf2 was mainly regulated by Keap1. Numerous researches[21,22] have found that Sesn2 could both directly and indirectly participate in the regulation of Nrf2 in the process of metabolism. However, Nrf2 was proved to be involved in many signal pathways, but the specific mechanism and functional effect were not completely understood.

We suggested that Sesn2 was one of the key regulators of Nrf2 during cerebral ischemia-reperfusion and neuronal OGD/R. In this work, we constructed Sesn2 siRNA lentivirus vector and transfected primary cultured cortical neurons of neonatal SD rats, and established OGD/R model. The effect of Sesn2 on neurons was observed. The expressions of Sesn2 and Nrf2 were accessed. The MCAO rat model with interference of Sesn2 siRNA was conducted. The relationship between Sesn2 and neural function, morphological changes, neuronal damage, Nrf2 expression was explored. The mechanism of Sesn2 was explored through *in vivo* and *in vitro* experiments.

Materials and Methods

Animals and Groups

Male SD rats weighing 200-250 g were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were randomly divided into groups according to Sesn2 interference and OGD/R establishment. Control group, vector group (blank plasmid transfected group), Sesn2 overexpression group, curcumin-treated group, vector + OGD/R group, Sesn2 overexpression + OGD/R group, curcumin treatment + OGD/R group. This study was approved by the Animal Ethics Committee of Hangzhou Fuyang Hospital of Traditional Chinese Medicine Animal Center.

Reagents

Sesn2 gene interference and over-expression of lentivirus were constructed by Shanghai GenePharm Biotechnology Co., Ltd. (Shanghai, China). 2,3,5-triphenyltetrazolium chloride (TTC stain) was purchased from Sigma Company (St. Louis, MO, USA). LDH kit, SOD kit (WST-1 method) and MDA kit were purchased from Shanghai GeneChem Biotechnology Co., Ltd. (Shanghai, China). Curcumin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Neuronal Survival Rate and Cell Viability

Cell viability was determined by cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Neurons were cultured in 96-well plates, and 10 μL of CCK8 were added in each well. After incubation for 2 h, the absorbance of each well was measured at 450 nm. The displayed value represented the cell viability. Activity value in the negative control group was set to 100% of survival rate. Ratio of activity value in other groups to the one in the negative control group was the survival. Lactate dehydrogenase (LDH) leakage rate assay was based on the instructions provided by Shanghai GeneChem Biotechnology Co., Ltd. (Shanghai, China). Blank wells, standard wells, determination wells and control wells were prepared according to the requirements. Cells were placed at room temperature for 3 min. Supernatant in each well was extracted, absorbance value of each well at 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated three times.

SOD and MDA Detection

Superoxide dismutase (SOD): SOD was accessed using WST-1 method. Blank wells, standard wells, determination wells and control wells were prepared according to the requirements. Cells were incubated at 37°C for 20 min. Absorbance value of each well at 450 nm was detected by a microplate reader.

Malondialdehyde (MDA): MDA was determined by thiobarbituric acid method. According to the instructions provided by Shanghai GeneChem Biotechnology Co., Ltd. (Shanghai, China), blank wells, standard wells, determination wells and control wells were prepared. Absorbance value of each well at 532 nm was detected by a microplate reader (1 cm of optical path, set zero by distilled water).

Establishment of OGD/R Model

After neurons were cultured *in vitro* for 6 days or lentiviral transfection for 3 days, 0.01 M PBS was used to wash for three times. Sugar-free Dulbecco's Modified Eagle Medium (DMEM) was replaced and placed in a three-gas incubator for 1.5 h. After 1.5 h of OGD, Neurobasal/B27 medium (Gibco, Grand Island, NY, USA) was washed

once, changed to Neurobasal/B27 medium again and placed in a normal incubator for reoxygenation for 24 h.

Establishment of Middle Cerebral Artery Infarction Model

Establishment of the rat model of middle cerebral artery occlusion was made according to the Longa method. The model of middle cerebral artery occlusion (MCAO) in adult male Sprague-Dawley (SD) rats was established to simulate the process of cerebral ischemia-reperfusion injury in animals. Chemical synthases of Sesn2 interference fragments were constructed; 48 h before modeling, lateral ventricle injection was performed.

Assessment of Neurological Deficits

The 5 level standard method of Longa was used to score the nerve function score at 24 h after the operation in rats. 0 points: no neurological deficit symptoms; 1 point: can't fully extend the contralateral forepaw; 2 points: turn to the opposite side when walking; 3 points: tilt to the opposite side when walking; 4 points: can't walk on their own, loss of consciousness. Rats with 0 points and loss of consciousness were removed; higher score indicated more serious neurological deficits.

Measurement of Infarct Size

Six rats in each group were randomly selected, and fresh brain tissue was taken after over anesthesia (the cerebellum and cortical surface coverage in the *pia mater* were removed). The brain tissue was then placed in the brain groove and cut into slices for approximately 2 mm along the coronal position. Slices were placed in 2% TTC solution, incubated in a 37°C water bath for 10 min in the dark, fixed with 4% formaldehyde and taking pictures. Infarction area was white, non-infarction area was red. Finally, Image J software (Rawak Software, Inc., Hamburg, Germany) was used to analyze and calculate the area of cerebral infarction.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysates were used to extract total protein after transfection for 24 to 48 h. 6 rats were randomly selected from each group. After anesthesia, the brains were collected. 100 mg of brain tissue from the cerebral infarction were weighed. The protein was extracted after adding RIPA, phenylmethylsulfonyl fluoride (PMSF, Merck, Millipore, Billerica, MA, USA) and phosphatase inhibitor homogenate in the proportion of 100:1:1. Nucleoprotein extraction was using a nuclear protein kit. The sample volume was determined and the primary antibodies of Sesn2, Srx1, Trx1 and Nrf2 (purchased from CST, 1:1000, Danvers, MA, USA) were added after conventional electrophoresis and incubated overnight at 4°C. The membrane was washed and then labeled with horseradish peroxidase (HRP) (Cell Signaling Technology, Danvers, MA, USA, goat anti-rabbit IgG, 1:5000) for 2 h at room temperature. After washing, the membranes were developed with enhanced chemiluminescence (ECL) imaging (Shanghai Biyuntian Biotechnology Co., Ltd. Shanghai, China). The determination of integral optical density of each band of the system was carried out by using the gel imaging analysis (IOD) with β-actin as a reference value. The relative expression of protein was calculated by the ratio of IOD to IOD in each band.

Statistical Analysis

We used statistical product and service solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) for data analysis, GraphPad Prism5.0 (Version X; La Jolla, CA, USA) for image editing. All data are expressed as mean ± SEM. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ considered statistically significant; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Effect of Sesn2 Interference on Neuronal Activity

Primary neurons of higher purity were cultured in the cerebral cortex of neonatal SD rats. OGD/R model was established to simulate cerebral ischemia-reperfusion *in vitro*. Cultured neurons were infected with Sesn2 interfering lentivirus and Sesn2 over-expressing lentivirus. Compared with the control group, Sesn2 mRNA level was significantly decreased in the Sesn2-interference group and increased in the Sesn2-overexpression group (Figure 1A). It illustrated that the model was established successfully, which provided a stable model and experimental basis for the follow-up study. Compared with the control group, the OGD/R group significantly decreased the survival rate, LDH leakage rate and MDA were significantly increased, whilst SOD decreased significantly

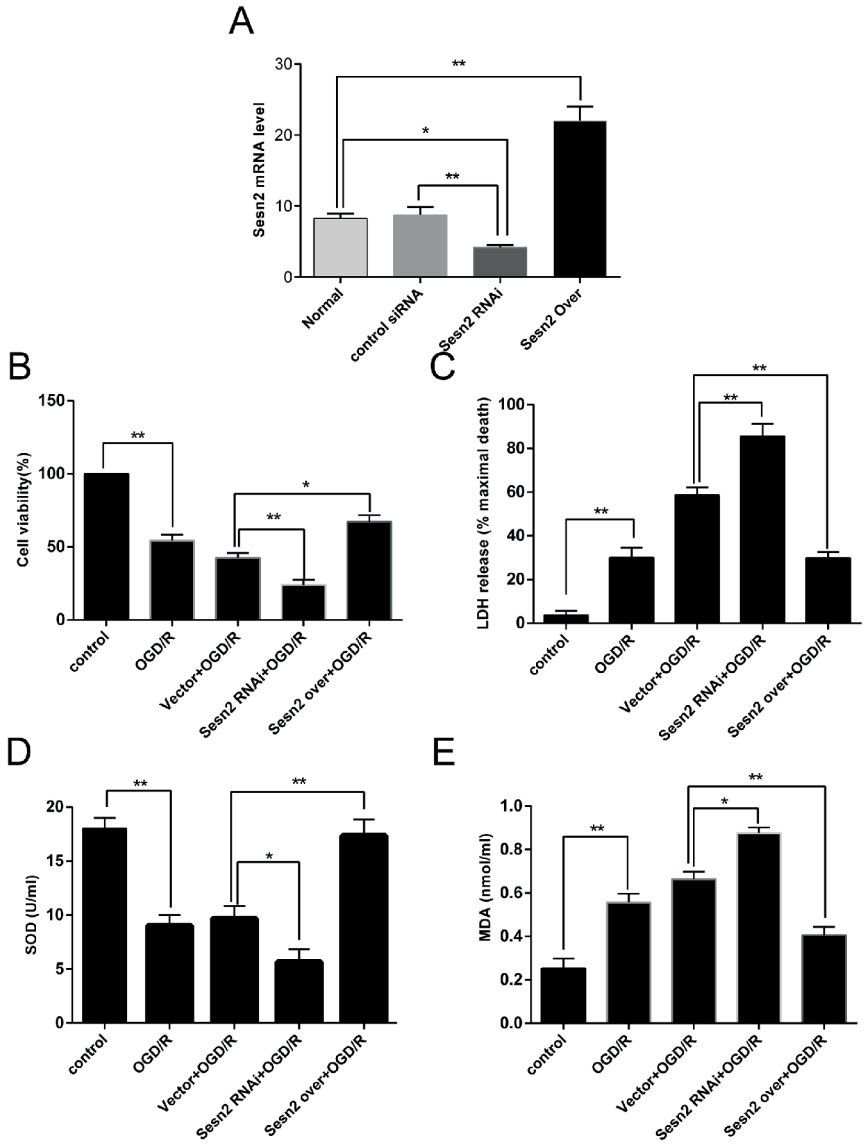

Figure 1. Effects of Sesn2 interference and overexpression on survival rate and activity of OGD/R neurons. ***A,*** Lentiviral interference efficiency of neurons. After Sstn2-siRNA and overexpression of virus were stably transfected into neuronal cells, changes in Sesn2 mRNA level were analyzed using Real-time qPCR (n = 6). *p<0.05, ** p<0.01. ***B,*** CCK8 was used to detect the level of neuronal cell viability. ***C,*** Cell injury was detected by LDH kit (n = 6). *p<0.05, **p<0.01. ***D,*** Effect of Sesn2 interference on neuronal SOD level and neuronal MDA level (n = 6 per group; *p<0.05; **p<0.01).

(Figure 1). These results indicated the successful construction of the cerebral ischemia-reperfusion model in SD rats. Sesn2 interference significantly increased neuronal damage and decreased survival. SOD level was significantly lower, while MDA level was significantly higher. Overexpression of Sesn2 obtained the opposite results. It is suggested that overexpressed Sesn2 significantly reduced oxidative stress in cultured primary neurons, thereby aggravating cerebral injury.

Effect of Sesn2 RNAi on Neuronal Nrf2 Expression

Western blot was used to determine the effect of Sesn2 on the expression of Nrf2 in neurons. There was no significant difference between Nrf2 and nuclear Nrf2 in all groups before establishing OGD/R (Figure 2A and 2B). Compared with vector + OGD/R group and OGD/R group, the total Nrf2 (Figure 2C) and nuclear Nrf2 (Figure 2D) in Sesn2 overexpression + OGD/R group were signi-

ficantly increased (p<0.01). This change was consistent with those in the curcumin + OGD/R group (curcumin + OGD/R group as a positive control) (p<0.01). Compared with vector + OGD/R group and OGD/R group, the total Nrf2 (Figure 2C) and nuclear Nrf2 (Figure 2D) were significantly decreased in Sesn2 RNAi + OGD/R group (p<0.05).

OGD/R model was established after Sesn2 interference or overexpression in neurons, and changes of Srx1 and Trx1 expression in each group were detected (Figure 2E). Compared with OGD/R group and vector + OGD/R group, the expressions of Srx1 and Trx1 in Sesn2 overexpression + OGD/R group (showed as Sesn2 + OGD/R group in the figure) were significantly higher, which were lower in the interference + OGD/R group (p<0.01) (Figure 2E).

Effect of Sesn2 on Neurological Function and Oxidative Stress in MCAO Model

The Longa scoring system was used to evaluate neurological deficits in MCAO rats (6 per group). Compared with sham group, MCAO group showed abnormal neurological function (Figure 3A). The animal model of cerebral ischemia-reperfusion was established. The model of MCAO was successfully established and was suitable for subsequent testing. In contrast to the scramble + MCAO group, neurological deficits and infarct size were significantly increased in the Sesn2 RNAi + MCAO rats (p < 0.01) (Figure 3A. C). There was no statistical difference between the MCAO group and the scramble + MCAO group. In sham group, the brain water content was 76.5 ± 0.68% (Figure 3B). The water content of Sesn2 RNAi + MCAO group was significantly higher than that of MCAO group and scramble + MCAO group. There was no statistical difference between the MCAO group and the scramble + MCAO group (Figure 3B). In the SOD and MDA detections, compared with MCAO group, after Sesn2 interference in MCAO group, the SOD decreased significantly, MDA was significantly increased (Figure 3D and E).

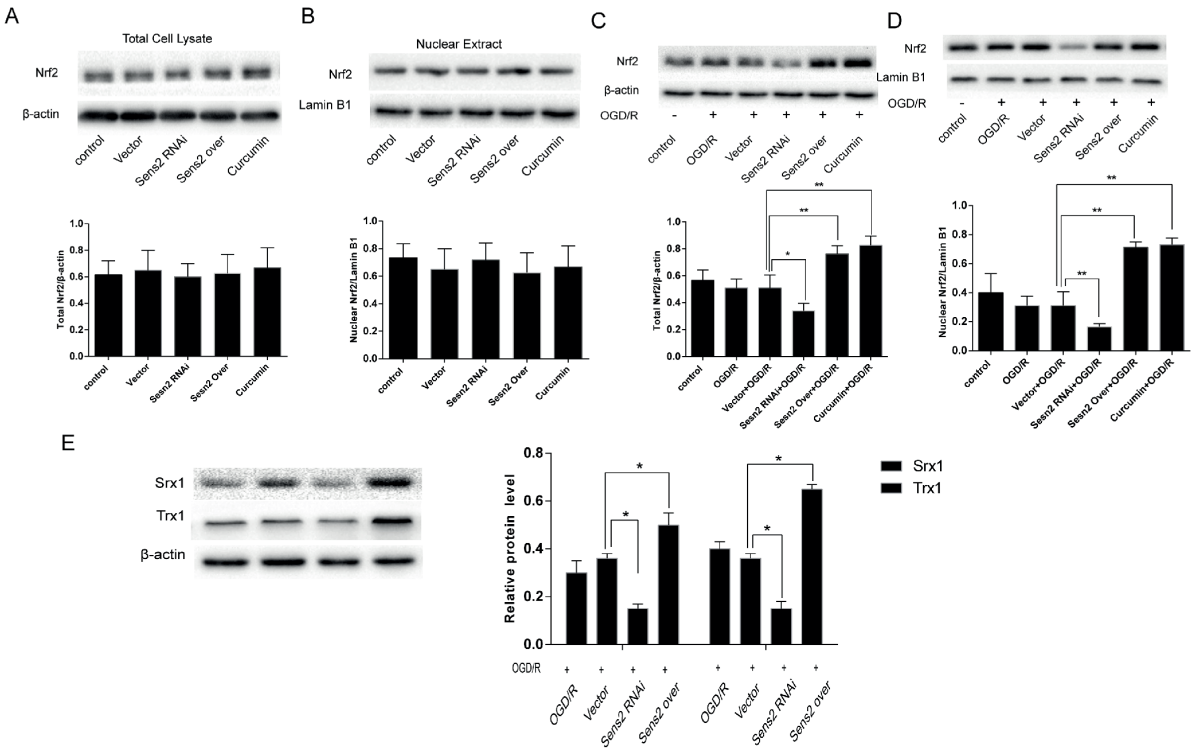

Figure 2. Effect of Sesn2 interference on neuronal Nrf2 expression. Western blot was used to detect the effect of Sesn2 on Nrf2 expression in neurons. ***A-B,*** Before establishing OGD/R, ttotal Nrf2 and nuclear Nrf2 in each group showed no significant difference. ***C-D,*** Compared with vector + OGD/R group and OGD/R group, the total Nrf2 (Figure 2C) and nuclear Nrf2 (Figure 2D) of neurons in Sesn2 over + OGD/R group were significantly increased (p<0.01). The total Nrf2 (Figure 2C) and nuclear Nrf2 (Figure 2D) in the Sesn2 RNAi + OGD/R group were significantly lower (p<0.05). Curcumin + OGD/R group as positive control. ***E,*** OGD/R model was established after Sesn2 interference or Sesn2 overexpression in neurons. Western Blot was used to detect the protein expressions of Srx1 and Trx1 in each group. Bar graphs showed the relative protein levels of Srx1 and Trx1 standardized with β-actin. N = 6 per group; *p<0.05; **p<0.01.

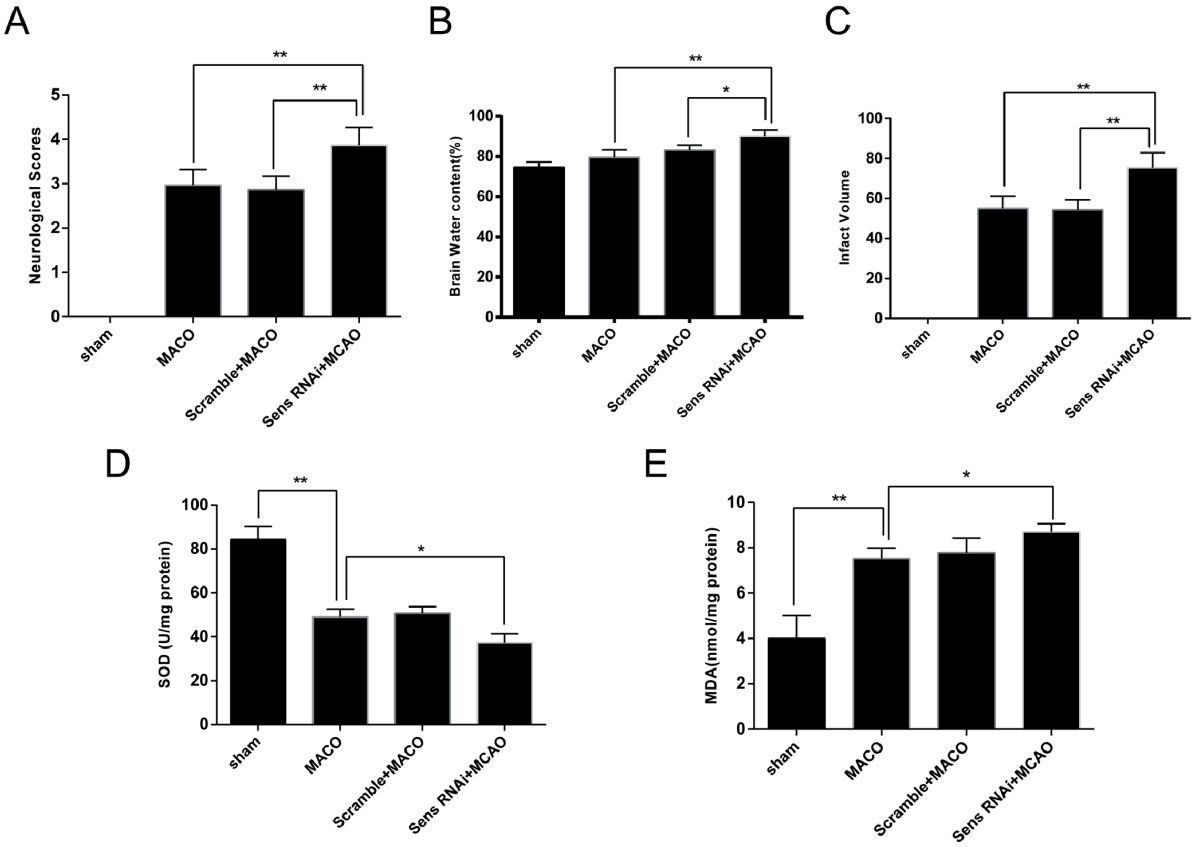

Figure 3. Effect of Sesn2 interference on neurological deficit, infarct size, brain water content and oxidative stress damage in rats. ***A-C*,** Assessment of neurological deficit in MCAO rat model by Longa score system (6 in each group). Compared with scramble + MCAO group, neurological deficits and infarct size were significantly increased in Sesn2 + MCAO rats. ***B*,** Water content in Sesn2 interference + MCAO group was significantly higher than MCAO group and scramble + MCAO group. (**$p<0.01$, n = 6). ***D-E*,** Effect of Sesn2 siRNA transfection on SOD and MDA levels. Compared with Sham group, the activity of SOD in MCAO group decreased significantly ($p<0.05$) and the MDA value increased significantly ($p<0.05$). Compared with MCAO group, SOD activity in Sesn2 + MCAO group was significantly decreased ($p<0.05$) and MDA was significantly increased ($p<0.05$).

Effect of Sesn2 RNAi on the Expressions of Total and Nuclear Nrf2 in Rat Cortical Neurons

We used Western blot to detect the expressions of total Nrf2 and nuclear Nrf2 in cortical neurons. In contrast to the MCAO group and the scramble + MCAO group, the total Nrf2 and nuclear Nrf2 were significantly reduced in the Sesn2 interference + MCAO group (Figure 4A, 4B). There was no significant difference between MCAO group and scramble + MCAO group. Before modeling, there was no significant difference in total Nrf2 and nuclear Nrf2 expression (Figure 4C, 4D). Compared with MCAO group and scramble + MCAO group, the levels of Srx1 and Trx1 in Sesn2RNAi + MCAO group were significantly decreased ($p<0.01$) (Figure 4E).

The above results suggested that the *in vivo* expressions of SOD, MDA, Nrf2, Srx1 and Trx1 were similar to those of *in vitro* results. After Sesn2 interference, SOD decreased significantly, while MDA increased significantly, total Nrf2 and nuclear Nrf2 decreased, Srx1 and Trx1 levels were significantly reduced. Results further confirmed that, Sesn2 interference was involved in the regulation of Nrf2, increased neuronal oxidative stress damage and promoted neuronal death. Sesn2 can effectively inhibit the above process.

Discussion

Sestrin2 belongs to the Sestrins family of proteins, the study found that the gene encoding Sestrins is located on the 6 chromosome q arm 21 sites. Mammalian Sestrins contains three subtypes: Sestrin1, Sestrin2 and Sestrin3[23]. Sestrins has the antioxidant effect, Sestrin2 has the strongest anti-

oxidant capacity, and is studied most extensively. Sestrin2 has been identified as an evolutionarily highly conserved, an important antioxidant protein molecule that had the ability in the regulation of cell proliferation and cell viability[24]. Sestrin2 expression in resting cells is relatively low, but when the environment and metabolism abnormalities occur, such as DNA damage, hypoxia, endoplasmic reticulum stress, lack of energy, starvation and other circumstances, Sestrin2 expression is up-regulated, mainly through the activation of some transcription factors, including P53, FoxO (Foxhead box O3), CCAAT, ATF4 (activating transcription factor 4), AP-1 (activator protein 1), etc.[8,25].

Studies[26] found that when Sestrin2 was seriously damaged in brain tissue, the expression of Sestrin2 can be significantly increased by HIF1α (hypoxia inducible factor α) and exerted a protective role. With the extension of cerebral ischemia in a certain range, the expression of Sestrin2 gradually increased. When the ischemic time was up to 24 h, the level of Sestrin2 reached the highest level, which further promoted the phosphorylation of RpS6, inhibited the mTOR (mammalian target of rapamycin) complex and exerted anti-oxidative stress role[27]. Brain derived neurotrophic factor, BDNF protected cerebral cortical neurons by increasing Sestrin2 expression and activating NO/PKG/NF-κB pathway[28].

Nrf2 is a transcription factor that has been found to be very sensitive to oxidative stress in recent years and greatly involved in the anti-oxidative stress of the body[29]. It forms Keap 1-Nrf2-ARE pathway together with the cytoplasmic protein chaperone Keap 1 and the antioxidant response sequence elements (ARE), which regulated the expression of many antioxidant enzymes in the body. Since Nrf2 played a central role in the body's anti-oxidative stress, its role in organ IRI has been studied more and more in recent years.

Insufficient oxygen and glucose supply in neurons result in stroke, which further leads to

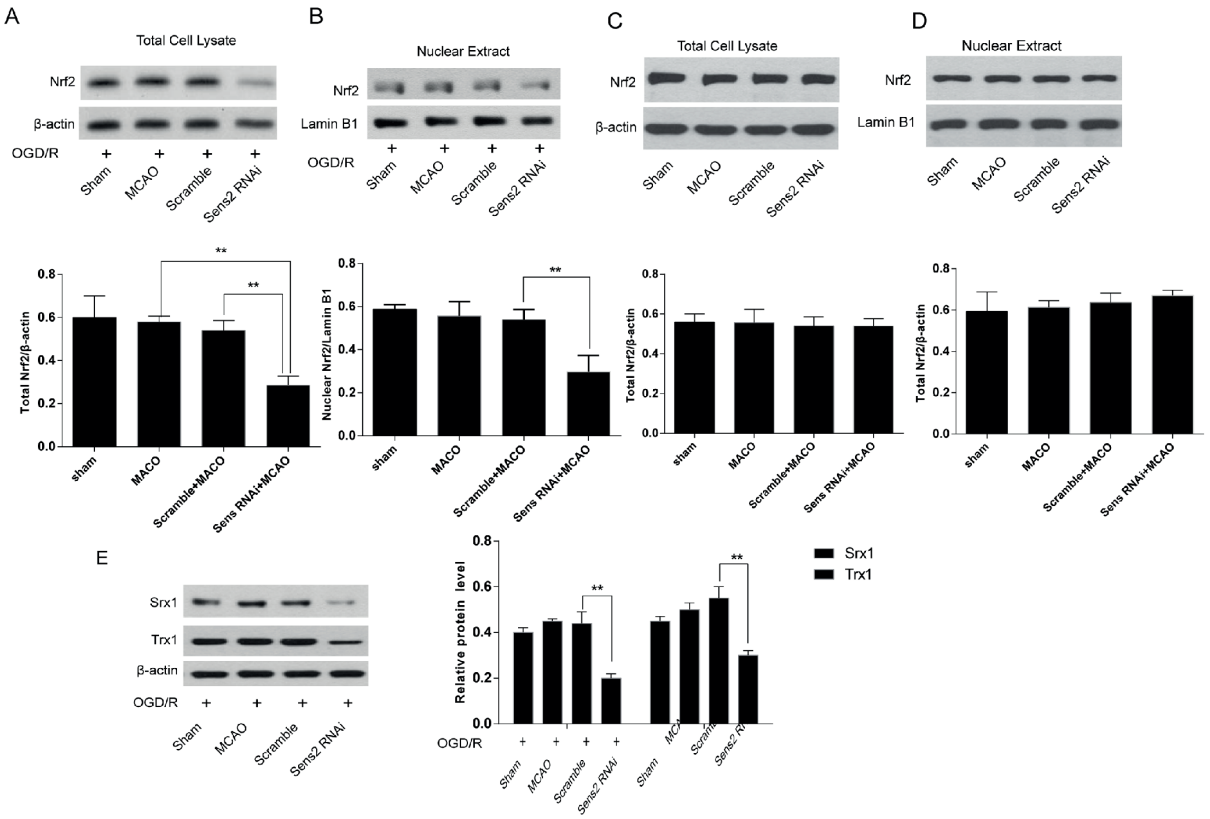

Figure 4. Effect of Sesn2 interference on total Nrf2 and nuclear Nrf2 expression in rat cortical neurons. ***A-B*****,** Effect of Sesn2 on the expression of Nrf2 in the cortex of OGD/R model was detected by Western blot. The expressions of total Nrf2 and nuclear Nrf2 in cortical neurons were examined. ***C-D*****,** Effect of Sesn2 on cerebral cortex expression in Nrf2-untreated OGD/R model was detected by Western blot. The expressions of total Nrf2 and nuclear Nrf2 in cortical neurons were examined. ***E*****,** Effect of Sesn2 interference on the expressions of Srx1 and Trx1 in cerebral cortex was detected by Western blot. N = 6 per group; *$p<$ 0.05; **$p<$ 0.01.

damage of mitochondria-based organelles, and even neuronal death. We simulated the process of ischemia-reperfusion in human brain tissue *in vitro* and selected the OGD/R model of primary neuronal mixed gas culture. Using lentivirus as a carrier, siRNA-mediated interference technology was used to transfect primary cultured neurons in OGD/R model. *In vitro* experiments found that Sesn2 significantly increased neuronal damage after injury, and decreased the survival rate. SOD level was significantly lower, while MDA level was significantly higher. Overexpression of Sesn2 changed in the opposite results. At the same time, we used rat MCAO model to verify the effect of Sesn2 interference on neuronal damage *in vivo*. After Sesn2 interference in MCAO, for rat neurons in ischemic cortex, the antioxidant capacity was significantly reduced, the damage was significantly increased. The above experiments pointed out that in the process of oxidative stress injury, Sesn2 interference damaged the cultured primary neurons. Overexpression of Sesn2 significantly reduced oxidative stress.

We further explored the Sesn2 interference aggravated the damage mechanism of OGD/R neurons. Sesn2 interference can reduce the level of Nrf2 expression. Its specific mechanism was probably involved in the regulation of Nrf2 by promoting the transport of Nrf2 from the nucleus to the cytoplasm or the promotion of Nrf2 decomposition. It aggravated neuronal oxidative stress damage, and promoted neuronal death. Sesn2 overexpression can effectively inhibit the above process. Srx1 and Trx1 were Nrf2 downstream proteins and were also very important anti-oxidants in neurons[30]. Scholars[31,32] have shown that curcumin was a very stable and efficient regulator to increase the expression of Nrf2. In the experiment, we used the curcumin treatment group as a positive control group. In our *in vitro* experiments, we found that overexpression of Sesn2 can enhance the expression of both Nrf2 and Nrf2, which was similar to curcumin treatment, and significantly increased the expression of Srx1 and Trx1. Sesn2 interference may inhibit the transmembrane protein, leading to the nuclear Nrf2 transport to the cytoplasm or nuclear Nrf2 decomposition, the total Nrf2 and nuclear Nrf2 expression levels were reduced. Expressions of Srx1 and Trx1 were also decreased. *In vivo* analysis of Sesn2 overexpression was not performed because of low transfection rates. Western blot indicated that Sesn2 interference can also promote the reduction of total Nrf2 and nuclear Nrf2 expression and significantly reduce the expressions of Srx1 and Trx1. It increased oxidative stress *in vitro* and *in vivo* model of neuronal damage.

Therefore, we deduced that from the Nrf2/ARE signal transduction pathway, Sesn2 interference can change the distribution of Nrf2 in and out of nucleus and promote the transfer of Nrf2 from nucleus to cytoplasm. The above phenomenon was through reducing the levels of Srx1 and Trx1, the downstream of Nrf2, thus aggravating neuronal oxidative stress injury.

We suggested that Sesn2 interference reduced the expression of Nrf2 during cerebral ischemia and reperfusion, worsening oxidative stress in neurons. Our result provided a new direction for the treatment of stroke.

Conclusions

We showed that Sesn2 promoted the transfer of nuclear Nrf2 to the cytoplasm, it decreased the expressions of Nrf2 and its downstream proteins, Srx1 and Trx1. Meanwhile, it increased the cerebral ischemia-reperfusion injury by changing the distribution of Nrf2.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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