Clematichinenoside Facilitates Recovery of Neurological and Motor Function in Rats after Cerebral Ischemic Injury through Inhibiting Notch/NF-κB Pathway

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Purpose: The present study was to observe the therapeutic efficiency of Clematichinenoside (AR) on cerebral ischemic injury in rats, especially on neurological and motor function recovery and to explore the underlying mechanism. *Methods:* Following middle cerebral artery occlusion/reperfusion (MCAO/R) surgery, rats were treated orally with 32, 16, and 8 mg/kg AR respectively for 14 days during which cerebral injury was evaluated and proinflammatory factors tumor necrosis factor-α and interleukin-6 as well as neurotrophic factors brain-derived neurotrophic factor and Neurotrophin-3 levels were determined with ELISA kits. Immunohistochemical analysis on number of neurons and reactive astrocytes in the hippocampus was to demonstrate the effect of AR on neuronal survival. Motor, learning, and memory recovery were assessed by Morris water maze, passive avoidance experiment, and rotatory rod test. Neuroprotection and anti-inflammation-related Notch and nuclear factor-κB (NF-κB) signaling pathways were analyzed by PCR and Western blot techniques on mammalian achaete-scute homologs1, Notch-1, intracellular Notch receptor domain, Jagged-1, transcription factor hairy, enhancer of split1 (Hes1), as well as the nuclear import of NF-κB in hippocampus. *Results:* AR administration reduced cerebral injury in rats exposed to MCAO/R and after treatment of AR for 14 days, proinflammatory reaction was inhibited, with neuronal survival rate raised and motor function recovery facilitated. PCR and WB analysis of Notch/NF-κB signaling pathway revealed the inhibitory effect of AR on pathway related components. *Conclusions:* AR is beneficial to recovery of neurological and motor function in rats after cerebral ischemic injury via inhibiting Notch/NF-κB pathway.

Key Words: Clematichinenoside—middle cerebral artery occlusion reperfusion—Notch/NF-κB—neuronal survival—motor functional recovery

Abbreviations: AR, Clematichinenoside; MCAO/R, middle cerebral artery occlusion reperfusion; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; BDNF, Brain-derived neurotrophic factor; NT-3, Neurotrophin-3; DG, dentate gyrus; Mash1, mammalian achaete-scute homologs-1; NICD, intracellular Notch receptor domain; Hes1, hairy and enhancer of split 1; Ngn1, Neurogenin-1; NF-κB, Nuclear factor-κB; bHLH, basic-helix-loop-helix; API, active pharmaceutical ingredient; TTC, 2, 3, 5-triphenyltetrazolium chloride; DAB, 3,3'-Diaminobenzidine; BCA, Bicinchoninic acid

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Introduction

Ischemic stroke is caused by a sudden interruption of local arterial blood supply to the brain tissue, accounting for approximately 70% of all strokes.[1] Free radical generation and consequent oxidative stress in stroke lead to brain damage, which is accompanied by activation of inflammatory responses.[2] Proinflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are released by damaged neurons,[3] which activate more inflammatory cells, resulting in astrocyte activation and numerous neuronal damages, especially hippocampal neurons that are particularly sensitive to the microenvironment.[4] Activated astrocytes proliferation further extend to the ischemic core, forming a glial scar within 2-4 weeks.[5] Glial scar is a marker of late ischemic stroke that blocks neuronal repair.[6] Neuronal damage is accompanied by irreversible damage of cognitive manifestations and neurological functions in hippocampus,[7] causing damage to spatial learning and memory. There is still an unmet need for neuroprotective treatment on ischemic stroke, especially chronic stroke.

The Notch signaling pathway is one of the most important pathways involved in long-term neuronal survival. The Notch-1 receptor is mainly expressed in the hippocampus which can affect memory recovery.[8] Once activated, the Notch receptor binds to the ligand (mainly Jagged and Delta) to form a dimer, which is cleaved into Notch intracellular domains (NICD) by γ-secretase. After ischemic stroke, the activated Notch signaling pathway insults neuronal repair and survival by regulating downstream transcriptional genes mainly including the basic-helix-loop-helix family composed of the proneuronal factors Mash1, Math, Ngn, and the inhibitor hairy and enhancer of split (Hes).[9,10] Studies found that activation of Notch signaling pathway can also promote nuclear factor-Kb (NF-κB) pathway after cerebral ischemia,[11,12] a key pathway that controls inflammation and based on recent research neuronal survival and memory in the adult brain.[13] After ischemia onset, Notch induces free NF-κB to translocate into the nucleus and activate the expression of the pro-inflammatory cytokine genes, releasing a large number of inflammatory cytokines including IL-1β, IL-6, TNF-α, and NO, which contribute to further brain injury.

Neurotrophic factors also play an important role in neuroprotection. brain-derived neurotrophic factor (BDNF) promotes cell proliferation, differentiation[14] and plays an important role in learning and memory.[15] Neurotrophin-3 (NT-3) maintains the survival of neural stem cells and promotes their proliferation and differentiation.[16] Overexpression of neurotrophic factors can inhibit Notch signaling pathway and regulate expression of Hes1, Mash1, and Ngn1.[17] It is suggested that neurotrophic factors interact with Notch and NF-κB signaling pathways to regulate neuronal survival during the pathogenesis of ischemic stroke.

Clematichinenoside (AR) is a pentacyclic triterpenoid saponin extracted from the Chinese medicinal material *Clematis chinensis* (*C chinensis*) Osbeck with a content of more than 90% and clear impurity composition (Fig 1).[18-20] *Clematis manshurica* (*C manshurica*) Rupr was an important origin of *C chinensis* Osbeck, an anti-inflammatory and antiarthritic traditional Chinese medicine of a long history. AR in this study was extracted from the roots and rhizomes of *C manshurica* Rupr with a purity of 95.3%.

Cumulative evidence has demonstrated the anti-inflammatory and antiarthritic properties of AR. As for its anti-cerebra ischemia effect, our laboratory's previous studies found that: 3 days' protective effect of AR on cerebral ischemic injury was related to balancing anti- and proinflammatory factors,[21] regulating Treg cells,[22] reducing the adhesion of endothelial cells,[23] and protecting blood-brain barrier[24] related to decreased NF-κB activation without investigating the in-depth mechanism. However, the therapeutic effects of relatively long-term administration of AR in ischemic stroke, especially on motor functions and learning/memory recovery have not been reported so far. Therefore, we hypothesized that 14 days' therapeutic administration of AR may enhance the learning and memory and exercise capacity of cerebral ischemia/reperfusion rats through anti-inflammatory and neuroprotective functions with a possible mechanism of inhibiting Notch/NF-κB pathways.

Methods

Animals and Treatment

SPF male Sprague-Dawley rats (250-280 g) were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd. in a temperature-controlled room (22°C ± 2°C) with a 12-hour light-dark cycle and a relative humidity of 60% ± 10%. Rats were allowed free access to food and water prior to the experiment and fasted for 12 hours prior to surgery. Rats were randomly divided into 6 groups: sham group, middle cerebral artery occlusion/reperfusion (MCAO/R) model group, AR (32 mg/kg) group, AR (16 mg/kg) group, AR (8 mg/kg) group,[24] and aspirin (16 mg/kg) group. To assess infarct size, cerebral edema, neurological deficits, water maze, darkness test, rotarod test, and ELISA analysis, n = 6 or 8 per group. For immunohistochemistry, real-time PCR and Western Blot, n = 3-5 per group. All animals were treated according to the institutional guidelines of China Pharmaceutical University (Nanjing, China). All efforts were made to minimize the suffering of animals and to reduce the number of animals used.

Preparation of AR

AR (provided by the Department of Traditional Chinese Medicine, Chinese Academy of Pharmacy, China

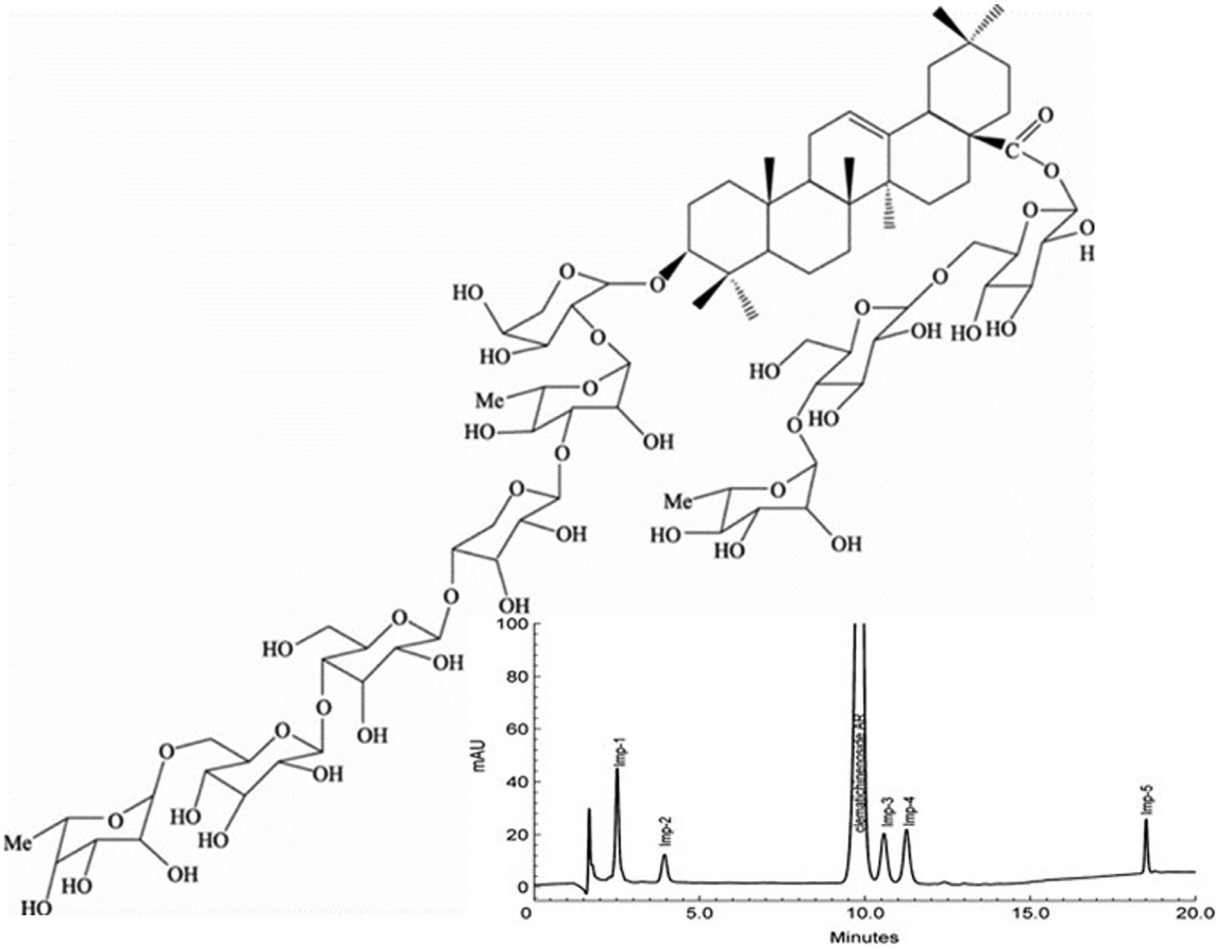

Figure 1. *Structural formula of Clematichinenoside (AR) and its typical chromatogram. (Entire structure: 3-O-β-{[O-α-L-rhamnopyranosyl-(1→6)-O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-O-β-D-ribopyranosyl-(1→3)-α-O-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]oxy} oleanolicacid 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester, $C_{82}H_{134}O_{43}$; molecular weight: 1806).*

Pharmaceutical University, CAS Registry Number: 761425-93-8) applied in this project was extracted from the roots and rhizomes of *C manshurica* Rupr, with a purity of 95.3% in a single batch of 30 g, and was dissolved in saline. The roots of *C chinensis* (050318) were obtained from Bozhou City, Anhui Province of China. A voucher specimen (050318) has been deposited in the China Pharmaceutical University. In brief, 500 kg of *Clematis manchuriensis* were taken and extracted by 10 times as much water and heat reflux for 3 times, each time for 2 hours. After cooling and filtering, preprocessed D101 macroporous resin column was used to elute the extraction with water, 20% and 50% ethanol sequentially and 50% ethanol elution fluid was collected, concentrated and desiccated to get total saponins, which were then refluxed with anhydrous ethanol for 2 hours and crystallized overnight after hot filtration. Afterwards, abandon the filtrate to collect sediment. Crude product of AR was obtained after drying, which was refined with acetone-water crystallization dried to acquire active pharmaceutical ingredient of AR. Content of the bulk drug was performed by high-performance liquid chromatography determination with octadecyl silane-bonded silica as filler, water as mobile phase A, and acetonitrile as mobile phase B through gradient elution at detection wavelength of 203 nm, column temperature of 30°C. Use one-point external standard method to calculate the content and the total yield was above .8%. The extraction and content determination method of this product was accurate and reproducible

Drug Administration

Aspirin (enteric-coated tablets, purchased from Fu'an Pharmaceutical group Yantai Justawore pharmaceutical Co. Ltd.) were used as a positive control with each tablet containing 25 mg of active ingredient. .5% sodium carboxymethyl cellulose was used to dissolve aspirin tables. Rats were given different doses of AR (32 mg/kg, 16 mg/kg, and 8 mg/kg) and Aspirin (16 mg/kg) by intragastric administration immediately after MCAO/R surgery according to previous studies,[23,24] and the volume was 0.5 mL/100 g once a day for the following 14 days.

MCAO Treatment

MCAO/R injury was served as an ischemic stroke model in our experiment as described previously.[25] Briefly, a silicone-coated monofilament nylon suture (about .26 mm in diameter, Beijing Cinontech Co., Ltd.) was inserted to seal the origin of the right MCA. The filaments were pulled out after 2 hours. The body temperature was maintained at 37°C ± .5°C throughout the experiment. The sham group was induced by the same operation without blocking of the MCA. All other groups underwent MCAO/R surgery. Experimental flow chart is shown in Figure 2A.

Neurological Deficiency Evaluation

After 30 minutes of the 3rd, 7th, and 14th day of drug administration, the neurological deficits of the animals were graded according to Bederson's method.[26] The scoring criteria were as follows: 0 = normal activities without neurologic deficiency; 1 =left forepaws with flexion, adduction and affixed to chest when lifted by the tail; 2 = decreased resistance to lateral push; 3=unilateral circling when free to walk; 4 = no spontaneous walking and flaccid paralysis. A higher score meant poorer neurological function.

Measurement of Infarct Size

After the neurological deficit score was measured at the 7th day and 14th day, another batch of rats was sacrificed. The whole brain was removed for coronary resection. After cut into 5 pieces, the brain slices were quickly placed in phosphate buffer containing 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC, Sangon Biotech Co., Ltd.) and incubated at 37°C for 15 minutes. The area of the left hemisphere and the unstained area (un-infarcted area) of the right hemisphere were calculated, and the percentage of infarction was calculated as follows using Image J (National Institutes of Health)[27]:

Percentage of cerebral infarction area = (left hemisphere area − right inferior brain infarct size)/left hemisphere area × 100%.

Evaluation of Brain Edema

The stained brain tissue is weighed immediately after staining to get the wet weight. Then the brain is placed in an oven at 110°C to dry to constant weight (dry weight), and the brain water content is calculated as follows[28]:

Brain tissue water content (%) = (1 − dry weight/wet weight) × 100%.

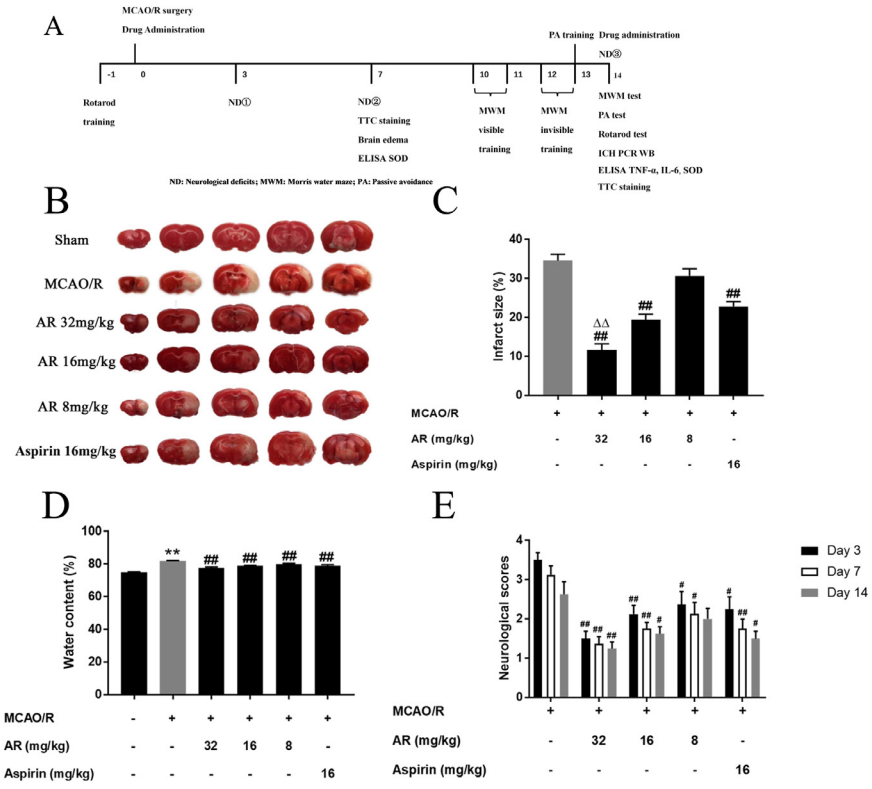

Figure 2. *Effects of Clematichinenoside (AR) on the outcome of neurological function after cerebral ischemia/reperfusion in rats for 3 days, 7 days, or 14 days. (A) Experiment arrangement diagram; (B and C) Representative TTC staining images and quantification of infarct volume 7days after stroke; (D) brain water content; (E) neurofunctional scores. (One-way ANOVA followed by Bonferroni post hoc for neurological scores, One-way ANOVA followed by Bonferroni post hoc for the others; n = 8/group.) Data are represented as mean ± S.E.M. *P < .05, **P < .01 versus sham group; #P < 0.05, ##P < 0.01 versus model group; ΔP < 0.05, ΔΔP < 0.01 versus aspirin group.*

Detection of SOD, TNF-α, and IL-6 in Serum and Hippocampus by ELISA

The blood of 1 batch of rats was collected from the left common carotid artery after the 7th and 14th day of AR administration. The blood samples were centrifuged at 4000 rpm and 4°C for 10 minutes in a refrigerated centrifuge, and the supernatant of seventh day was collected. After blood collection, the rats' hippocampus on the ischemic side of was taken out and rinsed with normal saline and the tissue homogenate was prepared on an ice water bath with the 1:9 ratio of hippocampus and normal saline. The homogenate samples were then centrifuged at 3000 rpm and 4°C for 20 minutes, and the supernatant was collected. Serum was used for SOD detection at 7th and 14th day for initial pharmacodynamics. And serum as well as hippocampus were used for detection of TNF-α and IL-6 at fourteenth day by ELISA kits (Neobioscience products) according to the kit instructions.

Immunohistochemistry

After 30 minutes at the 14th day of administration, the rats were sacrificed and the whole brain was taken out and placed in a 4% formaldehyde solution for fixation, and standard methodology of immunohistochemistry was used. Briefly, paraffin-embedded brain sections were prepared and then the samples were incubated with the primary antibody NeuN or GFAP (Abcam, 1:200) at 4°C overnight. In the next day, 50 μL of EnVision reagent was added to each section and incubated for 30 minutes at room temperature. Then, 50 μL of freshly prepared developer DAB was added to each section and the section was dyed with hematoxylin dye, rinsed, and dehydrated with ethanol. Transparently treat the brain tissue with xylene which was sealed with a neutral gum. Finally, photograph under the digital pathology slice scanner. The number of positive cells (brown) in the hippocampus dentate gyrus (DG) area of each sample was calculated using software image J (National Institutes of Health).

Morris Water Maze Test

Morris water maze test was performed for 5 days from the 10th day of administration to detect learning and memory in rats, during which the first and second days were the visible training session with the flag inserted in the water maze platform in the fourth quadrant of the water maze, the third and fourth days were the invisible training session without the flag on the platform and the fifth day was the test session with neither the flag nor the platform. The drug was continually administrated every day during the water maze experiment, and the experiment was performed 30 minutes after administration.[29] Rats were placed in the water from the 4 quadrants every day. Within the 90 seconds, the rats may find the platform and allowed to stay on the platform for 10 seconds. Record the time from the entry of the water to the climb (the escape latency) and the number of crossings. If the rat failed to find the platform within 90 seconds, the incubation period was recorded as 90 seconds. On the fifth day of the experiment, the platform was removed. The trajectory of free swimming in the 90 seconds, the percentage of the time spent in the fourth quadrant and the number of crossing times through the original platform spot were recorded.

Passive Avoidance Test

After 13th days of drug administration, the passive avoidance test[30] was performed to detect memory impair in rats. The experimental device consisted of a bright box and a dark box. The rat was placed in the device for 5 minutes and allowed to move freely. The rat would enter the dark room by instinct. A 36 V, 50 Hz alternating current was applied to the copper grid at the bottom of the darkroom, and the rat may escape to the bright room by electric shock. After 30 minutes of administration on the 14th day, the rats were placed in the bright room back to the hole and subjected to an electric shock when entering the dark room. The number of times the rats entered the dark room within 5 minutes (the number of errors) and the time to enter the darkroom for the first time (escape latency) were recorded.

Rotarod Test

The exercise balance ability of the rats was tested on an accelerating rotarod.[31] One day prior to MCAO/R surgery, the rats were placed on the stationary rotarod for 30 seconds. Then adjust the pole speed to 4 rpm to ensure that animals can be maintained on a rotating pole for 1 minute. After a 10 minutes rest, rats were placed on a rotating rod accelerated from 4 to 40 rpm in 5 minutes and were trained to stay more than 2 minutes on the rod. At the 14th days after MCAO/R surgery, rats were placed on a rotating rod that accelerated from 4 to 40 rpm in 5 minutes for 3 times, and the time until they dropped from the rod was recorded.

Real-Time PCR

At the end of the water maze experiment, the rats were sacrificed and the hippocampus of the ischemic brain was taken for the determination of BDNF, NT-3, Hes1, Mash1, Notch-1, and Jagged-1 mRNA in the hippocampus. Real-time PCR was performed as previously described.[32] Briefly, total RNA from ischemic brain hippocampus was extracted using Trizol reagent (Takara). Total RNA was then reverse-transcribed into cDNA using the HiScript II One Step qRT-PCR SYBR Green kit (Takara) according to the manufacturer's instructions. Real-time PCR was performed using quantitative PCR (Mastercycler, Eppendorf) in the presence of a fluorescent dye (SYBR Green I). The

comparison threshold cycle (CT) value method was used to determine the amount of product, and Equation $2^{-\Delta\Delta CT}$ was used to determine the fold change. mRNA levels were normalized to GAPDH of the same sample and shown as fold change compared to the mock group.[29] Primer (Sangon Biotech Co., Ltd.) is as follows: BDNF: F: GGG TGA AAC AAA GGTG GCT GT, R: GGA TTC AGT GGG ACT CCA GA; NT-3: F: GAT CCA GGC GGA TAT CTT GA, R: AGC GTC TCT GTT GCC GTA GT; Hes1: F: GGA GAG GCT GCC AAG GTT TT, R: AGG CGA CAC TGC GTT AGG A; Mash1: F: AGG CCC TAC TGG GAA TGG A, R: CCC TGT TGC TGA GAA CAT TGA; Notch-1: F: AGA GCT TTT CCT GTG TCT GTC C, R: CGG TAC AGT CAG GTG TGT TGT T; Jagged-1: F: GGG CCA GAC TGC AGG ATA AAC, R: CGC CGT GCC CTT TGT GGA G; GAPDH: F: CCC ATC ACC ATC TTC CAG, R : ACA GTC TTC TGA GTG GCA.

Western Blot Analysis

To determine the protein content of NICD and Jagged-1 in the hippocampus of ischemic brain tissue and the nuclear import of NF-κB p65 subunit, total protein, and cytoplasmic/nuclear protein extracts were prepared as described previously[33] and determined by BCA. After 30 minutes of the last administration, the rats were sacrificed. The hippocampus on ischemic side of the rat was removed. Each sample was lysed on the ice by adding appropriate amount of cell lysate (RIPA: PMSF (V: V) = 98:2)) for 30 minutes. After centrifugation at 12,000 rpm for 15 minutes, the resulting supernatant was total protein for the determination of NICD and Jagged-1. Cytoplasmic and nuclear protein was prepared as the manufacturer advised (Beyotime Institute of Biotechnology). Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with primary antibody of rabbit monoclonal anti-NICD (Abcam; 1:1000), rabbit polyclonal anti-Jagged-1 (Abclonal; 1:1000), or mouse monoclonal anti-NF-κB primary antibody (Cell Signaling Technology; 1:1000) overnight at 4°C. The blot was conjugated with the corresponding goat antirabbit secondary antibody (for NICD or Jagged-1; Beyotime Institute of Biotechnology; 1:1000) or goat antimouse secondary antibody (for NF-κB; Beyotime Institute of Biotechnology, 1:1000). The blot was visualized by enhanced chemiluminescence detection and normalized to rabbit monoclonal anti−β-actin (for total proteins or cytosol proteins; Beyotime Institute of Biotechnology; 1:1000) or Histone3 (for nuclear proteins; Proteintech; 1:1000). Gel-Pro analyzer 4 (JS-860B, Shanghai Peiqing Science & Technology Co., Ltd.) was used to quantify the bands.

Statistical Analysis

Data were expressed as Mean ± Standard Error of Mean (S.E.M). The comparison between the groups was performed by the 1-way analysis of variance method or 2-way analysis of variance (for neurological scores or latency time evaluations in Morris maze exploration experiment) method in the statistical software SPSS 20.0. The Bonferroni test was used if the variance between the groups was equal and the Dunnett's T3 if not. Statistical analysis results $P < 0.05$ were considered be significant. Pictures were analyzed using Image J, Photoshop CS5, Gel Pro Analyzer 4 and GraphPad Prism 5.0.

Results

Clematichinenoside (AR) Improved Neurological Outcome in Rats after Cerebral Ischemia/Reperfusion

The effect of AR on cerebral injury outcome of rats after cerebral ischemia/reperfusion was determined by the measurement of brain infarct size, brain water content, and neurological scores.

At seventh day, in a MCAO/R rat model, infarct size in the model group was 34.6% ± 4.6%, whereas administration with AR (8, 16, 32 mg/kg) significantly and dose-dependently reduced infarct size to 30.7% ± 5.1%, 19.4% ± 4.1%, and 11.7% ± 4.4%, respectively ($P > .05$, $P < .05$, and $P < .01$ versus model group). The infarct size in rats after treatment with 16 mg/kg Aspirin obviously decreased to 22.8% ± 3% ($P < 0.01$, Fig 2, A) with 32 mg/kg AR significantly better than 16 mg/kg aspirin. Similarly, 7 days after MCAO/R, cerebral water content of the 3 doses of AR decreased significantly (Fig 2, D). Neurological score was evaluated on the 3rd, 7th, and 14th day after MCAO/R, and AR treatment dose-dependently reduced the behavioral scores after MCAO/R (Fig 2, E).

At 14th day, cerebral infarction was accompanied by apparent tissue loss in the MCAO/R group, with the infarction rate up to 50.91% ± 15.83%, with almost no tissue loss observed in the AR group and the infarction area reduced to 11.06% ± 3.52%, significantly different from the model group ($P < .01$, Fig 3).

There was no significant difference in water content among each group due to tissue atrophy at 14 days.

SOD detection of oxidative stress confirmed that both 7 and 14 days of AR administration could significantly increase the level of SOD reduced after cerebral ischemia. The above results indicate the long-term efficacy of AR.

AR Regulated the Levels of Cerebral Ischemia-Related Inflammatory Cytokines and Neurotrophic Factors 14 Days after Cerebral Ischemia/Reperfusion in Rats

Long-term anti-inflammatory and neuronal protective effect of AR were preliminarily examined with ELISA kits of inflammatory cytokines TNF-α, IL-6, and neurotrophic factors BDNF and NGF. The results showed that at 14th day of administration, 32 mg/kg and 16 mg/kg AR,

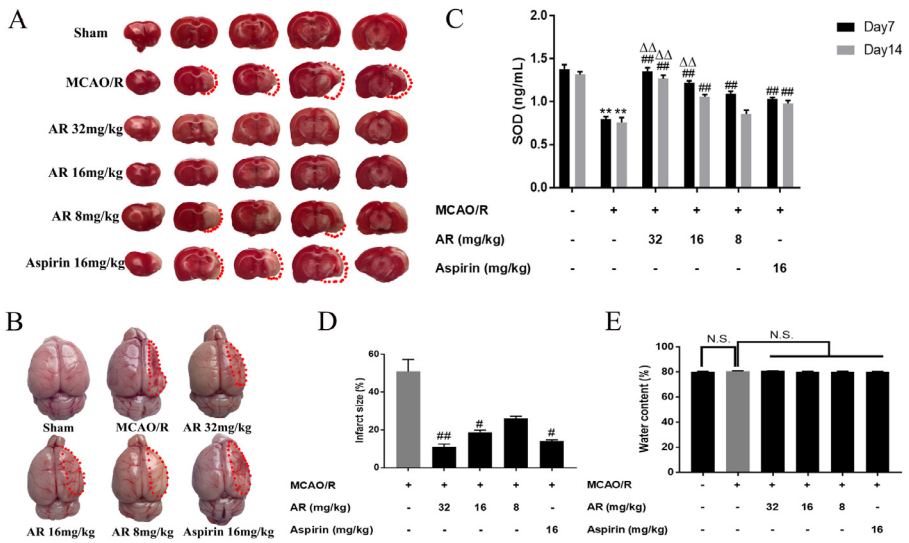

Figure 3. *Effects of Clematichinenoside (AR) on the brain injury and oxidative stress after cerebral ischemia/reperfusion in rats for 7 days or 14 days. (A, B, and D) Representative TTC staining and brain atrophy images and quantification of infarct volume 14 days after stroke; (C) serum SOD content 7 days or 14 days after stroke; (E) brain water content. (One-way ANOVA followed by Bonferroni post hoc; n = 6/group.) Data are represented as mean ± S.E.M. *P < 0.05, **P < 0.01 versus sham group; #P < .05, ##P < 0.01 versus model group; ΔP < 0.05, ΔΔP < 0.01 versus aspirin group.*

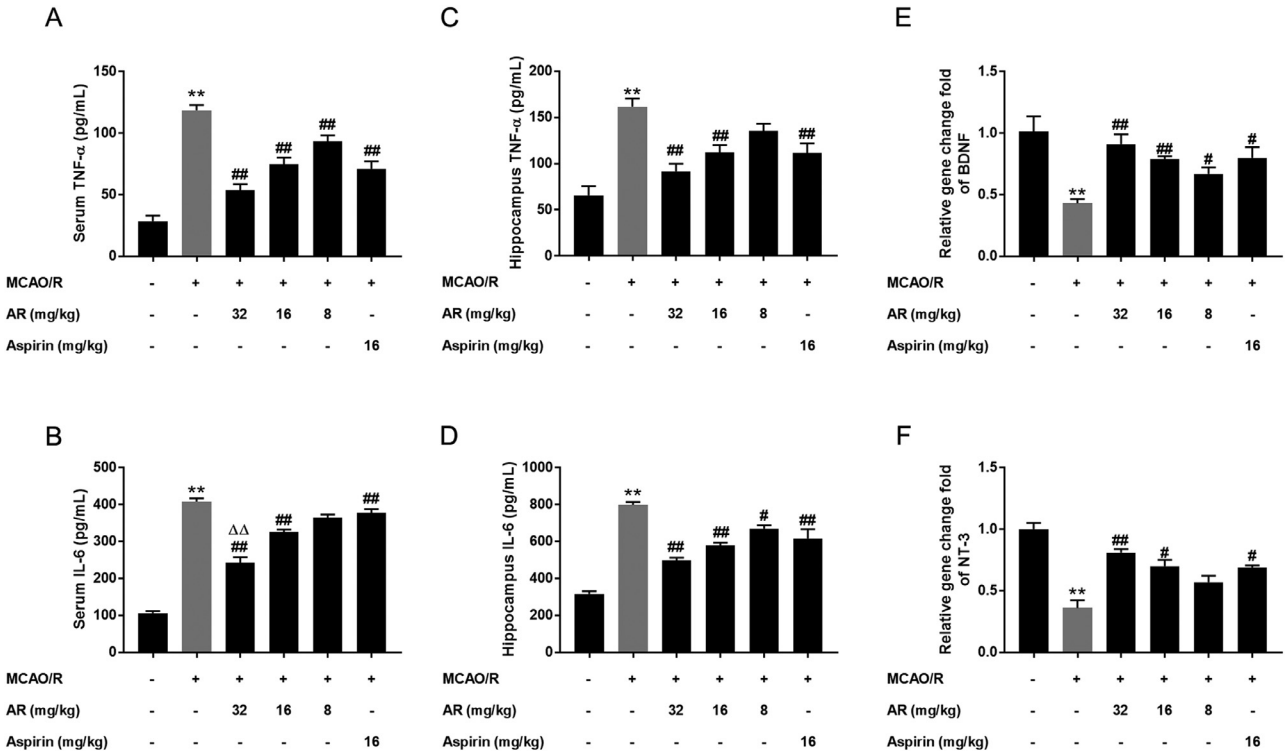

Figure 4. *Effect of AR on content of TNF-α and IL-6 in serum and hippocampus and mRNA levels of BDNF and NT-3 in hippocampus 14 days after cerebral ischemia/reperfusion in rats (One-way ANOVA followed by Bonferroni post hoc; n = 8/group.) Data are represented as mean ± S.E.M. *P <0.05, **P < 0.01 versus sham group; #P < .05, ##P < .01 versus model group; ΔP < 0.05, ΔΔP < .01 versus aspirin group.*

similar to aspirin, reduced the increased levels of TNF-α and in serum and hippocampus of MCAO/R rats ($P < .01$, Fig 4), and the effect of AR in the reduction of serum IL-6 was better than that of aspirin ($P < .01$, Fig 4, B). At the same time, 32 mg/kg AR markedly induced the releasing of BDNF and NT-3 (Fig 4, E,F). These results verified the anti-inflammatory and neuronal protective effects of AR 14 days after cerebral ischemia in rats, whose potential mechanism, we hypothesized, might relate to Notch and NF-κB signaling pathway.

AR Regulated Neurons and Astrocytes in Hippocampus 14 Days after Cerebral Ischemia/Reperfusion in Rats

14. Days' AR Increased the Number of Neurons in Hippocampus DG Area of Rats with Cerebral Ischemia/Reperfusion

To investigate whether AR can promote neuron survival after 14 days of administration, we performed immunohistochemical staining in the hippocampus of rats. As shown in Figure 5A and C, MCAO/R had serious damage to DG neurons ($P< 0.01$). Compared with the model group, AR 32 mg/kg, 16 mg/kg, and aspirin 16 mg/kg groups significantly rescued the number of neurons in the DG area of the hippocampus of rats after MCAO/R ($P< 0.01$), with the rescuing effect of AR on neurons in the CA1 region the most obvious (Fig 5, B).

14. Days' AR Reduced the Number of Reactive Astrocytes in Hippocampus of Rats with Cerebral Ischemia/Reperfusion

The number of hippocampal astrocytes in the MCAO/R group dramatically increased compared with the sham group ($P< .01$). A total of 32, 16, and 8 mg/kg AR and 16 mg/kg aspirin significantly reversed the number of hippocampal astrocytes in all dose groups ($P< 0.01$, $P< 0.01$, $P< 0.05$, $P < 0.01$, Fig 6). We also found that glial scar (a hallmark in late-stage of ischemic stroke, which

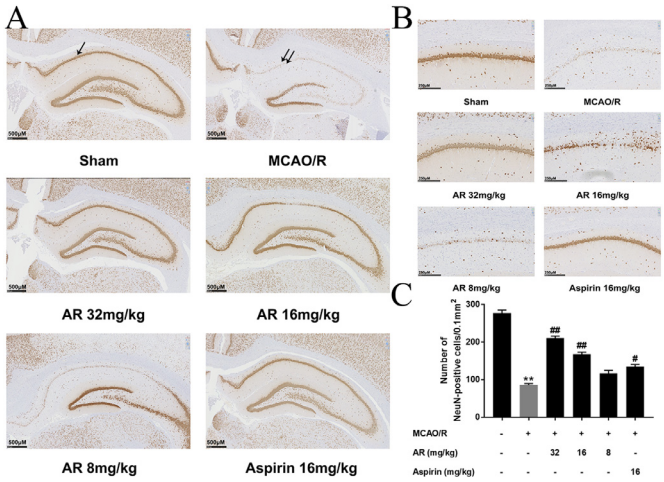

Figure 5. *Effect of AR on the number of neurons in hippocampus 14 days after cerebral ischemia/reperfusion in rats. (A and B) representative immunohistochemistry images of hippocampal DG and CA1 area NeuN-positive neurons 14 days after stroke. (C) Quantification of NeuN-positive neurons in DG area. (One-way ANOVA followed by Bonferroni post hoc; n = 3-5/group.) Data are represented as mean ± S.E.M. *P < .05, **P < 0.01 versus sham group; #P < 0.05, ##P < 0.01 versus model group.*

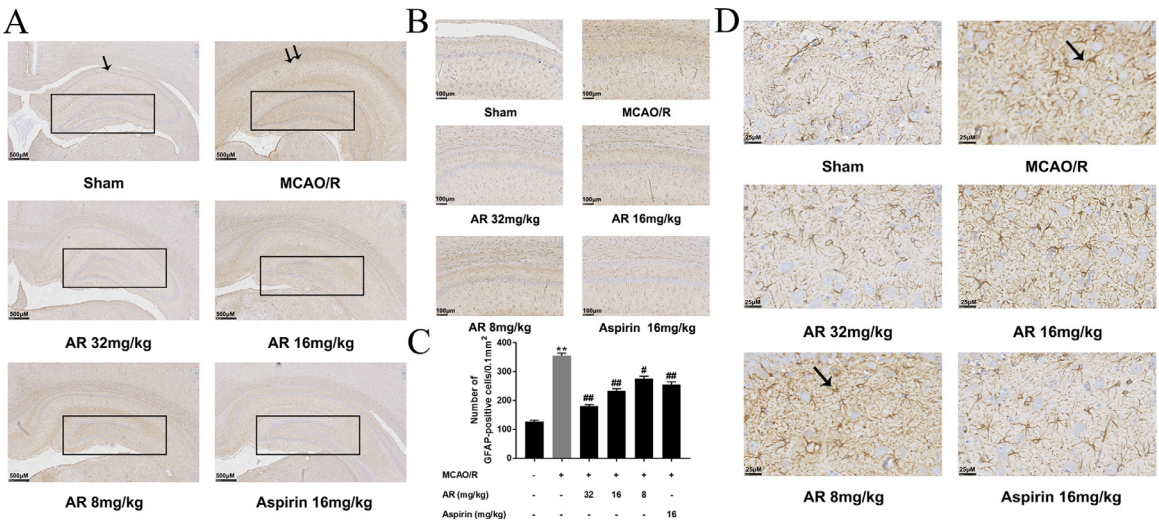

Figure 6. *Effect of AR on the number of astrocytes in hippocampus 14 days after cerebral ischemia/reperfusion in rats. (A and B) Representative immunohistochemistry images of hippocampal DG and CA1 area GFAP-positive neurons 14 days after stroke. (C) Quantification of GFAP-positive neurons in DG area. (One-way ANOVA followed by Bonferroni post hoc; n = 3-5/group.) (D) Representative glial scar images of hippocampal DG area. Data are represented as mean ± S.E.M. *P < 0.05, **P <0.01 versus sham group; #P < 0.05, ##P < 0.01 versus model group.*

hinders axonal regeneration and neuronal repair)[5-7] was formed at 14 days after MCAO/R injury (Fig 6, D), with 32 mg/kg, 16 mg/kg groups, and aspirin 16 mg/kg group significantly decreasing this immunoreactivity of reactive astrocytes.

AR Improved Learning, Memory, and Motor Abilities 14 Days after Cerebral Ischemia/Reperfusion in Rats

The effects of AR administration on the recovery of learning, memory, and motor ability after cerebral ischemia were verified by behavioral experiments.

In water maze test, the number of crossings and the percentage of the time spent on the quadrant IV of the MCAO/R group mice were significantly lower than those in the sham group ($P < .01$), while those in the 32 mg/kg AR group were remarkably higher ($P < 0.01$, Fig 7, A,B).

During the training period, there was no significant difference in the training latency on the visible or invisible platforms (Day 1 and Day 3) in each group, indicating that the ability of all rats to recognize targets was normal ($P > 0.05$, Fig 7, D). On Day 2, rats spent significantly reduced time to climb onto the platform ($P < 0.01$) in 32 mg/kg AR group compared to MCAO/R group, similar to results of the hidden platform experiment (Day 4, $P < 0.05$).

In passive avoidance experiment to test the influence of 14 days' AR on MCAO/R rats' susceptibility to memory injury, rats of MCAO/R group spent less time in the bright box and entered more frequently to the dark side than those of the sham group. Thirty two milligrams per kilogram AR group stayed longer and made significantly less mistakes compared to the MCAO/R group ($P < 0.01$, $P < 0.05$, Fig 7, E,F).

The above results showed that 14 days' administration of 32 mg/kg AR was helpful for the recovery of learning, memory and motor ability after MCAO/R in rats. In rotate experiment, the MCAO/R rats fell down earlier than rats of sham group ($P < 0.01$). The latency time to fall from the accelerating rotarod of 32 mg/kg and 16 mg/kg AR groups extended significantly compared to MCAO/R group ($P < 0.01$, $P < 0.05$, Fig 7, G).

These data were in accordance with the results from the above experiments, revealing that AR attenuated ischemic outcome and improved learning, memory and motor functions.

AR Inhibited the Expression of Notch Signaling-Related Components in Hippocampus 14 Days after Cerebral Ischemia/Reperfusion in Rats

In order to explore whether AR played a role in neuroprotection and motor function recovery through inhibiting Notch signaling pathway, a vital pathway for neuronal survival and functional recovery after cerebral ischemia injury, we used RT-PCR and Western blot methods to detect expression of components essential to this pathway. The results showed that 32, 16, and 8 mg/kg AR were similar to aspirin at 16 mg/kg in reducing the expression of Notch-1 and Jagged-1 in a dosage-dependent manner compared with the MCAO/R group ($P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.01$, Fig 8, A,B). And AR 32 mg/kg significantly reduced the protein levels of Notch inducing NICD and Jagged-1 in the hippocampus

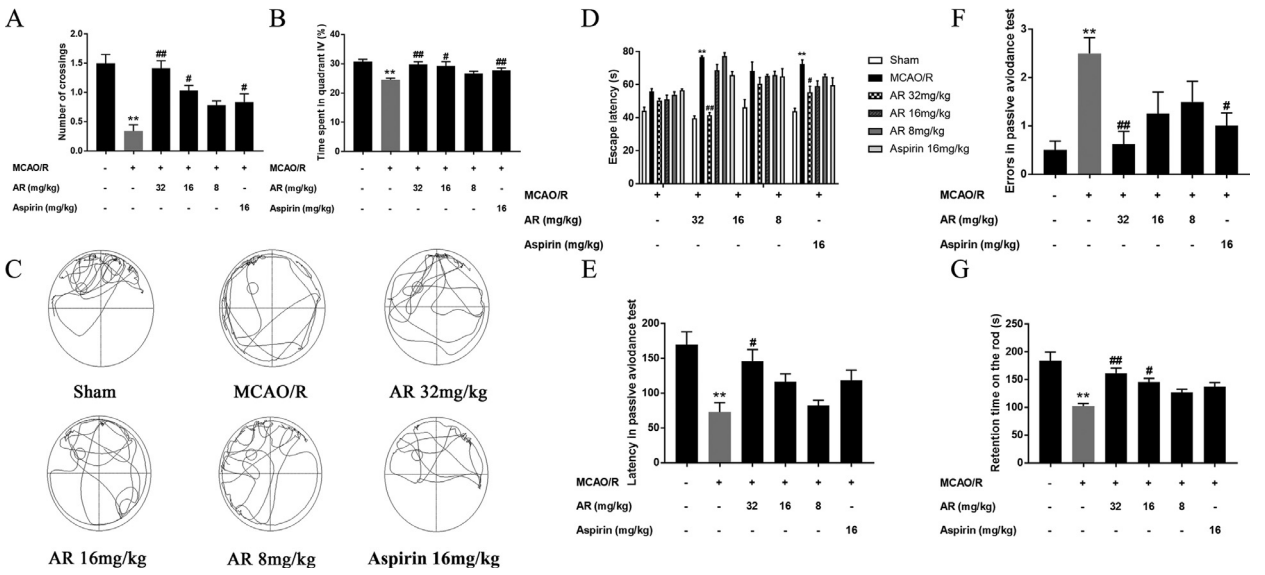

Figure 7. *Effect of AR on water maze test, rotate experiment, and passive avoidance 14 days after cerebral ischemia/reperfusion injury in rats. (A) The number of crossings in water maze; (B) the percentage of the time spent on the quadrant IV in water maze; (C) water maze trajectory representative maps; (D) escape latency in water maze; (E) latency in passive avoidance test; (F) errors in passive avoidance test; (G) retention time on the rod (Two-way ANOVA followed by Bonferroni post hoc for escape latency, One-way ANOVA followed by Bonferroni post hoc for the others; n = 8/group). Data are represented as mean ± S.E.M. *P < 0.05, **P < 0.01 versus sham group; #P < 0.05, ##P < 0.01 versus model group.*

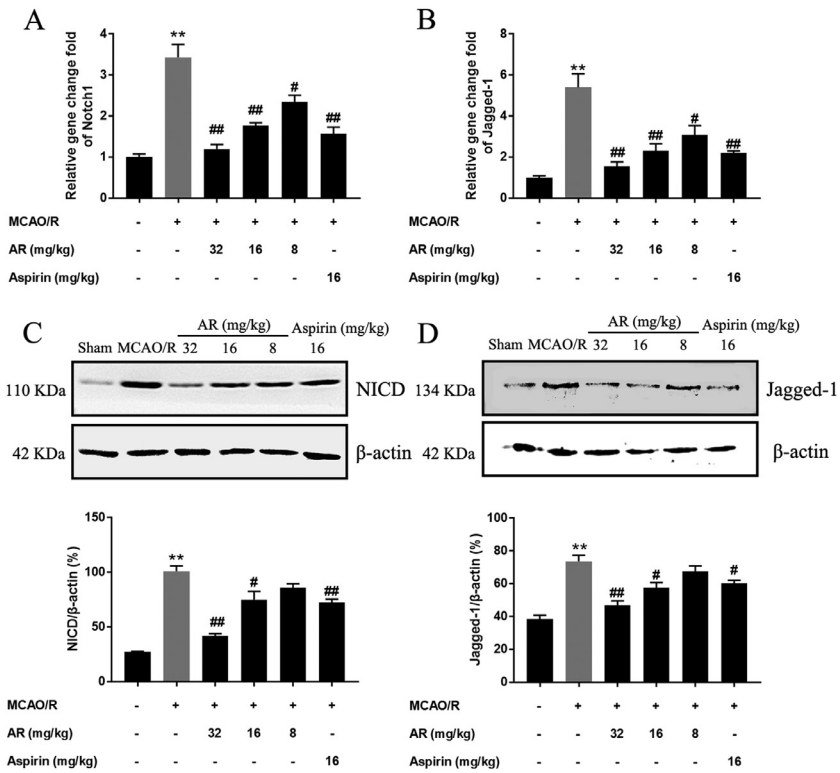

Figure 8. *Effect of AR on mRNA levels of (A) Notch1, (B) Jagged-1 and protein contents of (C) NICD, (D) Jagged-1 in hippocampus 14 days after cerebral ischemia/reperfusion in rats. (One-way ANOVA followed by Bonferroni post hoc; n = 3-5/group.) Data are represented as mean ± S.E.M. *P < 0.05, **P < 0.01 versus sham group; #P< 0.05, ##P < 0.01 versus model group.*

of rats after MCAO/R ($P < 0.01$, Fig 8, C,D). Among them, 32 mg/kg AR had the most obvious effect, and 16 mg/kg AR had the same effect with aspirin. This part further proved that AR may alleviate cerebral ischemia reperfusion injury in rats by inhibiting Notch signaling pathway.

AR Inhibited Notch/NF-κB Interaction in Hippocampus 14 Days after Cerebral Ischemia/Reperfusion in Rats

Mash1 and Hes1, as transcription factors in Notch signaling, play opposite roles in neuronal survival after cerebral ischemic injury,[9] in which ubiquitination and degradation of Mash1 by Notch-1 indicates more neuronal death while Hes1 is deleterious for neuronal survival and proliferation. The expression of downstream genes was proven to be able to induce NF-κB signaling pathway, which may further lead to inflammatory reactions and endanger neurons after ischemic stroke.[34] In this study, MCAO/R group showed less Mash1 and more Hes1 expression compared with sham group ($P< 0.01$, Fig 9, A,B), which can be reversed by AR treatment to different degrees. Meanwhile, NF-κB p65 subunit piled up more significantly in MCAO/R group and all AR dose groups lessened its nuclear import dose dependently, presumably reducing inflammatory reaction in conformity with reduced TNF-α and IL-6 releasing mentioned above.

Discussion

In this study, we reported for the first time the alleviating effect of 14 days' AR intragastric administration on cerebral ischemic injury in MCAO/R rats and explained that the mechanism of this protective effect may be related to the continuing inhibition of Notch/NF-κB signaling pathway to save neuronal loss and inhibit reactive astrocytes in later phase of ischemic stroke, which attenuated inflammatory response and rescued neuronal survival, consequently recovering motor, learning, and memory impairment in MCAO/R rats.

The administration dosage of AR was determined according to previous experiments[24] with the treatment period extended to 14 days. Studies have found that aspirin had a neuroprotective effect on cerebral ischemia aside from being an antiplatelet drug,[35] and the mechanism involved both NF-κB and Notch inhibition.[36,37] Therefore, we used aspirin (16 mg/kg) as a positive control.

The results showed that AR treatment significantly reduced infarct area, brain edema, and improved neurological function at seventh day of administration (Fig 2). Meanwhile, brain tissue loss was obviously observed in the MCAO/R rats, and after AR administration of 14 days, brain infarction rate as well as brain atrophy apparently decreased, with SOD level increased both in 7th and 14th day. Releasing of proinflammatory cytokines

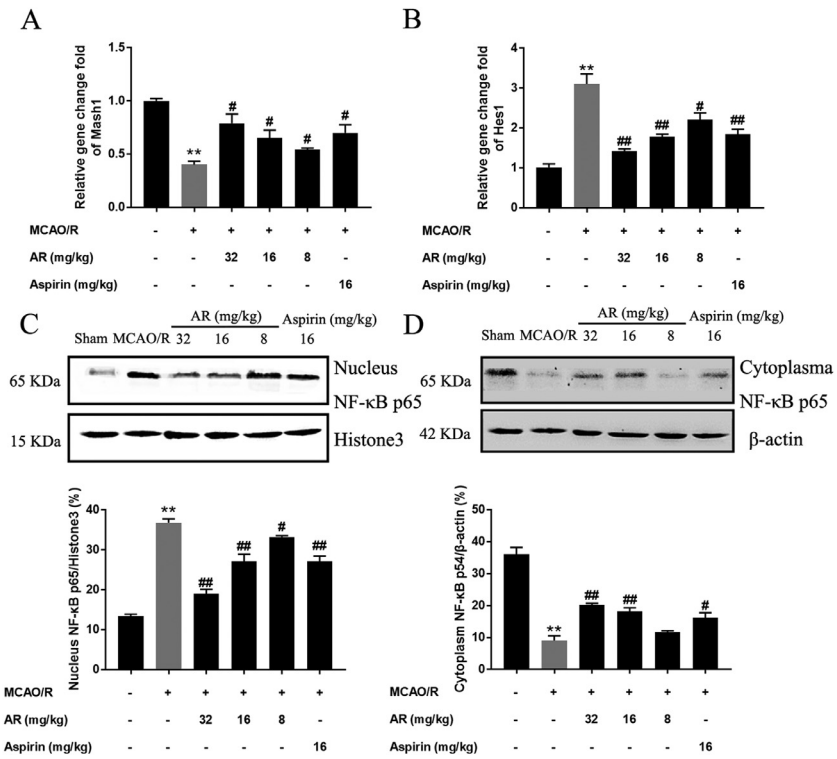

Figure 9. *Effect of AR on Mash1 and Hes1 expression and nucleus entry of NF-κB p65 subunit 14 days after cerebral ischemia/reperfusion in rats. (One-way ANOVA followed by Bonferroni post hoc; n = 3-5/group.) Data are represented as mean ± S.E.M. *P < 0.05, **P < 0.01 versus sham operation group; #P < 0.05, ##P < 0.01 versus model group.*

TNF-α and IL-6 significantly decreased while BDNF and NT-3 levels markedly increased, with 32 mg/kg and 16 mg/kg AR comparable to aspirin (Fig. 3 and 4). Therefore, we suggested that AR improved ischemic outcomes and played a long-term antioxidant stress, anti-inflammatory, and neuronal protective role, and speculated that the underlying mechanism was related to Notch and NF-κB pathways.

In ischemic stroke, hippocampal neurons are particularly important for spatial learning and memory. Therefore, it is of great significance to modify hippocampal neuronal injury and improve the cognitive and motor functions after ischemic stroke. Number of neurons and astrocytes in the hippocampal DG area of MCAO/R rats 14 days after AR administration showed that AR significantly maintained the number of hippocampal neurons in DG area ($P < 0.05$), especially in the CA1 area (Fig 5). At the same time, AR could reduce active astrocytes and glial scar after ischemic stroke which impeded nerve repair (Fig 6). Therefore, 14 days' treatment of AR rescued neuronal survival in cerebral ischemia/reperfusion rats. Besides, 32 mg/kg and 16 mg/kg AR could remarkably improve the performance of MCAO/R rats in Morris water maze, dark-avoid experiment and rotary rod experiment, promoting the recovery of learning, memory and motor functions (Fig 7).

Studies have shown that activation of the Notch signaling pathway enhances inflammatory responses and is

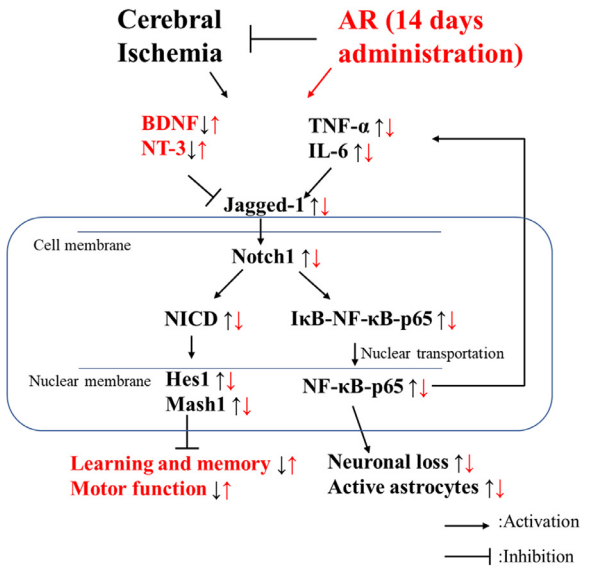

Figure 10. *The possible effects of Clematichinenoside (AR) on Notch/NF-κB pathways after cerebral ischemia/reperfusion.*

detrimental in neuronal survival following cerebral ischemia.[38] This may be related to NF-κB signaling pathway typically through lower nuclear transcription of NF-κB p65 subunit.[39] NF-κB pathway plays a key role in focal cerebral ischemia. Dissociation and degradation of I-κB induces transport of NF-κB from cytoplasm to nucleus, mediating harmful inflammatory effects, and

upregulating the expression of pro-inflammatory factors such as TNF-α and IL-6, which aggravate cerebral ischemia injury and promote neuronal necrosis and apoptosis.[40]

In our study, PCR and WB results showed that AR significantly downregulated the mRNA expression and protein expression of Notch-1, NICD, and Jagged-1 in the hippocampus of MCAO/R rats 14 days after administration (Fig 8). Similarly, AR enhanced the expression of Mash1, a preneuronal factor, and inhibited the expression of downstream Hes1, which acted synergistically with reduced NF-κB protein in nucleus ($P < 0.05$) and increased cytoplasmic NF-κB ($P < 0.05$, Fig 9), thus protecting the neurons in rats with cerebral ischemia, further attesting AR's anti-inflammatory effect and implied the relationship between Notch and NF-κB signaling. As a result, we speculated that AR could inhibit the Notch/NF-κB signaling pathway, inhibit the inflammatory response, and promote the survival of neurons.

Conclusions

Current research demonstrated that, as shown in Figure 10, 14 days' treatment of AR could reduce the prognosis of cerebral ischemia/reperfusion rats through Notch/NF-κB signaling pathway, improve the subacute stage and long-term learning, memory, and motor ability, as well as regulating neuronal survival after cerebral ischemia. The potential of AR in the treatment of cerebral ischemia reperfusion injury was further explored.

Author's Contributions

Conception and Design: Dan Xu, Nian Xia, Yun-man Li.

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Analysis and Interpretation of data: Dan Xu, Nian Xia.

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Administrative, Technical, or Material support: Ya-hui Hu, Wei-rong Fang, Yun-man Li.

Study Supervision: Wei-rong Fang, Yun-man Li.

Conflicts of Interest: The authors declare no conflict of interest.

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