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Original Research

Astragalus Polysaccharide Promotes Neuronal Injury Repair via the Notch1/NFκB Signaling Axis in the Ventroposterior Thalamic Nucleus in Rats with Focal Cerebral Ischemia

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Abstract

Background: Ischemic stroke is the most common form of stroke and the second most common cause of death and incapacity worldwide. Its pathogenesis and treatment have been the focus of considerable research. In traditional Chinese medicine, the root of Mongolian astragalus has been important in the treatment of stroke since ancient times. Astragalus polysaccharide (APS) is a key active ingredient of astragalus and offers therapeutic potential for conditions affecting the neurological system, the heart, cancer, and other disorders. However, it is not yet known how APS works to protect against ischemic stroke. **Methods**: Rats were subjected to middle cerebral artery occlusion (MCAO) to imitate localized cerebral ischemia. Each of four experimental groups (normal, sham, MCAO, and MCAO+APS) contained 12 adult male Sprague-Dawley (SD) rats selected randomly from a total of 48 rats. Following successful establishment of the model, rats in the MCAO+APS group received intraperitoneal injection of APS (50 mg/kg) once daily for 14 days, whereas all other groups received no APS. The Bederson nerve function score and the forelimb placement test were used to detect motor and sensory function defects, while Nissl staining was used to investigate pathological defects in the ventroposterior thalamic nucleus (VPN). Immunohistochemical staining and Western blot were used to evaluate the expression of Neurogenic locus notch homolog protein 1 (Notch1), hairy and enhancer of split 1 (Hes1), phospho-nuclear factor-κB p65 (p-NFκB p65), and nuclear factor-κB p65 (NFκB p65) proteins in the VPN on the ischemic side of MCAO rats. **Results**: APS promoted the recovery of sensory and motor function, enhanced neuronal morphology, increased the number of neurons, and inhibited the expression of Notch1/NFκB signaling pathway proteins in the VPN of rats with cerebral ischemia. **Conclusion**: After cerebral ischemia, APS can alleviate symptoms of secondary damage to the VPN, which may be attributed to the suppression of the Notch1/NFκB pathway.

Keywords: cerebral ischemia; astragalus polysaccharide; VPN; Notch1; Hes1; p-NFκB p65

1. Introduction

Ischemic stroke refers to cerebrovascular diseases that are caused by obstruction of cerebral artery blood flow leading to local brain tissue hypoxic-ischemic necrosis and to neurological defects. It is the second-leading cause of death and disability worldwide, with an increasing incidence [1,2]. Neuropathological studies have shown that the ipsilateral thalamus suffers secondary injury following middle cerebral artery occlusion (MCAO), which may result in thalamic shrinkage and reduced sensorimotor function after ischemia [3]. In a prior study, we also discovered that after cerebral ischemia, ventroposterior thalamic nucleus (VPN) neurons were harmed and diminished, which may be a significant contributing cause to the inadequate recovery of neurological function in clinical stroke patients.

Astragalus is the root of the leguminous plant astragalus mongholicus, and has long been used in Traditional Chinese medicine (TCM). It is the principal ingredient in the Buyang Huanwu decoction, a traditional TCM remedy for treating strokes. Previous pharmacological studies have shown that astragalus polysaccharide (APS), a key active ingredient of astragalus, can reduce the inflammatory response and immune dysfunction caused by cerebral ischemic injury, while also enhancing the index of immune organs and inhibiting the release of pro-inflammatory cytokines [4,5]. The Notch signaling axis is one of the most crucial pathways contributing to the long-term survival of neurons. Hairy and enhancer of split 1 (Hes1) and nuclear factor-κB (NFκB) are downstream target genes in this pathway that are induced by activation of Notch signaling [6]. The expression level of Hes1 correlates directly with the expression of Neurogenic locus notch homolog protein 1 (Notch1) [7]. NFκB is a key transcription factor that induces cell proliferation and differentiation, inflammatory immunity, and apoptosis, as well as playing a significant part in the pathological process of ischemic stroke [8,9]. The current study used a rat model of MCAO. APS was administered and its neuroprotective effect on secondary injury to the VPN after cerebral ischemia was investigated in this model, together with its effect on the Notch1/NFκB signaling pathway. We hope that the findings provide experimental support for the clinical use of APS.

2. Materials and Methods

2.1 Animals

Specific pathogen free (SPF) healthy male Sprague-Dawley (SD) rats (n = 48) with a body weight of 200 ± 20 g were purchased from the Qinglongshan Animal Experimental Center [SCXK, (Hangzhou, Zhejiang, China), 2019-0002]. Animals were placed in an experimental animal room of the Wannan Medical College and maintained at 24 ± 2 °C with a light/dark cycle of 12 h/12 h. Free feeding and drinking were allowed, and adaptive feeding was undertaken for one week. Following that, 12 rats were randomly assigned to each of four groups: normal, sham, MCAO, and MCAO+APS. The Laboratory Animal Welfare and Ethics Committee of Wannan Medical College evaluated and approved these animal studies (WNMC-AME-2023134).

2.2 Establishment of the MCAO Model

The MCAO model was established using the suture-occluded method [10]. Intraperitoneal injection with 1.5% pentobarbital sodium (2 mL/kg, AM00469, Beijing Chemical Reagent Company, Beijing, China) was first used to anesthetize rats. They were then fixed in a supine position on the operating table under a shadowless lamp and the neck skin was disinfected with iodophor. The layers of tissue were separated to expose the right common carotid artery (CCA) and a glass minute hand was used to carefully separate the vagus nerve and CCA. The separation was continued to the bifurcation of the internal carotid artery (ICA) and external carotid artery (ECA), the CCA and distal ECA were ligated, and a spare wire was set up at the proximal end. Ophthalmic scissors were used to make a tiny incision in the ECA so that a threaded plug could be inserted (A2-2432, Beijing Cinonech Co. Ltd, Beijing, China). The ECA was then cut off and the thread was looped into the ICA through the stump of the ECA. The insertion depth was 18.5 ± 0.5 mm until slight resistance was noted. The tip of the cord was passed through the beginning of the middle cerebral artery and the cord ligated. The incision was then sutured to avoid infection during and after the operation. In the sham group, the blood vessels were separated without being ligated. The room temperature was kept at 22 °C during the operation to allow animals to recover from anesthesia under temperature-controlled conditions. After they awoke from surgery (usually about 2 hours), neurological impairment was evaluated according to the Bederson scoring criteria [11]. Rats with scores of 1–3 were included in the study. Following successful establishment of the model, the MCAO+APS group was given an intraperitoneal injection of 50 mg/kg APS (purity >90%, A7970, Solarbio, Beijing, China) once a day for 14 consecutive days, as suggested in the literature [12]. The study designs are displayed in Fig. 1A.

2.3 Neurobehavioral Evaluation

Neurological impairment in rats was assessed 2 hours after surgery and on days 1, 3, 7, and 14. Neurological function was classified into four levels: 0, no neurological impairment; 1, the forepaw on the opposite side of the tail suspension test could not be fully extended; 2, the resistance of the opposite front paw to thrust decreased; 3, turned to the opposite side after placing.

The forelimb placement test was used to assess the comprehensive ability of visual and proprioceptive perception of rats on days 3, 7, and 14. A square grid (3 cm × 3 cm) was placed horizontally at a height of 1 meter above ground. On the frame, rats were gently pushed from behind to encourage them to crawl from one side of the grid surface to the other within 2 minutes. During the crawling process, one point was awarded if the forelimbs fell into the grid. The number of times the hemiplegic forelimbs fell into the net during the 2-minute crawl was recorded.

2.4 Nissl Staining

Following treatment, 6 rats in each group were injected intraperitoneally with 1.5% sodium pentobarbital (3 mL/kg) and 4% paraformaldehyde was then infused before brain extraction. Brain tissue was fixed in 4% paraformaldehyde. After dehydration with a sucrose gradient, the tissue was embedded using optimal cutting temperature compound (OTC) in a –20 °C frozen microtome (HM525NX, Thermo Fisher Science, Waltham, MA, USA). It was then cut into 20 µm slices and washed three times with phosphate buffered saline (PBS) for 5 min each before use. Filtered Nissl staining solution was then added dropwise to the tissue slices containing the VPN, and these were placed in a 37 °C incubator for 30 minutes. Excess staining agent was washed off with double distilled water and the sections were dehydrated, made transparent and sealed. Images were observed and recorded at 400× magnification. The morphology and quantity of Nissl-positive neurons in each visual field was examined using ImageJ software (1.41r, National Institutes of Health, Bethesda, MD, USA).

2.5 Immunohistochemical Staining

Frozen sections of brain tissue containing VPN were baked in an incubator in a 60 °C incubator for 2 hours. H_2O_2 (3%) was added to remove endogenous peroxidase and the slices were incubated for 10 minutes at 37 °C. To repair the antigen, the slices were boiled in 10 mM sodium citrate buffer (pH 6.0) for 2 minutes at high temperature and for 14 minutes at low temperature. Non-specific antibody binding was first blocked by incubating with 5% goat serum (AR0004, Boster Biotechnology, Wuhan, Hubei, China) for 30 min. Incubation with rabbit anti-Notch1 (1:200, 10062-AP, Proteintech, Wuhan, Hubei, China), rabbit anti-Hes1 (1:200, DF7569, Affinity Biosciences, Cincinnati, OH, USA), and rabbit anti-p-NFκB p65 (1:200, AF2006,

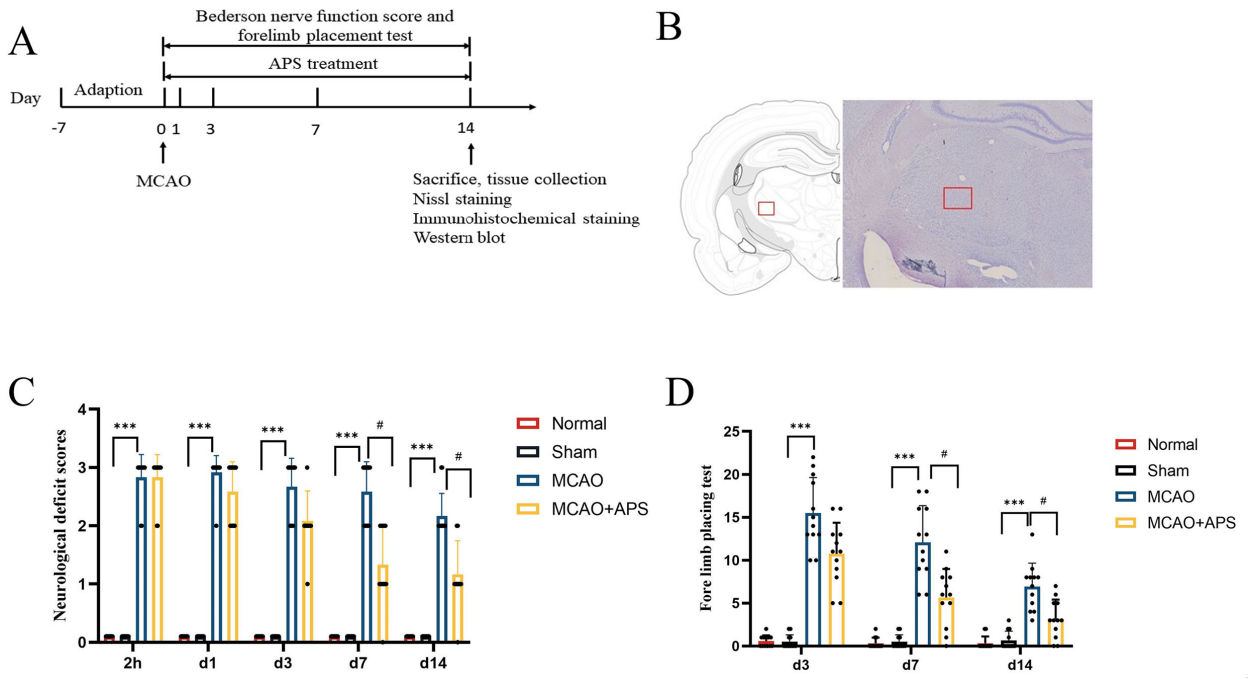

Fig. 1. Experimental design and behavioral tests. (A) Schematic of the study design. (B) Schematic diagram of statistical area for counting positive cells by Nissl and immunohistochemical staining. (C) Bederson nerve function score; n = 12 per group; ***$p < 0.001$, compared with the sham group; $^{\#}p < 0.05$, compared with the MCAO group. (D) Forelimb placement test; n = 12 per group; ***$p < 0.001$, compared with the sham group; $^{\#}p < 0.05$, compared with the MCAO group. MACO, middle cerebral artery occlusion; APS, Astragalus polysaccharide.

Affinity Biosciences, Cincinnati, OH, USA) was conducted at 4 °C. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and 3,3′-diaminobenzene (PA 110, TIANGEN Biotech, Beijing, China) were then used for staining and color development. The slices were permeated with xylene and sealed with neutral gum. ImageJ software was used to examine the expression of Notch1, Hes1, and phospho-nuclear factor-κB p65 (p-NFκB p65) proteins and to obtain a positive cell count.

2.6 Western Blot

The remaining 6 rats from each group were anaesthetized by intraperitoneal injection with 1.5% pentobarbital sodium. After decapitation, the brain was removed and placed on an ice box. According to a Stereotaxic map of rat brain described by Paxinos *et al.* [13], a surgical blade was used on an ice box to cut 2.16 and 3.84 mm behind the anterior fontanel of the brain tissue to collect contains VPN area of the brain tissue, and then the VPN was extracted with microscopic tweezers as described in Fig. 1B. Proteins from the VPN were extracted using radio immunoprecipitation assay (RIPA) lysis buffer, phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors. The sample protein (40 ug) was run for two hours on an sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis according to the molecular weight of the target protein. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using a continuous flow of 300 mA for 1.5 hours. The membranes were blocked for two hours before incubation overnight at 4 °C with primary antibodies against Notch1, Hes1, p-NFκB p65, NFκB p65 (1:1000, AF5006, Affinity Biosciences, Cincinnati, OH, USA), and GAPDH (1:1000, AF7021, Affinity Biosciences, Cincinnati, OH, USA). Following this, the membranes were incubated with goat anti-rabbit IgG (H+L) secondary antibodies (1:1000, A0208, Beyotime Biotechnology, Shanghai, China) for 1 hour at room temperature, washed, then analyzed with an ECL kit (P0018FS, Beyotime Biotechnology, Shanghai, China). GAPDH was used as the internal reference for Notch1 and Hes1, and NFκB p65 was used as the reference for p-NFκB p65. The strip gray values were quantified by ImageJ software.

2.7 Statistical Analysis

SPSS 26.0 software (IBM Corp., Chicago, IL, USA) was used for statistical analysis of the data, and GraphPad-Prism 9.0 (Dotmatics, Boston, MA, USA) for the creation of images. The measurement data were evaluated by a normality test and by a homogeneity test of variance. Data with a normal distribution were reported as the mean ± standard deviation. The K-W test was used to compare non-normally distributed data, with $p < 0.05$ used as the threshold for statistical significance.

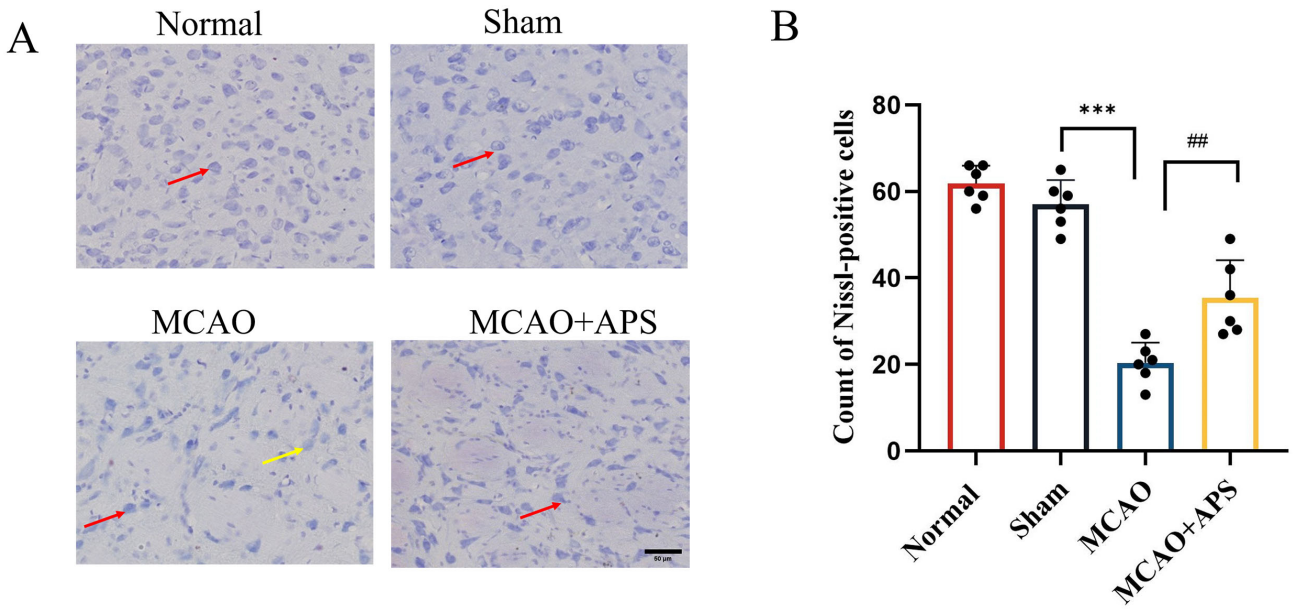

Fig. 2. Effect of APS on histopathological changes to ventroposterior thalamic nucleus (VPN). (A) Representative Nissl staining images of each group. (400×, 50 um scale) Red arrows show Nissl-positive neurons and yellow arrows show damaged neurons; n = 6 per group. (B) Statistical analysis of the number of Nissl-positive neurons in each group. ***$p < 0.001$, compared with the sham group; $^{\#\#}p < 0.01$, compared with the MCAO group.

3. Results

3.1 APS Induced the Restoration of Sensory and Motor Function in MCAO Rats

The Bederson nerve function score revealed that rats in the normal group and in the sham operation group had no neurological function defects (Fig. 1C). In contrast, the MCAO group had severe neurological damage and considerably greater neurological impairment scores than those of the sham group at 2 hours after cerebral ischemia and at days 1, 3, 7, and 14 ($p < 0.001$). The neurological function scores of the MCAO+APS group were lower than those of the MCAO group on days 7 and 14 ($p < 0.05$).

The forelimb placement test showed that rats in the normal and sham operation groups would grasp the grid line tightly when crawling, and almost never fall into the grid (Fig. 1D). Rats in the MCAO group showed a significantly higher number of hemiplegic forelimb falls into the grid on days 3, 7 and 14 compared to the sham group ($p < 0.001$). Rats in the MCAO+APS group showed significantly fewer hemiplegic forelimb falls into the grid on days 7 and 14 than those in the MCAO group ($p < 0.05$).

3.2 APS Effectively Improved Neuronal Injury in the VPN

Nissl staining revealed that neurons in the VPN of rats in the normal and sham groups showed a complete morphology, structure, and quantity, with clear outline, centered nuclei, and complete Nissl bodies (Fig. 2A,B). However, in the MCAO group, the volume of neurons in the affected side of the thalamus was smaller, the edge of the cell membrane was blurred, and both necrolysis and nuclear pyknosis were observed. Furthermore, the number of Nissl-positive neurons was significantly lower in the MCAO group than in the sham group ($p < 0.001$). The morphology of neurons in the MCAO+APS group was mostly normal, and the number of Nissl-positive neurons was higher than in the MCAO group ($p < 0.01$).

3.3 APS Inhibited the Expression of Notch1, Hes1 and p-NFκB p65 in the VPN of MCAO Rats

Immunohistochemistry was used to investigate the expression of Notch1, Hes1, and p-NFκB p65 in the right VPN of rats in each group (Fig. 3). Scattered positive cells showed a brown-yellow color. There were no discernible differences in expression between the normal and sham groups ($p > 0.05$). The MCAO group showed a higher number of positive cells for Notch1, Hes1, and p-NFκB p65 than the sham group ($p < 0.001$). However, the MCAO+APS group showed decreased expression of Notch1, Hes1, and p-NFκB p65 compared to the MCAO group ($p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively).

The results obtained with Western blot analysis mirrored those from immunohistochemistry (Fig. 4). The relative expression levels of Notch1 and Hes1 in the VPN were significantly higher in the MCAO group than in the sham group ($p < 0.01$), as was the expression of p-NFκB p65/NFκB p65 ($p < 0.05$). However, Notch1 and Hes1 expression in the VPN from the MCAO+APS group was significantly lower than that of the MCAO group ($p < 0.01$), while the expression of p-NFκB p65/NFκB p65 was also lower ($p < 0.05$).

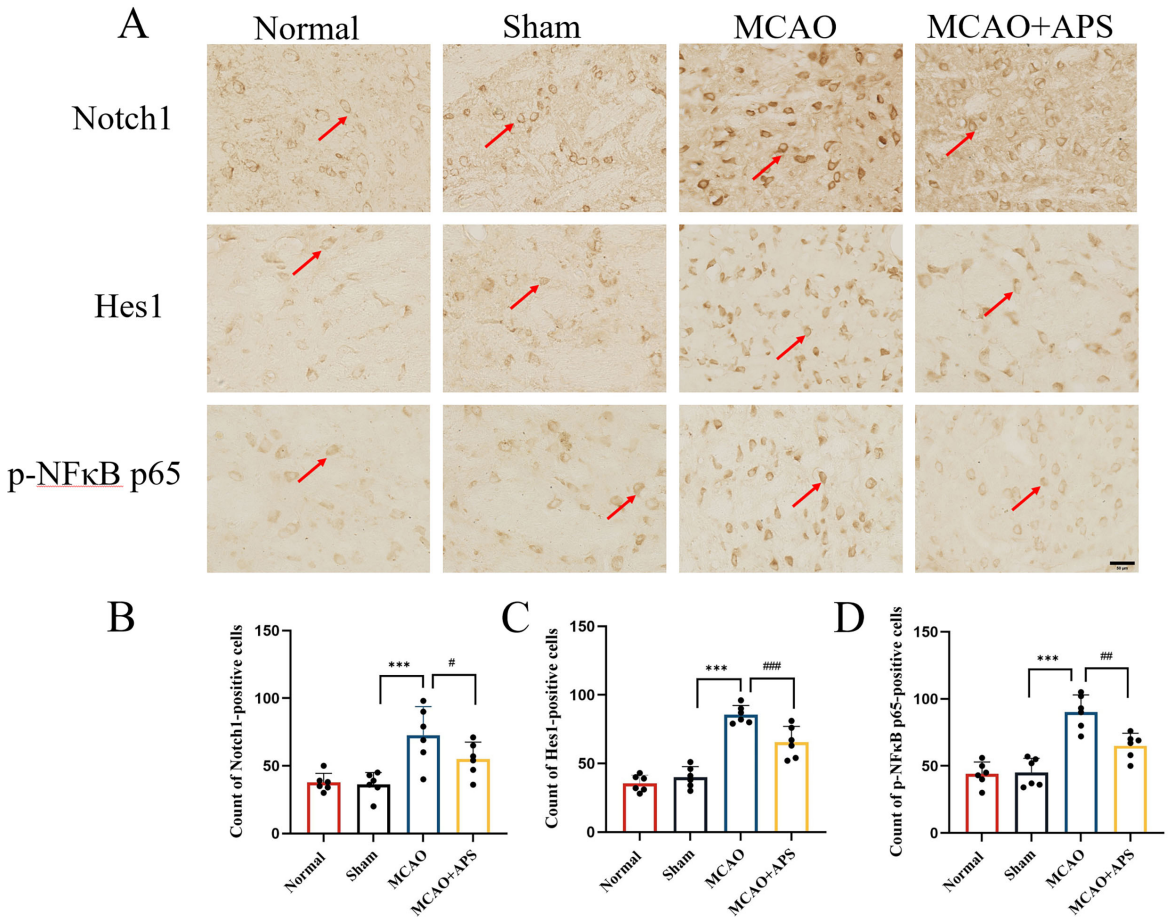

Fig. 3. Immunohistochemistry was used to investigate the effect of APS on the expression of Notch1, Hes1, and p-NFκB p65 in the VPN of rats with cerebral ischemia. (A) Representative immunohistochemical staining of Notch1, hairy and Hes1, and p-NFκB p65 of rats in each group (400×, 50 um scale); Red arrows show positive neurons. (B) The average positive cell count for Notch1 expression in the MCAO+APS group. ***$p < 0.001$, compared with the sham group; $^{\#}p < 0.05$, compared with the MCAO group. (C) The average positive cell count for Hes1 expression in the MCAO+APS group; ***$p < 0.001$, compared with the sham group; $^{\#\#\#}p < 0.001$, compared with the MCAO group. (D) Average positive cell count for p-NFκB p65 expression in the MCAO+APS group; ***$p < 0.001$, compared with the sham group; $^{\#\#}p < 0.01$, compared with the MCAO group; n = 6 per group. Notch1, Neurogenic locus notch homolog protein 1; p-NFκB p65, phospho-nuclear factor-κB p65; Hes1, hairy and enhancer of split 1.

4. Discussion

The incidence of stroke continues to increase [14]. However, therapeutic options for cerebral ischemia are very limited, and the recovery of neurological function in clinical stroke patients is often not ideal [15]. The search for effective treatment methods and drugs for ischemic stroke has therefore been a popular area of research. The MCAO model used in this study was demonstrated to be successful by the increased nerve defect score observed in these rats. Following APS treatment, the neurological deficit function score in the MCAO+APS group of rats decreased. Moreover, they experienced less falls of hemiplegic forelimbs into the grid, suggesting that APS can promote early recovery of sensorimotor function in rats. A previous study using a rat model of MCAO showed that APS could inhibit neuroimmune disorders and inflammation induced by cerebral ischemic injury, reduce the extent of edema in brain cells, and play a protective role in brain tissue [12]. This is consistent with the findings of the present study.

According to the literature and to previous laboratory studies, it is important to treat secondary damage in the thalamus following cerebral infarction. The thalamus is the sensory center under the cortex. In rat models of MCAO, secondary damage to the thalamus leads to cognitive dysfunction and sensory defects after focal cerebral infarction, with the damage occurring in the VPN [3,16–18]. Nissl staining in the present study showed damage to the morphological structure of neurons in the VPN region of the MCAO group, as well as a reduced number of Nissl-positive neurons. However, the morphological structure of cells in the ischemic region of the MCAO+APS group showed significant improvement compared to the MCAO group, together

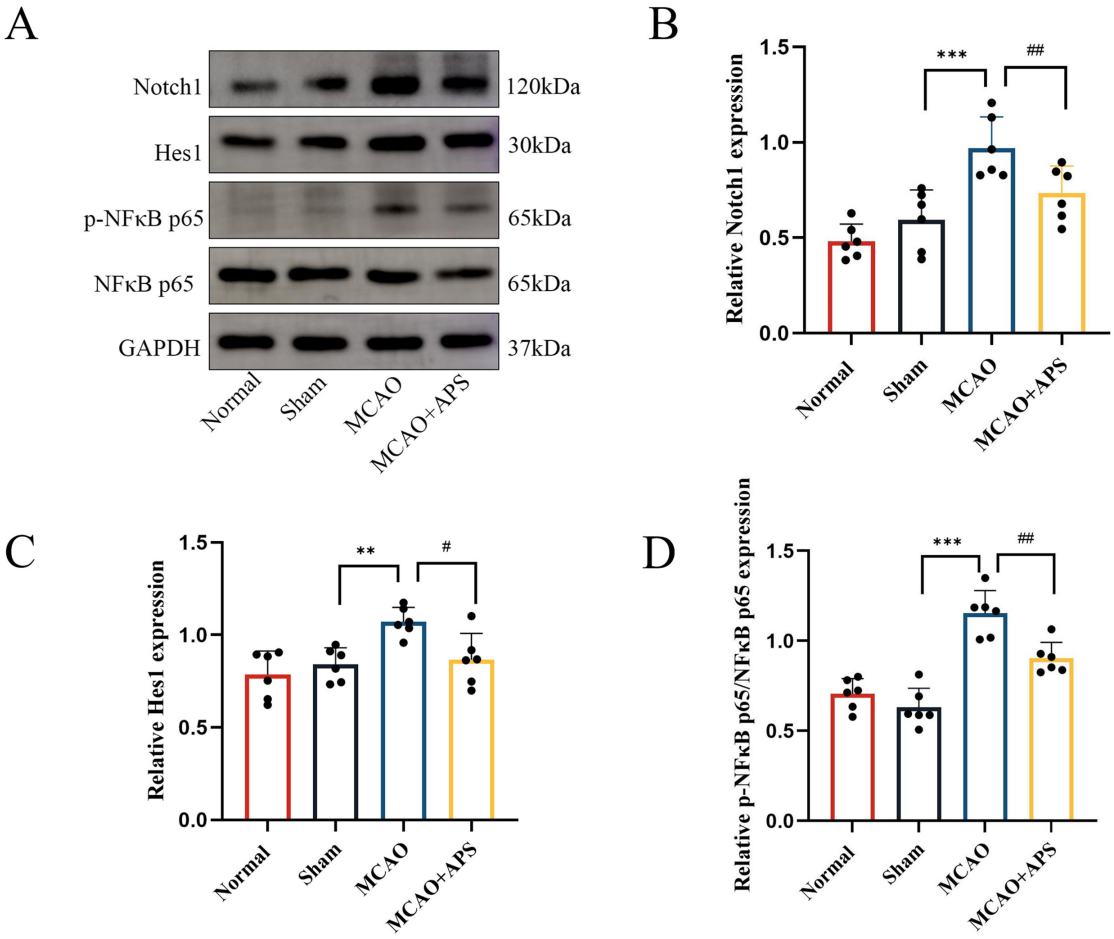

Fig. 4. Western blotting was used to determine the effect of APS on the expression of Notch1, Hes1, and p-NFκB p65 proteins. (A) Representative bands for Notch1, Hes1, p-NFκB p65, nuclear factor-κB p65 (NFκB p65), and GAPDH protein. (B) Quantitative results for Notch1, (C) Hes1, and (D) p-NFκB p65 protein; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with the MCAO group; n = 6 per group.

with an increased number of Nissl-positive neurons. These results suggest that APS could help to attenuate the damage to neurons caused by cerebral ischemia.

Inflammation occurs during the entire process of ischemic stroke, and the pathogenic mechanism of ischemic stroke injury is extremely complex [19]. The generation of free radicals and the resulting oxidative stress that occurs during ischemic stroke can cause brain damage and activation of an inflammatory response. Both the Notch signaling pathway and NFκB are associated with the inflammatory response after ischemic stroke [20,21]. The Notch intracellular domain is released into the cell and moves to the nucleus once the Notch signaling pathway is engaged, thereby inducing transcriptional activation and expression of Hes1 and NFκB [6]. Hes1 is commonly used as a biomarker for Notch1 pathway activation [22]. NFκB is a key transcription factor involved in the pathological development of ischemic stroke, cell proliferation and differentiation, as well as inflammatory immunity and apoptosis [14,23]. During cerebral ischemia, gamma-secretase is activated immediately and the Notch1 level increases, causing an increased level of p65 subunit in the p50/p65 dimer of NFκB. This induces leukocyte infiltration and increases the secretion of inflammatory factors such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, which then escalate the inflammatory response in the neuron [24]. Xu *et al.* [25] reported that Clematichinenoside could protect neurons in ischemic rats by inhibiting the Notch1/NFκB signaling pathway, thereby reducing the inflammatory response and the hence the extent of motor, learning and memory injury. The anti-inflammatory effect of Clematichinenoside is thought to be related to its suppression of Notch1/NFκB signaling pathway activation. Huang *et al.* [26] reported that APS causes liver cancer cell death by inhibiting the expression of Notch1. We predicted that the APS nerve protecting effect on cerebral ischemia rats would interfere with the Notch1/NFκB signaling pathway and so lessen inflammatory harm. In the present study, immunohistochemical and Western blot methods showed that Notch1, Hes1, and p-NFκB p65 expression levels in the VPN of MCAO experimental animals were higher than in the sham group. This indicates that thalamic ischemia may trigger activation of

the Notch1/NFκB signaling pathway. However, the APS-treated group showed lower expression levels for Notch1, Hes1, and p-NFκB p65 than the MCAO group. These findings imply that APS can reduce neuronal damage following cerebral ischemia, suppress Notch1/NFκB protein production in VPN during ischemia, and aid in the restoration of sensorimotor function in rats. Nevertheless, further experimentation is required to determine the optimal dosage of APS and to confirm that it prevents the release of downstream inflammatory factors in the Notch1/NFκB signaling pathway.

5. Conclusion

APS can promote the recovery of motor and sensory functions and attenuate secondary thalamic injury in rats with cerebral ischemia. This effect may be linked to the inhibition of Notch1/NFκB protein expression, thereby reducing neuronal injury in the VPN region of cerebral ischemia. These findings provide an experimental foundation for further work on the clinical application of APS for ischemic stroke.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HL, HM, and WL conceived and designed the experiment. WL and ZN conducted the experiments and analyzed the data. WL drew up the first draft. FW and HM analyzed the data and revised the manuscript. HL gave final approval to the upcoming release. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal studies were reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of Wannan Medical College (No.WNMC-AME-2023134).

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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