Original Paper

Inhibition of the SOCS1-JAK2-STAT3 Signaling Pathway Confers Neuroprotection in Rats with Ischemic Stroke

Xiao-Lei Wang^a Chun-Mei Qiao^b Jiong-Ou Liu^a Chun-Yang

^aDepartment of Ultrasound, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot,

^bDepartment of Neurology, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, P.R. China

Key Words

Ischemic stroke • SOCS1 • JAK2 • STAT3 • Signaling pathway • Neuron

Abstract

Background: The present study aims to investigate the protective effects of the SOCS1-JAK2-STAT3 signaling pathway on neurons in a rat model of ischemic stroke. **Methods:** Our study was conducted using an ischemic stroke rat model. After the microglia were extracted, 40 neonatal Sprague-Dawley (SD) rats were assigned into the blank, AG490, model and negative control (NC) groups. The neurological function of all the rats was evaluated. Histopathological changes were observed. qRT-PCR and Western blotting were applied to measure the expression of genes and proteins in the SOCS1-JAK2-STAT3 signaling pathway and related to apoptosis. The TUNEL assay was employed to calculate the cellular morphology and apoptosis of neuronal cells. Cell viability was detected using the MTT assay. In addition, immunoassays were used to measure the contents of superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) as well as the levels of oxidative stress. **Results:** Compared with the blank group, the AG490 and model groups showed higher neurological function scores—the cytoplasm of the neurons were cavitated, the organelles were reduced with unclear margins, some of the neurons were necrotic, and apoptosis was increased. In addition, the NC and model groups inhibited decreased cell viability, lower mRNA and protein expression of SOCS1, SOCS3 and JAK2, and reduced SOD and GSH levels but higher mRNA and protein expression levels of STAT3, Bax and caspase-3 as well as increased protein expression of P-JAK2, P-STAT3 and activated caspase-3 (c-caspase-3). Moreover, the MDA levels were up-regulated in the NC and model groups. In contrast, opposing trends were found in the AG490 group compared with the NC and model groups. **Conclusion:** These data demonstrate that inhibiting the SOCS1-JAK2-STAT3 signaling pathway can reduce the loss of nerve function and apoptosis of neuronal cells, which provides a new target for the clinical treatment of ischemic stroke.

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Published by S. Karger AG, Basel

Chun-Yang Li

Department of Neurology, The Affiliated Hospital of Inner Mongolia Medical University
No.1, Tongdao North Road, Hohhot, The Inner Mongolia Autonomous Region, (P.R. China)
Tel. +86-0471-3451109, E-Mail chunyang1108@126.com

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Introduction

Stroke is a leading cause of death and the most common cause of permanent disability in the world, and treatment for stroke is compromised by limitation in available preventive or therapeutic methods [1, 2]. Ischemic stroke is characterized by the critical reduction of cerebral blood flow in the entire brain, which could result in selective neuronal injury in the hippocampus [3]. The majority of strokes are ischemic in nature because blocking blood circulation via either thrombosis or embolism causes tissue death in the affected areas [4]. Sources of inflammation, including atherosclerosis, autoimmune diseases, infection and surgery, influence vascular injury and increase the risk of stroke [5]. In addition, severe brain ischemia could lead to an initial inflammatory response in both the central nervous system (CNS) and the peripheral nervous system, and subsequent severe immune responses may induce infections that can be life-threatening to the patients [1]. Moreover, the extent of neuronal death could expand depending on the ischemic duration, which could last for several days after ischemic stroke even with successful reperfusion [2]. It has been observed that the activated Janus kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) pathway is closely related to brain injury and is involved in the regulation of cerebral ischemia/reperfusion injury and brain injury after subarachnoid hemorrhage (SAH) [6].

AG490 is a tyrosine kinase inhibitor that is widely applied to inhibit JAK2 and is regarded as a method of blocking STAT3 activation as well as downstream effectors [7]. As a central component of cardioprotection, STAT3 belongs to the STAT family of signal responsive transcription factors, which comprises seven members encoded by different genes and acts as transcriptional activators for a wide range of targeted genes [8, 9]. In higher eukaryotes, JAK/STAT signaling is one of the pathways that plays an essential role in immune regulation and immune-mediated diseases [10, 11]. The JAK-STAT pathway includes a family of receptor-associated JAKs that can phosphorylate tyrosine residues on STATs, and activation of the JAK-STAT pathway via ischemic preconditioning could result in adaptation to ischemic stress [10]. A previous study illustrated that cytokines activate JAK2 and subsequently phosphorylate STAT3 in cerebral ischemia and other neuroinflammatory diseases [12]. The suppressor of cytokine signaling (SOCS) protein family comprises eight members that are important in the molecular regulation of cytokine signaling [13]. SOCS1, also known as SSI-1 and CAS, has been thought to be the most important class of cytokine-inducible genes that negatively regulate signaling and can be rapidly induced by a variety of cytokines [14]. SOCS1 is induced by cytokine activation of the JAK/STAT pathway but also acts as a negative feedback regulator to inhibit JAK signaling [13]. It has been proven that SOCS proteins can better control inflammation during ischemic stroke and that SOCS1 expression in the brain could be a treatment option for acute brain injuries, including stroke and trauma [15]. Based on this evidence, our study aims to elucidate how to manipulate the SOCS1-JAK2-STAT3 signaling pathway to protect neurons, which could provide a new target for the clinical treatment of ischemic stroke.

Materials and Methods

Ethics statement

All the rats were tested in strict accordance with the local guidelines for the care and use of animals at The Affiliated Hospital of Inner Mongolia Medical University and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH) of the United States. They were treated as humanely as possible, and efforts were made to minimize pain.

Establishment of a rat model of ischemic stroke

Adult male Sprague-Dawley (SD) rats (n = 40, weighing 250~300 g) were used to establish a model of ischemic stroke. Neonatal SD rats (n = 10, less than 24 h, non-specific sex, housed together with the adult male rats) were selected for microglia extraction. The rats were purchased from PENGSHENG Bio

TECH Co. Ltd [License No: SCXK (SU) 2009-0001]; Certificate No: 2022065, Jiangsu, China]. Forty adult male rats were classified into the ischemic stroke model group (n = 30) and the blank group (n = 10). The rats were anesthetized with an intraperitoneal injection of 0.3% pentobarbital sodium and placed in a supine position. Then, a median incision was made in the anterior neck to access to the internal carotid artery on both sides. At a point 3 mm proximal from the junction of the internal carotid artery, an embolic coil was threaded in and through to the initiation site of the anterior cerebral artery. The incision was closed, and no bleeding occurred. Approximately 1 cm of the stump suture was left outside the skin, and 2 h after blood flow occlusion, the thread was pulled out to induce ischemia-reperfusion [16]. Meanwhile, the rats in the blank group had free access to food and water.

Neurological function score

At 1, 2, 3, 4, 5, 6, and 7 days after the surgery, the rats' neurological impairment was assessed using the Longa 5 criteria as follows: no obvious symptom of neurological disorders, 0 points; inability of full body flexion in the left forelimb, 1 point; rotation to the left, 2 points; toppling to the left while walking, 3 points; and conscious disturbance and unable to walk on its own, 4 points. A rat scoring over 1 point was considered a successful model [17].

Animal grouping

All the rats were assigned into the blank group, the model group (rats with ischemic stroke that received no additional treatment), the pathway inhibitor + model group (AG490 group, rats received hypodermic injection of AG490 [1 mg/rat] every two days from day 3 after establishment of the model until euthanasia), or the negative control (NC) group (rats received hypodermic injection of normal saline [1 ml/rat] every two days from day 3 after establishment of the model until euthanasia).

Transmission electron microscopy observation

The left frontal cortex was collected from all the rats in the blank group and sliced into 1-mm-thick sections. The sections were fixed in glutaraldehyde and preserved at 4°C. Then, following a rinse with phosphate-buffer saline (PBS) (10 min × 3 times; 0.1 mol/L), the sections were fixed with 1 g/L osmic acid. Afterwards, the sections were rinsed with PBS, dehydrated in an alcohol gradient (15 min) and acetone (100 g/L, 15 min × 2 times) followed by resin embedding, slicing and coloration with uranyl acetate and lead citrate. The sections were observed and photographed under a transmission electron microscope (H-7500, Hitachi Ltd., Tokyo, Japan).

Hematoxylin-eosin (HE)

Cerebral cortex tissue from rats in each group was extracted and fixed in Davidson's solution for 24 h followed by routine fixation, clearing, wax dipping and paraffin embedding. Then, the tissues were sliced into 3-μm-thick sections (10 slices), incubated for 1 h at 50°C, stained with hematoxylin-eosin and observed under a microscope.

APNEL

The preserved sections were rinsed with PBS three times (5 min per wash) and subsequently treated with a Diaminobenzidine (DAB) Horseradish Peroxidase Color Development Kit according to the manufacturer's instructions. The sections were first treated with horseradish peroxidase at room temperature for 10 min, incubated in a mixture of TdT and DIG-d-UTP (1 μL each) and labeling buffer (18 μL) at 37°C for 2 h, rinsed with 0.01 mol/L Tris buffered saline (TBS) and blocked for 30 min. Next, the sections were incubated with biotinylated anti-digoxin antibody (diluted at 1:100, 50 μL/slice) at 37°C for 30 min. Then, after the sections were rinsed with TBS, they were incubated in a mixture of the antibody diluent (1 μL) and SABC (10 μL) (50 μL/slice) at 37°C for 30 min followed by DAB coloration and hematoxylin staining, dehydration, hyalinization and sealing. An HPIAS-100 image analysis system was used for image analysis and calculation of the corresponding rate of positively stained cells and the number of the apoptotic cells.

Immunohistochemistry

The cerebral cortex tissue from rats in each group were removed and embedded in paraffin sections, which were baked for 20 min at 68°C. After conventional xylene dewaxing and dehydration with an alcohol

gradient, the sections were placed at room temperature for 15 min and then were washed 2-3 times with PBS. After a blocking solution of normal goat serum (NGS) was added, the sections were placed at room temperature for 20 min and were incubated with antibodies targeting SOCS1, SOCS3, JAK2 and STAT3 (1:500, PL Laboratories Inc., New York, USA) at 37°C for 1 h. PBS was used to wash the sections. Secondary antibodies (PL Laboratories Inc., New York, USA) were incubated with the sections at 37°C for 1 h, which were washed with PBS and developed with DAB. The staining intensity was observed under a microscope. After the sections were restained with hematoxylin for 2 min, they were dehydrated, cleared, mounted, and observed under a Puguang microscope. Yellow-brown staining in the cytoplasm was considered strongly positive, and pale yellow was considered weakly positive in assessing the distribution of SOCS1, SOCS3, JAK2 and STAT3 protein in brain cells. The sections were photographed using digital camera. Image-Pro software was used for quantitative analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The insular cortex tissues collected from the four groups were homogenized in RNeasy lysis buffer, and total RNA was extracted from the hippocampus tissues using RNA extraction kits (Qiagen, Crawfordsville, IN, USA). Microglia in each group were collected to obtain total RNA, which was dissolved in ultrapure water containing diethylpyrocarbonate (DEPC). An ultraviolet and visible light (UV/VIS) spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, USA) was used to measure the absorbance at 260 nm and 280 nm. Then, the RNA quality was identified, and the total RNA concentration was determined. Primers specific for SOCS1, JAK2, STAT3, SOCS3, Bax, Bcl-2 and caspase-3 were designed, and the primers were synthesized by Sangon Biotech in Shanghai (Table 1). Total RNA was reverse transcribed using a specific stem-loop reverse transcription primer in the TaqMan® MicroRNA Reverse Transcription Kit to synthesize cDNA. Each reverse transcription reaction was 10 µL. The reaction conditions were 16°C for 30 min, 42°C for 30 min and 85°C for 10 min. TaqMan® MicroRNA Assays were also designed, and the reaction conditions were an initial denaturation (95°C, 3 min) followed by 40 cycles of amplification (95°C, 15 s), annealing (60°C, 30 s) and extension (72°C, 30 s). The reaction system contained 10 µL of PCR forward primer (2 µL), PCR reverse primer (2 µL), DNA template (4 µL), ROX Buffer (50×) (1 µL), SYBR Premix Ex TaqII (25 µL) and dH₂O (16 µL). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and gene expression was calculated using the 2^{-ΔΔCt} method.

Western blotting

The insular cortex and microglia were treated with cell lysis buffer to extract total protein. Protein (20 µg) and loading buffer were mixed and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the protein was transferred to nitrocellulose membrane, which was blocked with PBS containing 0.5% bovine milk powder for 1 h at room temperature and incubated with primary antibodies (PL Laboratories Inc., New York, USA) at 4°C overnight. After 3 rinses with PBS, the membrane was incubated with HRP-conjugated secondary antibody (PL Laboratories Inc., New York, USA) for 1 h at room temperature. Next, the membrane was rinsed 3 times with PBS prior to development with an electrochemiluminescence (ECL) solution and imaging of the exposure using a gel imaging system (Bio-Rad, CA, USA). The gray value of the bands was analyzed.

Table 1. Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR). Note: SOCS, suppressor of cytokine signaling; JAK2, Janus kinase 2; STAT3, signal transducers and transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Name	Sequences
SOCS1	Forward 5'-CACGCACTTCCGCACATTCC-3' Reverse 5'-TCCAGCAGCTCGAAGAGGGA-3'
JAK2	Forward 5'-TTTGAAGACAGGGACCCTACACAG-3' Reverse 5'-TCATAGCGGCACATCTCCACA-3'
STAT3	Forward 5'-CACCCATAGTGAGCCCTTGGA-3' Reverse 5'-TGAGTGCAGTGACCAGGACAGA-3'
SOCS3	Forward 5'-ACCAGCGCCACTTCTTCACA-3' Reverse 5'-GTGGAGCATCATACTGGTCC-3'
Bax	Forward 5'-GTACAGCTCGGGCACTTTAG-3' Reverse 5'-TGCAGAGGATGATTGCTGAC-3'
Bcl-2	Forward 5'-ATGCCGGGTGAGGAACTCTTCA-3' Reverse 5'-GGTGGTGGAGGAACCTTTCA-3'
caspase-3	Forward 5'-CGGGATCTGTTTCTTTGCAT-3' Reverse 5'-GGACCTGTGGACCTGAAAAA-3'
GAPDH	Forward 5'-CAAGGTCATCCATGACAACTTTG-3' Reverse 5'-GTCCACCACCCTGTTGCTGTAG-3'

Protein expression levels of SOCS1, SOCS3, phosphorylated JAK2 (P-JAK2), and P-STAT3 (p-Tyr-705-STAT3) as well as the protein expression and activation levels of Bax, Bcl-2 and caspase-3 were measured with GAPDH used as an internal reference (Bioworld technology Inc., Minnesota, USA). The relative protein expression of a target gene was defined as the ratio between the gray values of the target band and the corresponding value of the GAPDH band.

Determination of oxidative stress

Tissue sample preparation: The insular cortex was added to homogenates at a ratio of 1:9. The mixture was blended with a homogenizer for 5 min, and after 15 min of centrifugation (2000 rpm/min), the enzymes in the tissues were extracted from the supernatants for testing. Cell sample preparation: collected cells were washed 1-2 times with ice-cold PBS or ice-cold normal saline. Immunoprecipitation (IP) was performed using anti-SOCS1 (P0013, conducted on ice), which were made by lysing cells in an EP tube and centrifuging (12,000 rpm/revolution) at 4°C. The supernatants were collected to determine the SOD, GSH and MDA content according to the instructions of a kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) using a spectrophotometer (uV2100, Japan).

Microglia culture and cells grouping

After immersion disinfection with 75% ethanol, the neonatal rats (approximately 2 days old) were transferred to a clean and sterile culture dish. Skulls of the rats were cracked to remove the brain tissues, without disturbing the brainstem. After repeated washes with D-Hank's solution, the tissues were placed into a penicillin vial. The fully homogenized tissues were treated with 0.125% trypsin for mechanical isolation and 10 min of static digestion. Then, the tissues were combined with DMEM/F12 high-glucose medium containing 10% fetal bovine serum, 100 mg/l streptomycin and 1×10^6 U penicillin to terminate the enzyme reaction. After the tissue suspensions were filtered with a 200 µm mesh sieve, they were centrifuged at 1000 r/min for 10 min. The cell pellets were seeded into a culture flask coated with poly-L-lysine. The samples were placed in an incubator at 37°C containing 5% CO₂. The culture medium was changed the following day. Subsequently, the medium was changed every 3 days. Approximately 14 days after initial culturing, the previous culture medium was removed and the cells were digested with trypsin. After centrifugation in cell dissociation buffer, the cells were washed and seeded into 96-well culture plates. Microglia were isolated and purified using mild digestion. Flow cytometry was applied to determine the purity of the microglial cells. After the microglia were seeded into 96-well culture plates, they were randomly assigned into the blank group, the model group, the AG490 group and the AG490+AG490 group. In the blank group, the samples were cultured for 12 h under normal conditions. In contrast, the culture medium in the model group was replaced with sugar-free DMEM, and the samples were incubated for 6 h in an environment containing 5% CO₂, 1% O₂ and 94% N₂. After oxygen and glucose deprivation, the model group was cultured in normal medium under normal conditions for another 12 h. Fresh normal media were added to the NC and the AG490 groups, which were treated with 20 µl normal saline or 10 µl AG490 (50 nmol/ml), respectively, and continuously cultured 12 h for further experiments.

Assay

The microglia were inoculated in 96-well culture-plates, and the culture medium was then removed. Each well was treated with 20 µl of MTT solution (5 mg/ml) (Sigma-Aldrich Chemical Company, St Louis, MO, USA). The microglia were cultured for another 4 h, and the solution in each well was replaced with 50 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemical Company, St Louis, MO, USA). The plates were shaken to fully dissolve the purple crystals. The optical density (OD) at 490 nm was measured using a microplate reader.

Statistical analysis

Data analysis was performed using SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as the mean ± standard deviations. Comparisons between two groups were conducted with the *t* test, and comparisons among multiple groups were done with one-way analysis of variance (ANOVA). For enumeration data, the comparisons between two groups were analyzed using the χ^2 test. *P* < 0.05 indicated statistical significance.

Results

Inhibition of the SOCS1-JAK2-STAT3 signaling pathway decreased the impaired neurological function of rats after surgery

The survival rate of all the rats was 100%. Rats that scored over 1 point were incapable of fully stretching the left forelimb and wobbled to the left with conscious disturbance of normal walking, which suggests that the model was well-established. The scores on days 1-7 in the blank group were 0, and there were no symptoms of nerve injury. After sub-grouping, we observed that compared with the blank group, the NC, model and AG490 groups presented significantly increased neurological function scores (all $P < 0.05$) (Fig. 1). The AG490 group had significantly lower scores than the NC group and the model group, and the neurological symptoms were alleviated (all $P < 0.05$), which suggests that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may contribute to the attenuation of neurological impairment.

Inhibition of the SOCS1-JAK2-STAT3 signaling pathway repaired injury of neuronal ultra-structure in rats

Under a transmission electron microscope (Fig. 2), the neurons in the blank group were round and rich in organelles in the cytoplasm. As shown in Fig. 2, mitochondria were well organized and large in number, the rough endoplasmic reticulum maintained its structural integrity, the dictyosome was abundant, the nucleus comprised a relatively large volume, the karyotheca and nucleopore were clearly visible and the euchromatin presented as evenly distributed fine particles. The NC group and the model group presented fewer organelles in the cytoplasm and vacuole-like mitochondria. The rough endoplasmic reticulum was expanded and degranulated, and the presence of free ribosomes was reduced. In addition, the NC group and the model group also presented karyopyknosis, fusion of the double-layer nuclear membrane, an agglomerated karyotheca and visible apoptotic bodies. After inhibition of the SOCS1-JAK2-STAT3 pathway, the AG490 group had increased organelles in the cytoplasm, a slightly expanded rough endoplasmic reticulum, increased number of free ribosomes, a clearly structured double-layer nuclear membrane, evenly distributed euchromatin and fewer apoptotic bodies compared with the NC and model groups. Hence, it was concluded that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may repair the neuronal ultra-structure injuries in model rats.

Histopathological changes in all the rats in the blank, NC, model and AG490 groups

As revealed by HE staining (Fig. 3), the blank group showed no histopathological changes. The neurons in the blank group were round and well structured and had clear cytoplasm, a well-structured nucleus and evenly distributed euchromatin. In contrast, the NC group and the model group had neurons presenting ruptured structures, severe edema, karyopyknosis, hyperchromatic nuclei, chromatin condensation, and observable necrocytosis. In comparison with the NC group and the model group, the AG490 group presented increased

Fig. 1. Neurological scores of rats in the blank, NC, model and AG490 groups after surgery. Note: *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; NC, negative control.

Fig. 2. Electron microscope observations of neuronal ultrastructures in rats from each group (Magnification $\times 100000$). Note: NC, negative control.

Fig. 3. Histopathological changes of all the rats from the blank, NC, model, and AG490 groups as detected using HE staining (Magnification $\times 200$). Note: NC, negative control; HE, hematoxylin-eosin.

ingly attenuated edema in the neurons with edema only observed surrounding a few neurons. Karyopyknosis was also present, but the chromatin was relatively evenly distributed. In addition, there was no observable necrocytosis in the AG490 group. Based on this result, it can be concluded that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may reduce the histopathological damage caused by ischemic stroke in rats.

Table 2. Comparison of the number of TUNEL-positive cells in blank, NC, model and AG490 groups. Note: *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; NC, negative control; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Group	Number of TUNEL-positive cells
Blank group	1.22 ± 0.79
AG490 group	$56.06 \pm 5.84^*$
Model group	$75.51 \pm 4.88^{* \#}$
NC group	$74.68 \pm 6.28^{* \#}$

Inhibition of the SOCS1-JAK2-STAT3 signaling pathway reduced neuronal apoptosis in brain tissues

The blank group occasionally presented neuronal apoptosis while the NC group and the model group showed a larger number of apoptotic neurons along with obvious karyopyknosis and condensation of fragmented chromatin. Compared to the NC group and the model group, the AG490 group had a significantly lower number of apoptotic neurons ($P < 0.05$), and only a few neurons were dark brown, which indicated that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may significantly contribute to the reduction of apoptotic neurons (Table

Comparison of SOCS1-, SOCS3-, JAK2- and STAT3-positive cells in rats from each group as detected by immunohistochemistry

The immunohistochemistry results are shown in Fig. 4. The blank group had many SOCS1- and SOCS3-positive cells but a small number of JAK2- and STAT3-positive cells. In contrast, the NC group and the model group had fewer SOCS1- and SOCS3-positive cells and more JAK2- and STAT3-positive cells. Interestingly, in the AG490 group, positive SOCS1 and SOCS3 expression was increased, and the number of JAK2- and STAT3-positive cells was significantly decreased compared with those in the NC group and the model group. Thus, we

Fig. 4. Immunohistochemical detection of SOCS1-, SOCS3-, JAK2- and STAT3-positive cells in the four groups (Magnification $\times 200$). Note: SOCS, suppressor of cytokine signaling; JAK2, Janus kinase 2; STAT3, signal transducers and transcription 3; NC, negative control.

Table 3. Expression of oxidative stress-related factors in rats from the blank, NC, model and AG490 groups. Note: *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; NC, negative control.

Index	Blank group	AG490 group	Model group	NC group
SOD (KU/L)	100 ± 5.59	$153.98 \pm 5.61^*$	$93.59 \pm 5.14^{*#}$	$112.01 \pm 8.78^{*#}$
GSH (μmol/L)	92.83 ± 3.82	$75.61 \pm 3.20^*$	$56.50 \pm 2.10^{*#}$	$65.45 \pm 2.19^{*#}$
MDA (nmol/L)	3.29 ± 0.09	$8.35 \pm 1.02^*$	$14.99 \pm 1.34^{*#}$	$13.86 \pm 0.96^{*#}$

that the inhibition of the SOCS1-JAK2-STAT3 signaling pathway may suppress JAK2 and STAT3 expression in cells.

Expression of genes and proteins related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis in rats among each group

Compared with the blank group, the NC group and the model group had significantly lower mRNA and protein expression levels of SOCS1, SOCS3 and bcl-2; significantly increased mRNA and protein expression levels of JAK2, STAT3, caspase-3 and bax and remarkably elevated expression levels of P-JAK2, P-STAT3 and activated caspase-3 (c-caspase-3) (all $P < 0.05$). In the AG490 group, the mRNA and protein expression levels of JAK2, STAT3, caspase-3 and bax were significantly lower; the expression levels of P-JAK2, P-STAT3 and c-caspase-3 were significantly reduced; and the mRNA and protein expression levels of SOCS1, SOCS3 and bcl-2 were notably increased (all $P < 0.05$). These results indicated that SOCS1 and SOCS3 expression is negatively correlated with JAK2 and STAT3 expression and that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may suppress the phosphorylation of JAK2

Fig. 5. Expression of genes and proteins related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis in rats from the blank, NC, model and AG490 group. Note: A, relative mRNA expression of genes related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis was quantified using the $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal reference, data are presented as the mean $\pm$ standard deviation, $n = 10$; B, quantification of the western blotting results, the detected phosphorylation site of STAT3 is Tyr-705; C, analysis of protein expression levels in rats from each group, the relative expression level of the treated bar chart and the internal control bar chart is considered the relative protein expression levels, data are presented as the mean $\pm$ standard deviation, $n = 10$; *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; SOCS, suppressor of cytokine signaling; JAK, Janus kinase 2; STAT3, signal transducers and transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

and STAT3, down-regulate the expression of c-caspase-3 and bax, and up-regulate bcl-2 expression. All of which contribute to suppressing neuronal apoptosis (Fig. 5).

Oxidative stress levels of rats in the blank, NC, model and AG490 groups

Measurement of oxidative stress levels showed that the NC group and the model group presented significantly lower SOD and GSH expression and significantly enhanced MDA expression compared with the blank group (all $P < 0.05$). In contrast, compared with the blank, NC group and the model group, the AG490 group had significantly elevated SOD and GSH expression and significantly decreased MDA expression (all $P < 0.05$), indicating that inhibition of the SOCS1-JAK2-STAT3 signaling pathway may attenuate oxidative stress injury in an ischemic model in rat brains (Table 3).

Inhibition of the SOCS1-JAK2-STAT3 signaling pathway increased cell viability

The microglia activity results are presented in Fig. 6. Compared with the blank group (OD value 0.984 ± 0.031), the microglial cells in the AG490 (0.7415 ± 0.078), model (0.415 ± 0.089) and NC (0.434 ± 0.042) groups showed severe damage with significantly lower cell survival rates ($P < 0.05$). There was no significant difference in cell viability between the model group and the NC group. Compared with the model group, the AG490 group exhibited significantly better cell viability ($P < 0.05$).

Fig. 6. Microglia activity in each group as detected by the MTT assay. Note: *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; NC, negative control.

Fig. 7. Expression of genes and proteins related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis in microglia from the blank, NC, model and AG490 groups. Note: A, mRNA expression of genes related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis in microglia from each group were calculated using the real-time PCR method with GAPDH as an internal reference, data are presented as the mean $\pm$ standard deviation, $n = 3$; B, identification of the western blotting results, the detected phosphorylation site on STAT3 is Tyr-705; C, analysis of protein expression in microglia from each group, the ratio of treated and the internal control bar graphs is considered the relative protein expression, data are presented as the mean $\pm$ standard deviation, $n = 3$; *, $P < 0.05$, compared with the blank group; #, $P < 0.05$, compared with the AG490 group; SOCS, suppressor of cytokine signaling; JAK2, Janus kinase 2; STAT3, signal transducers and transcription 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

Expression of genes and proteins related to the SOCS1-JAK2-STAT3 signaling pathway and apoptosis in microglia from the blank, NC, model and AG490 groups

The trends of mRNA and protein expression were essentially the same between the *in vitro* ischemia-reperfusion injury model and the *in vivo* cerebral ischemia-reperfusion injury model. The NC group and the model group had significantly lower mRNA and protein

Table 4. Expression levels of SOD, GSH and MDA in microglia from the blank, NC, model and AG490 groups. Note: *, $P < 0.05$, compared with the blank group; # $P < 0.05$, compared with the AG490 group; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde; NC, negative control.

Index	Blank group	AG490 group	Model group	NC group
SOD (U/mg)	245.83 ± 10.31	165.73 ± 5.94*	102.31 ± 5.14*#	97.01 ± 8.78*#
GSH (U/mg)	52.91 ± 3.82	33.01 ± 3.20*	25.08 ± 2.10*#	21.14 ± 2.19*#
MDA (mmol /mg)	3.14 ± 0.90	10.51 ± 1.02*	17.99 ± 1.34*#	18.05 ± 1.34*#

expression levels of SOCS1, SOCS3 and bcl-2; significantly increased mRNA and protein expression levels of JAK2, STAT3, caspase-3 and bax and remarkably elevated expression of P-JAK2, P-STAT3 and c-caspase-3 compared with the blank group (all $P < 0.05$). In the AG490 group, the mRNA and protein expression levels of JAK2, STAT3, caspase-3 and bax were significantly decreased; the expression levels of P-JAK2, P-STAT3 and c-caspase-3 were significantly reduced; and the mRNA and protein expression levels of bcl-2, SOCS1 and SOCS3 were notably increased (all $P < 0.05$) (Fig. 7).

Oxidative stress levels of microglia from the blank, NC, model and AG490 groups

The trends in the oxidative stress levels were essentially the same between the *in vitro* and *in vivo* ischemia-reperfusion injury models. The NC group and the model group presented significantly reduced SOD and GSH expression levels and significantly enhanced MDA expression compared with the blank group (all $P < 0.05$). In contrast, compared with the NC group and the model group, the AG490 group significantly increased SOD and GSH expression levels and significantly decreased MDA expression (all $P < 0.05$) (Table 4).

Discussion

Stroke is the leading cause of neurological disability in humans. Ischemic stroke has a high mortality rate and often leads to disability due to the occlusion of the main cerebral artery—this loss of blood flow seriously affects the physical and mental health of individuals who suffer from stroke. The current treatments for ischemic stroke mainly dependent on blood flow restoration, including either pharmacological thrombolysis or mechanical thrombectomy, which carry side effects that aggravate the injury to the ischemic brain tissue after reperfusion [18]. Therefore, it is urgent to find an effective treatment for patients suffering from ischemic stroke. Furthermore, a previous study demonstrated the relationship between ischemic stroke and the JAK2/STAT3 signaling pathway in an animal model [19]. Thus, our study aimed to investigate the role of the SOCS1-JAK2-STAT3 signaling pathway in protecting neurons from apoptosis in a rat model of ischemic stroke and to provide a new approach for treating patients with ischemic stroke.

Our study has shown that ischemic stroke could damage the neuronal ultrastructure in rats and that inhibiting the SOCS1-JAK2-STAT3 signaling pathway can reverse the damage and improve histopathological alterations after ischemic stroke in rats; furthermore, this inhibition significantly reduced the number of apoptotic neurons and ameliorated the neurological impairment and cerebral oxidative stress injury in a rat model of ischemic stroke. Oxidative stress, reflecting an imbalance between production of reactive oxygen species (ROS) and the ability to eliminate reactive intermediates, could repair the damage caused by an adequate antioxidant defense [20]. Stroke often occurs with neurodegenerative sequelae associated with the new connections and angiogenesis, and many neurons subjected to ischemia due to stroke die within a few hours, which illustrates that ischemic stroke may damage the neuronal ultrastructures [21, 22]. Consistent with this observation, cerebral infarction expands rapidly after ischemic injury, and inhibition of the Rho and Rho-ROCK signaling pathways has been discussed because these pathways can promote neuronal

differentiation of mouse neural stem cells, which is consistent with our study [23, 24]. In addition, some studies have shown that inhibition of the JNK/AP-1 pathway could reduce neuronal cell death and improve behavioral results after ischemic brain injury; additionally, mesenchymal stem cells (MSCs) could decrease the number of apoptotic cells by suppressing the TLR2/NF κ B signaling pathway, which verifies that inhibiting the related pathway could improve the impairment [25, 26]. Beyond that, another study found that inhibition of the STAT3 pathway promotes metastatic progression in some human tumors, which further confirms our results [27].

In addition, down-regulation of the SOCS1-JAK2-STAT3 pathway could decrease JAK2 and STAT3 expression in cells as well as the levels of phosphorylated protein, which demonstrates the negative correlation between SOCS1 and JAK2-STAT3 expression. Moreover, mRNA and protein expression levels of Caspase-3 and Bax could be reduced, whereas those of Bcl-2 could be increased after inhibition of the SOCS1-JAK2-STAT3 pathway. In conclusion, in these studies, the activation of caspase-3 and Bax and the down-regulation of Bcl-2 have been reported to be strongly associated with neuron apoptosis, as Bax expression results in activated caspase-3 and indirectly inhibits Bcl-2 expression via the formation of Bax/Bcl-2 heterodimers, which initiate cell apoptosis following cerebral ischemia/reperfusion injury [28]. As an important signaling pathway, the JAK2-STAT3 pathway can regulate a variety of cell biological activities such as the immune response, cell differentiation and cell growth [29]. The SOCS1 pathway could inhibit the kinase activity of JAK2 and abolish the release of associated chemokines by blocking the JAK/STAT pathway, which highlight the negative regulation of SOCS1 on this pathway [30]. Additionally, silencing SOCS3 is essential to suppressing STAT3 phosphorylation, and JAK2-STAT3 expression is down-regulated via inhibition of the JAK2-STAT3 pathway, which is in accordance with our results [29-31]. Similarly, SN could induce Bcl-2 expression by inhibiting the JAK2/STAT3 pathway and prevent apoptosis by blocking the activation of Caspase-3 expression, which proves that inhibition of the SOCS1-JAK2-STAT3 signaling pathway could decrease Caspase-3 expression and increase Bcl-2 expression [19]. Additionally, after silencing the SOCS1-JAK2-STAT3 signaling pathway, the expression of the anti-apoptotic suppressor gene Bcl-2 is significantly up-regulated, whereas the expression of the apoptotic gene Bax is decreased, which is similar to our findings [32]. In addition, our study has proven that inhibition of the SOCS1-JAK2-STAT3 signaling pathway could reduce oxidative stress injury in the brains of rats subjected to ischemic stroke. One study found that the role of the JAK2/STAT3 signaling pathway could induce oxidative stress injury in response to hydrogen peroxide, which is similar to our results [33]. Additionally, previous evidence has illustrated that treatment with hrACE2 could prevent the activation of the JAK2/STAT3/SOCS3 pathway resulting in ACE2 protecting against oxidative stress [34].

Conclusion

Our study indicates that the SOCS1-JAK2-STAT3 signaling pathway plays an essential role in ischemic stroke in which SOCS1-JAK2-STAT3 signaling could reverse neuronal ultrastructure damage and improve histopathological injury; furthermore, inhibition of SOCS1-JAK2-STAT3 signaling could reduce neuronal apoptosis and alter the expression of phosphorylated protein. However, the exact mechanism of this activity is still unclear; therefore, more studies are required to provide further evidence for translating this activity from clinical treatment of ischemic stroke.

Abbreviations

CNS (central nervous system); JAK2 (Janus kinase 2); STAT3 (signal transducers and activators of transcription 3); SAH (subarachnoid hemorrhage); SOCS (suppressor of

cytokine signaling); DAB (diaminobenzidine, HE (hematoxylin-eosin); qRT-PCR (quantitative real-time polymerase chain reaction); ECL (electrochemiluminescence).

Acknowledgements

This study was supported in part by a grant from National Natural Science Foundation of China (No.81360213). All authors thanked reviewers for their kind comments.

Disclosure Statement

None.

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