Effects of Zhuyu Annao on hippocampal neuronal apoptosis and cognitive disorder in a rat model of ischemic brain injury

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Abstract

Introduction: We aimed to determine the effects of Zhuyu Annao (ZYAN) on hippocampal neuronal apoptosis and cognitive disorder (CD) in ischemic brain injury (IBI).
Material and methods: The improved Rice-Vannucci method was used to prepare a rat model of IBI. Rats in the ZYAN group were intragastrically administered 12.50 g/kg of ZYAN 4 h after modeling; rats in the mock surgical group (MSG) and model group (MG) were administered an intraperitoneal injection of the same volume of normal saline twice daily.
Results: Compared with the MG, the area of cerebral infarction, pathological changes, neuron apoptosis index, and neurodeficit score were significantly lower in the ZYAN group ($p < 0.05$). The Morris water maze test showed that in the ZYAN group, the incubation period was significantly shortened and the number of platform crossings was significantly increased ($p < 0.05$). Furthermore, the rats in the ZYAN group showed significantly reduced protein and mRNA expression of cleaved caspase-3, Bax, p-53, and p38 mitogen-activated protein kinase (MAPK) in the hippocampus, whereas the protein and mRNA expression levels of Bcl-2 were significantly increased ($p < 0.05$).
Conclusions: ZYAN reduced the area of cerebral infarction, attenuated neuronal apoptosis, and alleviated CD in the rats with IBI. The beneficial effect of ZYAN in IBI may be mediated via inhibition of the p38 MAPK signaling pathway and caspase-3 cascade reaction.

Key words: Zhuyu Annao, ischemic brain injury, hippocampus, neuronal apoptosis, cognitive disorder.

Introduction

Ischemic stroke (IS) is one of the main causes of disability and death in adults. According to estimates, a case of symptomatic stroke is observed every 2 s; therefore, it is considered as one of the major public health issues [1, 2]. Currently, common treatment methods for IS include

thrombolysis, reduction of intracranial pressure, use of neuroprotective agents, and removal of hematoma. However, treatment efficacy is typically poor because of the short therapeutic time window and adverse effects of drugs; thus, approximately 70–80% of stroke survivors suffer from cognitive disorder (CD), which seriously affects their prognosis and quality of life [3–5]. Therefore, elucidation of the pathogenic mechanisms underlying post-IS neuronal damage and CD and development of more effective therapies are key research imperatives.

A correlation has been demonstrated between ischemic brain injury (IBI) and mitochondrial dysfunction, cell apoptosis, oxygen radical activation, and inflammatory reaction [6]. Necrosis and apoptosis are two forms of neuronal death, with apoptosis being the main form of neuronal death in IBI. Other studies have revealed that hyperphosphorylation of the hippocampal Tau protein is closely associated with CD [7]. Zhuyu Annao (ZYAN) is a traditional Chinese medicine (TCM)-formula granule manufactured by Jiangyin Tian Jiang Pharmaceutical Co. Ltd. It is based on the ZYAN pill developed by chief physician Wu Zihui (a distinguished veteran TCM practitioner in Guangxi). ZYAN has been widely used in the treatment of craniocerebral injury and CH encephaledema. Increasing evidence has shown that ZYAN may alleviate IBI through inhibition of cell apoptosis and anti-inflammatory action [8]. In a preliminary clinical study, neurological function defect score, cerebral hematoma absorption rate, and cerebral edema were significantly improved in the acute stage of hemorrhagic stroke; the total effective rate and basic cure rate were 92.50% and 47.50%, respectively [9]. The p38 mitogen-activated protein kinase (MAPK) signaling pathway and caspase-3 cascade reaction are the most common mechanisms involved in neuronal apoptosis. Based on these findings, we hypothesized that ZYAN plays a role in improving neuronal apoptosis in IBI through activation of the p38 MAPK signaling pathway and caspase-3 cascade reaction. In this study, we sought to explore new targets for ZYAN and elucidate its mechanism of action. In particular, we investigated the effects of ZYAN on hippocampal nerve cell apoptosis and CD in rats with IBI; additionally, we explored its potential neuroprotective effect to provide evidence for clinical practice.

Material and methods

Material

A total of 72 clean-grade male Sprague–Dawley rats (age: 18 months; weight: 270–300 g) were purchased from Laboratory Animal Centre, Chongqing Medical University. The rats were given ad libitum access to food and water and were housed in a controlled environment (temperature: 21–23°C; relative humidity: 40–60%). The rats were randomly assigned to a mock surgical group (MSG; n = 24), model group (MG; n = 24), or ZYAN group (n = 24) using the random number table method. All procedures were approved by the Animal Care and Use Committee of The First Affiliated Hospital of Guangxi University of Chinese Medicine.

Reagents used

The ZYAN was developed into a granule formula (*Hirudo* leech; woodlouse; earthworm, 10 g; peach seed, 10 g; *Campsis grandiflora*, 10 g; *Herba lycopi*, 10 g; wine-treated rhubarb, 10 g; *Rhizoma chuanxiong*, 10 g; *Polyporus umbellatus*, 10 g; and *Rhizoma alismatis*, 10 g) by Jiangyin Tian Jiang Pharmaceutical Co. Ltd. The Morris water maze system was purchased from Shanghai Jiliang Software Technology Co. Ltd.; triphenyltetrazolium chloride (TTC) from Shanghai Hualan Chemical Technology Co. Ltd.; Nissl staining solution from Beijing Solarbio Science and Technology Co. Ltd.; TUNEL assay kit from Roche; cell counting kit-8 from Dojindo; SDS-PAGE kit from Wuhan Servicebio Technology Co. Ltd.; BCA protein assay kit from Bio-Rad; phenylmethylsulfonyl fluoride, phosphorylase inhibitor, PIRA lysate, developer, and fixer kit for black and white film and paper and ECL from Wuhan Servicebio Technologies Co. Ltd.; polyvinylidene fluoride membrane from Millipore; and β-actin antibody, cleaved caspase-3 (activated), monoclonal antibody (rabbit anti-rat), Bcl-2 monoclonal antibody (rabbit anti-rat), Bcl 2-associated X protein monoclonal antibody (rabbit anti-rat), p53 (rabbit anti-rat), p-p38 monoclonal antibody (rabbit anti-rat), and MAPK monoclonal antibody (rabbit anti-rat) from Sigma.

Establishment of animal models and administration methods

The improved Rice-Vannucci method [10] was used to prepare the rat model of IBI. Except for the rats in the MSG, those in the MG and the ZYAN group were anesthetized via inhalation of diethyl ether. After anesthesia, the rats were placed in the supine position, and their neck skin was exposed and incised layer by layer to separate the tissues. Then, in the rats of all the groups, except those of the MSG, the common carotid arteries of both sides were exposed, ligated, and sutured. After successful modeling, the rats in the ZYAN group were intragastrically administered 12.50 g/kg of ZYAN twice a day through the mouth using a 20-gauge gavage needle. The rats in the MSG and MG were intraperitoneally administered

the same concentration of normal saline. All the groups were administered the indicated drugs for 7 consecutive days. The grouping and dosage administered to the rats are shown in Table I.

Table I. Grouping and dosage of rats

Group	*n*	Dosage
MSG	24	–
MG	24	–
ZYAN	24	12.50 g/kg

MSG – mock surgical group, MG – model group, ZYAN – Zhuyu Annao.

Neurologic deficit and behavioral test

Eight rats were selected from each group, and the Morris water maze (MWM) method was used to assess behavioral changes. The MWM apparatus consists of a circular pool measuring 120 × 60 cm, with a cylindrical 10 × 40 cm PMMA platform above it. The room and water temperatures were maintained at 24–26°C. MWM training was conducted 5 days before the modeling and four times daily 1, 2, 3, 4, 5, 6, and 7 days after the modeling. During each training, the rats were placed in the water with their head toward the pool wall, at a half radian of the quadrant farthest from the platform; the route taken by the rats in the pool before they found the platform in the water was observed, and this period was termed the incubation period (IP). The longest swimming time was < 90 s. The platform was removed after the rats found it. The rats were subsequently placed at the midpoint of the quadrant opposite to the platform denoted as the crossing platform to record the number of platform crossings performed by the rats within 90 s. In addition, the Garcia reporting method was used for scoring the nerve function 7 days after the administration of drugs, and the following aspects were evaluated: autonomic activity, symmetrical motion, symmetrical abduction of forelimbs, climbing, response to touching of body with a blunt rod, and response to touching of whiskers with a blunt rod. The scores ranged from 3 to 18 (18 representing normal function; the higher the scores, the lesser the nerve function defect).

Measurement of the area of cerebral infarction using TTC

After administration of the drugs for 7 days, eight rats in each group were anesthetized using an intraperitoneal injection of 10% chloral hydrate (3.50 ml/kg). Subsequently, the rats were decapitated, their brains were collected, and 2-mm-thick coronal brain slices were prepared. The slices were immersed in 2% TTC staining solution and subsequently incubated in the dark at 37°C for 20 min. PBS buffer solution was used to wash off the TTC solution, after which the slices were placed in 4% paraformaldehyde and fixed in the dark at 4°C. Images were captured after 24 h. Image J software was used to analyze the area of cerebral infarction; the percentage of the area of cerebral infarction was calculated using the following formula: area of cerebral infarction% = (left cerebral hemisphere size - size of noninfarcted right cerebral hemisphere) / 2 × size of left cerebral hemisphere × 100.

Preparation of tissue slices and Nissl staining

After administration of the drugs for 7 days, eight rats selected from each group were anesthetized. The rats were decapitated, and their brains were collected. The olfactory bulb, cerebella, and lower brain stem were removed. The brain tissues were immersed in 4% paraformaldehyde, fixed for 60 min, and soaked in 30% sucrose solution. Subsequently, 5-µm-thick slices were prepared and used for Nissl staining, immunohistochemical staining, and cell apoptosis detection. The Nissl staining revealed the pathological changes in the tissues, which were examined under a light microscope.

Detection of hippocampal neuron apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling staining method

As per the deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay kit's instructions, the tissues were stained using a DAPI working solution for 10 min at 37°C and washed three times with PBS buffer solution for 5 min. Subsequently, the slices were sealed and fixed. A fluorescence microscope was used to detect hippocampal neuron apoptosis; the number of TUNEL-positive (apoptotic) cells was observed under a high-power field (× 400). A total of 200 cells were counted, and the number of normal cells differing in their nuclei from those of the apoptotic cells was recorded.

Apoptosis index (AI) = number of TUNEL-positive cells / total number of cells × 100%. Five fields were randomly selected from each slice, and the mean AI value was used in the analysis.

Western blot

The brain tissues were excised and homogenized on ice. A protein extraction buffer was used for total protein extraction. Then, the BCA assay method was used to determine the protein concentration and calculate the sample loading quan-

tity. Separation and spacer gels were prepared, followed by SDS-PAGE electrophoresis, membrane transfer, sealing, and incubation with primary antibodies (cleaved caspase 3, Bcl-2, Bax, p53, p-p38, and MAPK all referring to goat anti-rat) and secondary antibodies (goat anti-rat IgG with fluorescence labeling, 800 pathway). Finally, grayscale values were detected using the Odyssey Infrared Imaging program (LI-COR Biosciences).

Fluorescence quantitative polymerase chain reaction

The brain tissues were removed, frozen in liquid nitrogen, and ground into powder. Total RNA was extracted with TRIZOL reagent for quantitative analysis. After determining that the A260/A280 was 1.8–2.0, 1 ng of the RNA sample, 1 µl of oligo (dT), RNase-free dH_2O, 0.5 µl of dNTP mixture, 2 µl of 5 × M-MLV buffer, 0.25 µl of RTase M-MLV, and 0.25 µl of RNase inhibitor were used to prepare the sample, which was then incubated at 42°C for 1 h, followed by 70°C for 15 min, then ice for 5 min. Subsequently, polymerase chain reaction (PCR) detection was performed. The PCR reaction mixture comprised 50 ng of cDNA, 0.5 µl of upstream and downstream primers, 25 µl of FastStart Universal SYBR Green Master (ROX), and RNase-free dH_2O, made up to a 50 µl sample volume. The reaction conditions were as follows: predenaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s, performed for a total of 40 cycles. $2^{-\Delta\Delta Ct}$ was used to calculate the mRNA levels of cleaved caspase-3, Bax, p53, p38 MAPK, and Bcl-2. The primer sequences used were as follows:

Cleaved caspase-3: Forward: 5′-GCAGCAGCCTCAAATTGTTGAC-3′, Reverse 5′-TGCTCCGGCTCAAACCATC-3′, 205 bp; Bax: Forward 5′-GGATGCGTCCACCAAGAA-3′, Reverse 5′-TCCCGGAGGAAGTCCATT-3′, 148 bp; Bcl-2: Forward 5′-CCTTTGTGTAACTGTACGGCC-3′, Reverse 5′-CTTTGGCAGTAAATAGCTGATTCGAC-3′, 270 bp; p53: Forward 5′-TATGGCTTCCACCTGGGCTT-3′, Reverse 5′-TCTTCCAGATACTCGGGATACAA-3′, 306 bp; p38MAPK:Forward5′-TGCCCGAACGTACCAGAAC-3′, Reverse 5′-CCTTTTGGCGTGAATGATGGA-3′, 142 bp; β-actin; Forward 5′-CTCCATCCTGGCCTCGCTG-3′, Reverse 5′-GCTGTCACCTTCACCGTTCC-3′, 268 bp. The primers were synthesized by Sangon Biotech (Shanghai) Co. Ltd.

Statistical analysis

All data analyses were performed using SPSS 25.0 statistical software. Continuous variables are presented as mean ± standard deviation (mean ± SD), and between-group differences were assessed using one-way ANOVA. For pair-wise comparisons, the least significant difference method was used in the case of homogeneous variance, whereas the Dennett T3 method was used in the case of heterogeneous variance. $P < 0.05$ was considered statistically significant.

Results

ZYAN improved neurodeficit score and spatial learning and memory ability

As shown in Figure 1 A, compared with the rats in the MSG, the neurodeficit score (NDS) was significantly lower in the MG; however, NDS was significantly higher in the ZYAN group compared with that in the MG ($p < 0.05$), suggesting that ZYAN may alleviate nerve defect in rats with IBI.

In the MWM test, the IP of the MG was significantly longer than that of the MSG on days 2 and 3; on day 7, the IP was significantly shorter in the ZYAN group than that in the MG ($p < 0.05$; Figure 1 B–D). Moreover, compared with the MSG, the number of platform crossings was significantly higher in the MG but was significantly lower in the ZYAN group compared with that in the MG ($p < 0.05$), suggesting that ZYAN may improve spatial learning and memory ability (LMA) in rats with IBI and promote the recovery of nerve injury.

ZYAN reduced the area of cerebral infarction

Approximately 48 h after the modeling, the rats were decapitated and their brains collected. The brains appeared abnormal (Figure 2 A); in the MG and the ZYAN group, both sides of the brain were asymmetrical. In addition, there was obvious edema and local whiteness in the right cerebral hemisphere; however, in the MSG, both sides of the brain were symmetrical, with no whiteness or edema. TTC staining revealed obvious infarction in the brain tissues of the MG and the ZYAN group but not in those of the MSG. As shown in Figure 2 B, C, compared with the MSG, the brain water content (BWC) and area of cerebral infarction were significantly higher in the MG and the ZYAN group ($p < 0.05$); however, the BWC and area of cerebral infarction were significantly lower in the ZYAN group than in the MG ($p < 0.05$), suggesting that ZYAN may reduce the cerebral edema volume and the area of cerebral infarction in rats with IBI.

ZYAN alleviated hippocampal nerve cell damage

According to the Nissl staining results obtained 48 h after the modeling, in the MSG, the nerve cells in the hippocampus CA1 and cortical areas were arranged in neat rows; in addition, pyramid-shaped

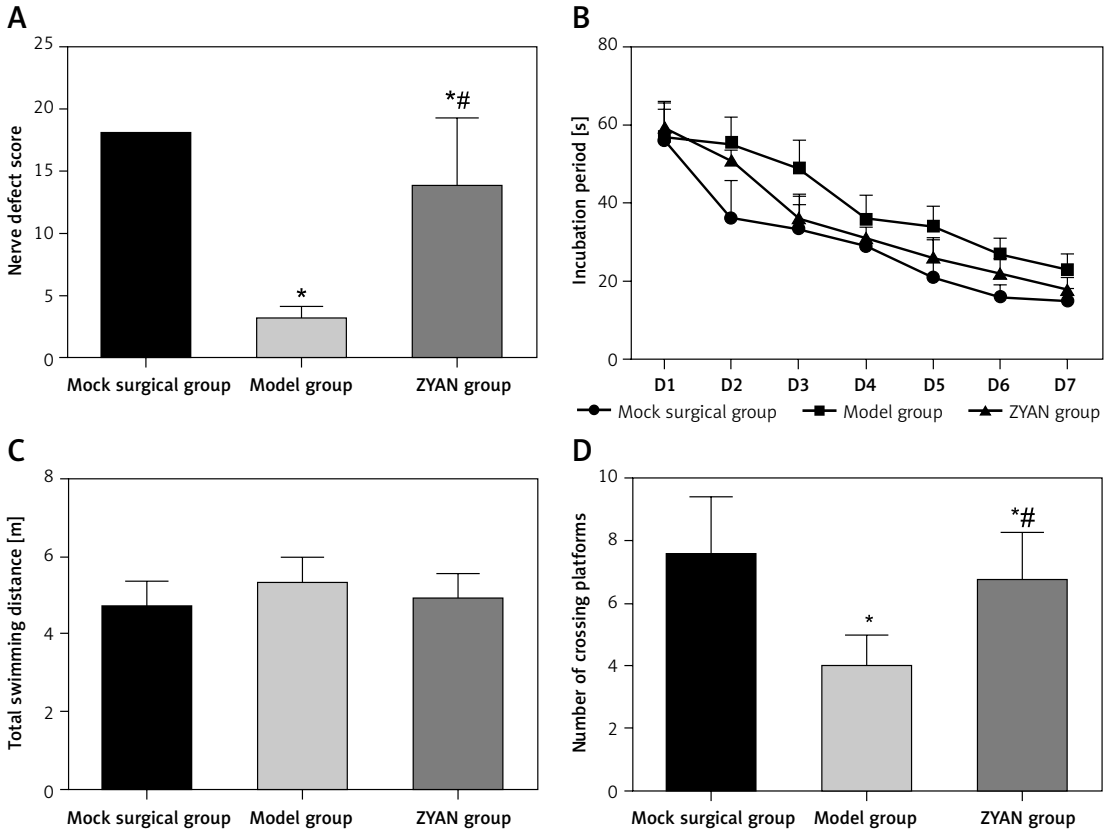

Figure 1. Effect of ZYAN on nerve function and spatial LMA in rats with IBI. **A** – NDS; **B** – IP results during the detection of spatial LMA through MWM; **C** – total swimming distance; **D** – number of platform crossings

[a]p < 0.05 refers to the comparison with MSG, [b]p < 0.05 refers to the comparison with MG. LMA – learning and memory ability, NDS – neurodeficit score, MWM – Morris water maze, IP – incubation period, MSG – mock surgical group, MG – model group, ZYAN – Zhuyu Annao.

cells with a clear structure were observed, and a violet Nissl body was observed in the cytosol. However, in the MG and the ZYAN group, the nerve cells in the hippocampus CA1 and cortical areas showed ischemic changes; there appeared to be cellular edema with a loose and irregular tissue structure. Moreover, the Nissl body was not observed in some nerve cells (Figure 3 A, B). Based on the statistical analysis, the number of Nissl-positive cells in the hippocampus CA1 and cortical areas were lower in the MG and the ZYAN group compared with that in MSG; however, the number of Nissl-positive cells was significantly higher in the ZYAN group compared with that in MG ($p < 0.05$, Figure 3 C, D), suggesting that ZYAN may reduce ischemic injury of hippocampal nerve cells and restore their normal structure in rats with IBI.

ZYAN inhibited hippocampal neuronal apoptosis

In Figure 4, the apoptotic cells show brown nuclear staining or deposits of brown particles. Compared with the MSG, the neuron count and AI in the hippocampus CA1 were significantly higher in MG and the ZYAN group ($p < 0.05$); however, the neuron count and AI in the ZYAN group were significantly lower than those in the MG ($p < 0.05$), suggesting that ZYAN may inhibit hippocampal neuronal apoptosis and promote the recovery of neuron injury.

ZYAN reduced expression of apoptosis-related proteins

As shown in Figure 5, compared with MSG, the protein expression levels of cleaved caspase-3, Bax, p53, and p38 MAPK in the hippocampus were significantly increased in the MG; however, the protein expression level of Bcl-2 in the MG was significantly reduced ($p < 0.05$). Compared with the MG, the protein expression levels of cleaved caspase-3, Bax, p53, and p38 MAPK in the hippocampus were significantly reduced and the protein expression level of Bcl-2 was significantly increased in the ZYAN group ($p < 0.05$), suggesting that ZYAN may reduce the levels of caspase-3, Bax, p53, and p38 MAPK proteins in the hippocampal tissues and increase the levels of Bcl-2, thereby inhibiting neuronal apoptosis.

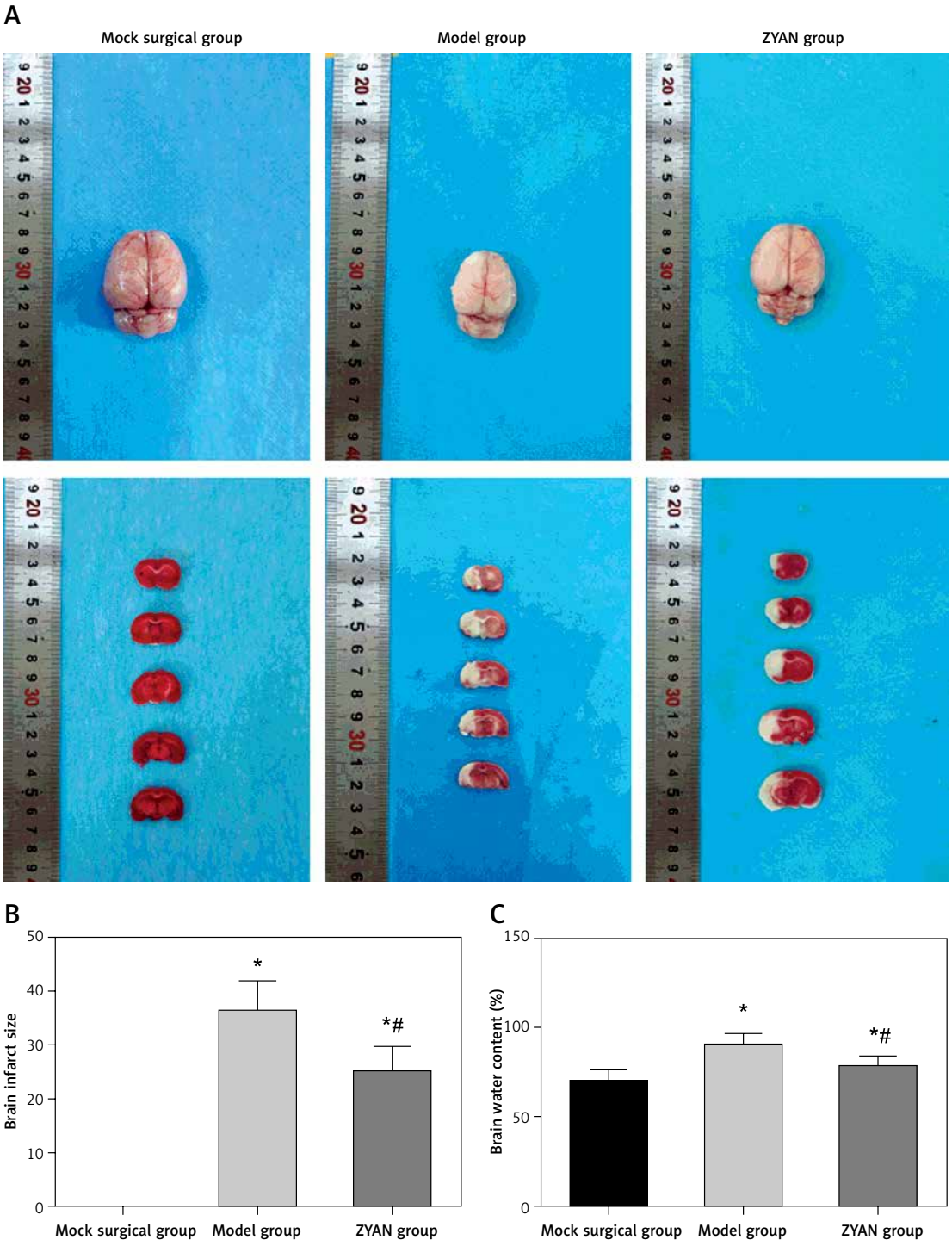

Figure 2. ZYAN reduces the BIS of rats. **A** – Gross morphological changes and TTC staining of brain tissues: the white area indicates infarcted area, whereas the red area indicates normal brain tissues; **B** – comparison of BIS; **C** – comparison of BWC. A, B, and C refer to MSG, MG, and ZYAN, respectively

˚p < 0.05 refers to the comparison with MSG, #p < 0.05 refers to the comparison with MG. TTC – triphenyltetrazolium chloride, BWC – brain water content, MSG – mock surgical group, MG – model group, ZYAN – Zhuyu Annao.

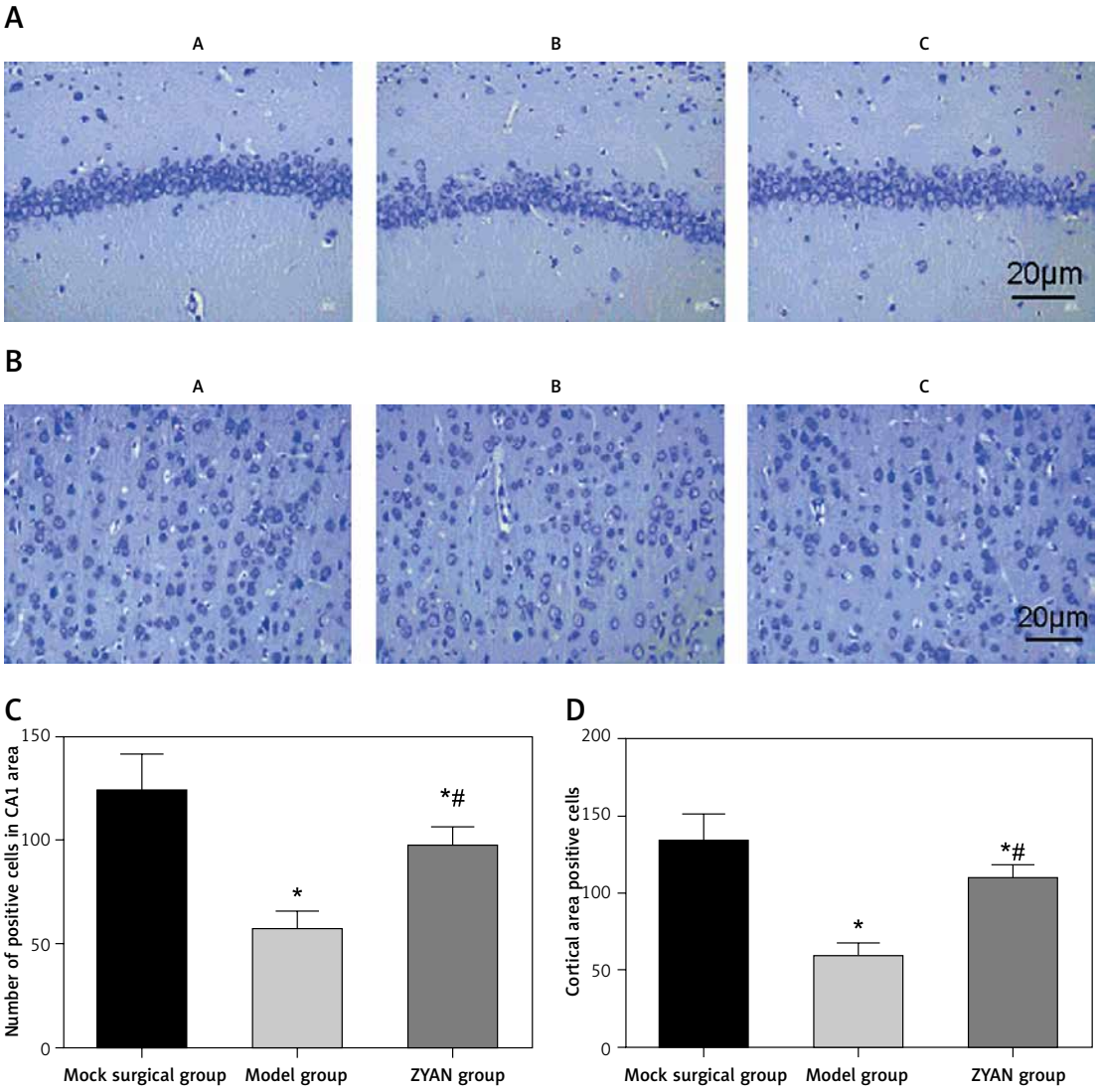

Figure 3. Pathomorphology of the hippocampus CA1 area and cortical area in all groups (Nissl staining × 400). **A** – Nissl staining of hippocampus CA1 area; **B** – Nissl staining of cerebral cortex; **C** – number of positive cells in the hippocampus CA1 area; **D** – number of positive cells in the cerebral cortex. A, B, and C represent MSG, MG, and ZYAN, respectively, scale: 20 μm

$^{*}p < 0.05$ refers to the comparison with MSG, $^{\#}p < 0.05$ refers to the comparison with MG. MSG – mock surgical group, MG – model group, ZYAN – Zhuyu Annao.

ZYAN inhibited expression of apoptosis-related genes

As per the fluorescent quantitative (FQ)-PCR results, the mRNA expression levels of cleaved caspase-3, Bax, p53, and p38 MAPK in the hippocampus were significantly increased and that of Bcl-2 was significantly reduced in the MG ($p < 0.05$). Compared with the MG, the mRNA expression levels of cleaved caspase-3, Bax, p53, and p38 MAPK in the hippocampus were significantly lower and that of Bcl-2 was significantly higher in the ZYAN group ($p < 0.05$), suggesting that ZYAN may inhibit the expression of apoptosis-related genes in the hippocampal neurons. These results were consistent with the Western blot results (Figure 6).

Discussion

IS can cause damage to the neurons and white matter, which is the main reason for death and neurologic deficit in patients with IS [11]. Currently, a large number of basic and clinical studies on IS are ongoing [12]. Although the pathogenesis of IS has been understood to a certain extent, the pathophysiological process of IS involves complex cascade reactions in time and space; thus, there is a paucity of effective treatment modalities for IS. In this study, we investigated the effects of ZYAN on cognitive impairment and apoptosis of the hippocampus nerve cells in IBI. The results showed that ZYAN reduced neuronal apoptosis and alleviated cognitive impairment in the rats with IBI.

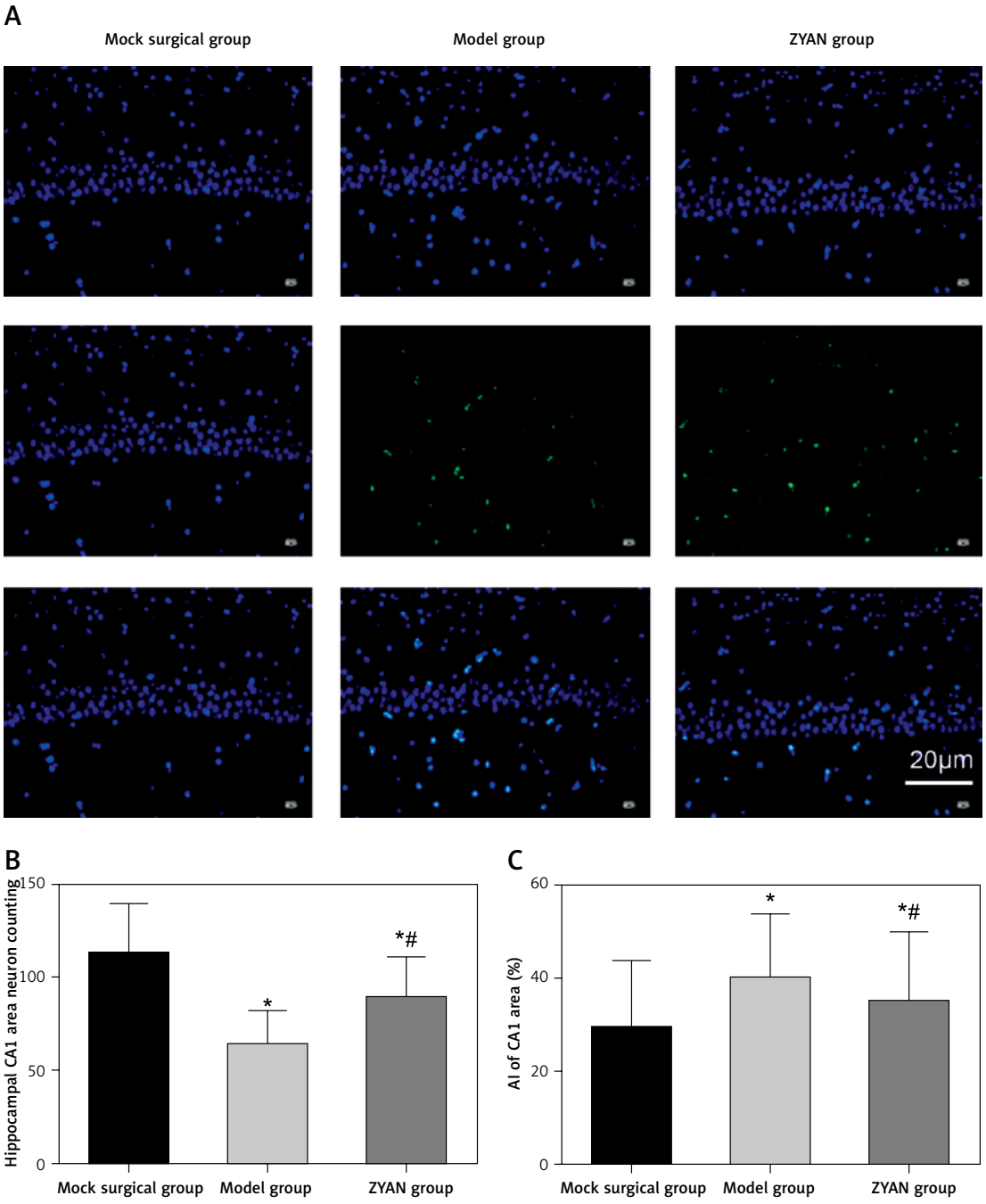

Figure 4. Detection of cell apoptosis in the hippocampus CA1 area of all groups using the TUNEL method (× 400). **A** – Cell apoptosis in the hippocampus CA1 area; **B** – neuron count in the hippocampus CA1 area; **C** – AI in the hippocampus CA1 area. A, B, and C represent MSG, MG, and ZYAN, scale: 20 μm

[a]p < 0.05 refers to the comparison with MSG, [b]p < 0.05 refers to the comparison with MG. MSG – mock surgical group, MG – model group, ZYAN – Zhuyu Annao.

Chen *et al.* [13] stated that Toll-like receptor 4 (TLR4) mediates the inflammatory injury caused by CH. In addition, ZYAN exhibited neuroprotective effects by reducing TLR4 expression to alleviate encephaledema and neuronal apoptosis. In this study, we established a rat model of cerebral ischemia (CI) and assessed the effects of ZYAN on the model. Compared with the MSG, the MG exhibited deterioration in CD, with a significant increase in the area of cerebral infarction, hippocampal nerve cell damage index, and hippocampal nerve cell AI; however, ZYAN significantly alleviated CD, with a decrease in the area of cerebral infarction, hippocampal nerve cell damage index, and hippocampal nerve cell AI. These findings indicate a neuroprotective effect of ZYAN in IBI, which is consistent with the abovementioned reports. However, the mechanism by which ZYAN

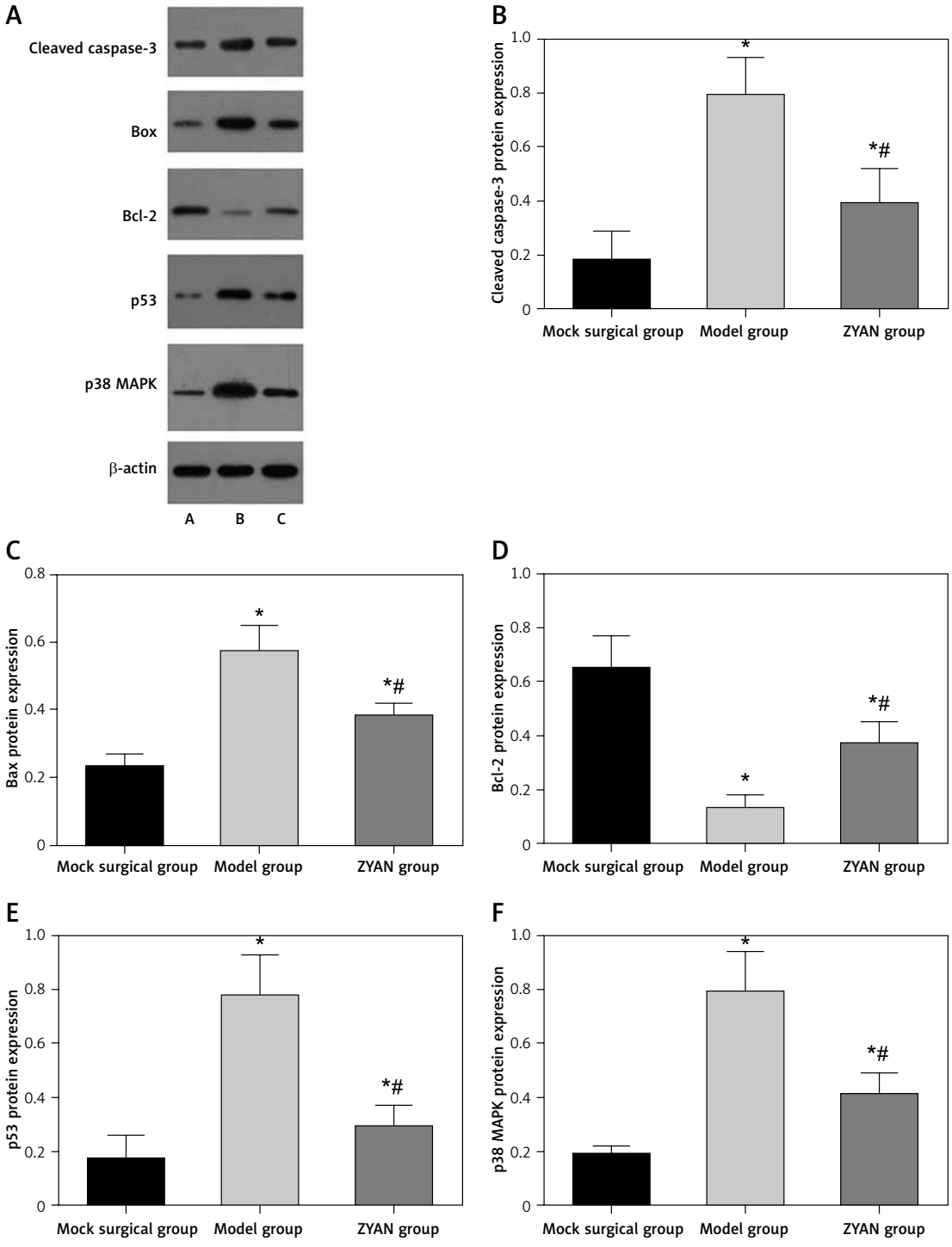

Figure 5. Western blotting results showing the expression of apoptosis-related proteins in the hippocampus of all groups. **A** – Histogram illustrating the expression levels of apoptosis-related proteins; **B** – cleaved caspase-3 protein expression; **C** – Bax protein expression; **D** – Bcl-2 protein expression; **E** – p53 protein expression; **F** – p38 MAPK protein expression. A, B, and C represent MSG, MG, and ZYAN

[a]p < 0.05 refers to the comparison with MSG, [b]p < 0.05 refers to the comparison with MG. MSG – mock surgical group, MG – model group.

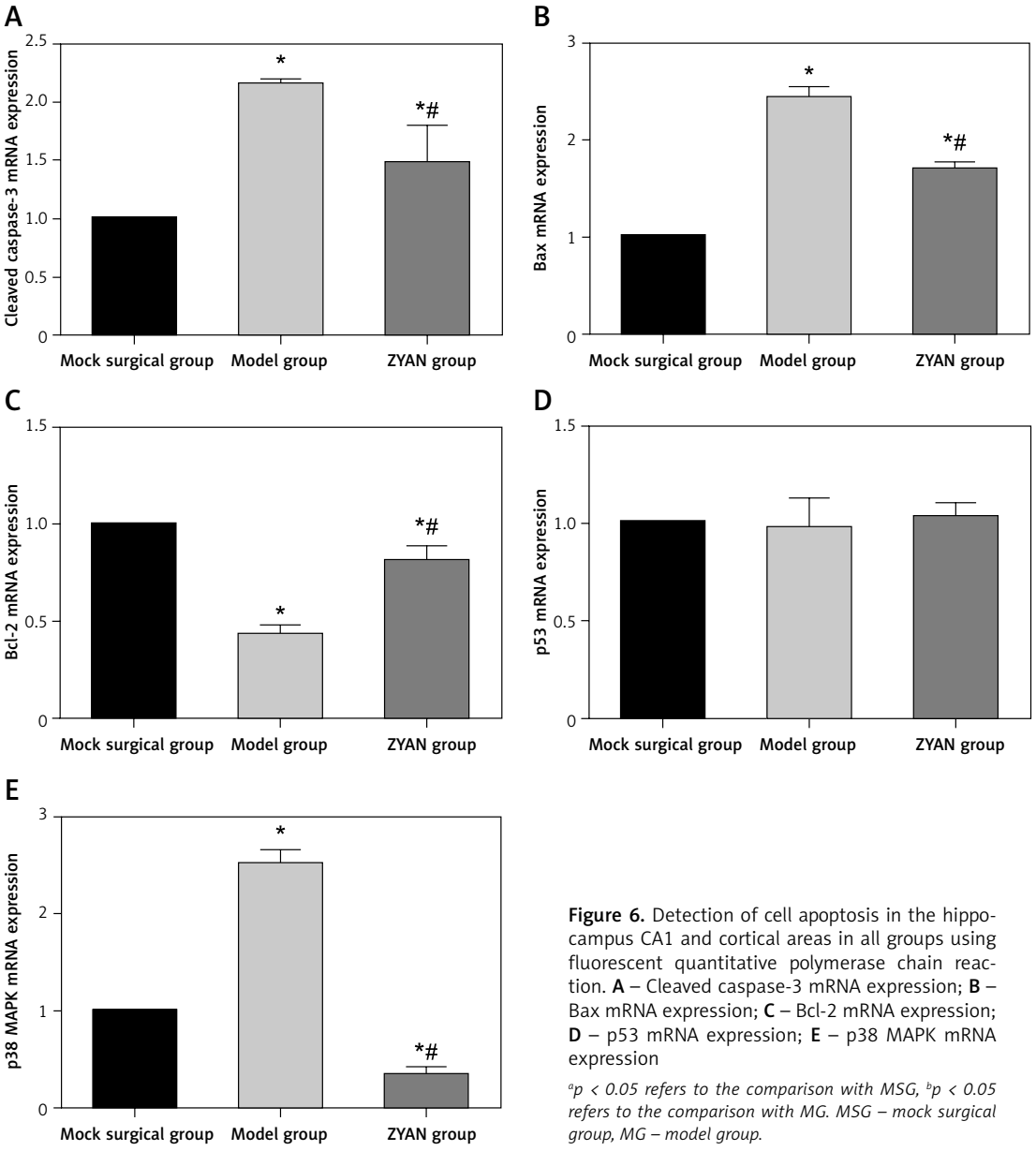

Figure 6. Detection of cell apoptosis in the hippocampus CA1 and cortical areas in all groups using fluorescent quantitative polymerase chain reaction. **A** – Cleaved caspase-3 mRNA expression; **B** – Bax mRNA expression; **C** – Bcl-2 mRNA expression; **D** – p53 mRNA expression; **E** – p38 MAPK mRNA expression

[a]p < 0.05 refers to the comparison with MSG, [b]p < 0.05 refers to the comparison with MG. MSG – mock surgical group, MG – model group.

alleviates neuronal apoptosis and improves CD remains unclear.

CI-mediated cell apoptosis is broadly categorized as occurring via exogenous or endogenous pathways. The exogenous pathway of cell apoptosis is mediated via a cascade reaction involving cysteinyl aspartate specific proteinase (caspase)-8, caused by activation of death receptors on the cell surface. The endogenous pathway, also known as mitochondria-mediated cell apoptosis, is the main pathway of neuronal apoptosis in CI; it mainly refers to the cell apoptosis caused by the activation of caspase-3 after mitochondrial stimulation [14, 15]. Cell apoptosis is accomplished mainly via caspase activation. Caspase-3 can permeate the cell nucleus by hydrolyzing the arrestin of deoxyribonucleases, leading to activation of key factors downstream of the caspase cascade reactions, thereby resulting in cell apoptosis. Simultaneously, caspase-3 can dissociate from Bcl-2, causing a Bax/Bcl-2 imbalance, which promotes neuronal apoptosis [16–18]. Furthermore, neuronal apoptosis is also regulated by the p38 MAPK signaling pathway; activation of this pathway can directly activate Bax and transport it to the outer mitochondrial membrane, thereby mediating the entry of cytochrome C into the cytoplasm, activating the caspase-3 cascade reaction [19, 20]. Liu *et al.* [21] found that changes in mitochondrial function are closely related to neuronal apoptosis. In addition, the more severe the neuronal apoptosis is, the more obvious is the increase in the expression of the mitochondrial apoptosis-related genes caspase-3, Bax, and Bcl-2 [22]. Liang [23] found

that the Annao pill significantly reduced microglial cell activation and the expression of NF-κBp65 and caspase-3 after ICH; in addition, it increased the expression of a brain-derived neurotrophic factor and synaptophysin. These changes exert a protective effect on hemorrhagic brain tissue. He *et al.* [24] reported that the Naoyian granule could inhibit DNA fragmentation in nerve cells and reduce neuronal apoptosis. These studies suggest that TCM preparations can significantly reduce neuronal apoptosis and play a protective role against brain injury. In our study, compared with MG, the ZYAN group showed significantly lower protein expression of cleaved caspase-3, Bax, p-p38, and MAPK in the hippocampus, and significantly higher protein expression of Bcl-2; these findings were consistent with those of FQ-PCR. Therefore, we hypothesized that ZYAN inhibits neuronal apoptosis by inhibiting the p38 MAPK signaling pathway and caspase-3 cascade reaction. ZYAN contains *Hirudo*, woodlouse, *C. grandiflora*, peach seed, earthworm, *R. chuanxiong*, wine-treated rhubarb, and other ingredients. In previous studies, chemicals contained in the abovementioned components, such as hirudin [25], ligustrazine [26], and chrysophanol [27], have been found to improve brain injury and brain edema caused by CI. The results of this study are consistent with those of previous studies.

However, some limitations of our study should be considered when interpreting our results. In this study, a rat model was developed, and TTC staining was used as the classic method to detect the areas of cerebral infarction in the rats. The coordinates where the infarcts were the most obvious in each group were selected. Due to individual differences in the rats and an error in experimental operation, it was difficult to ensure the same infarcts in the same coordinates of the brain. Therefore, the most obvious ischemic lesions of the rat brain were selected for comparison. Due to the effect of photography, dyeing, and photo pixels, the image may only show a local whitish discoloration and no obvious infarction lesions. Therefore, the results of this experiment may not be very convincing. Moreover, the relationship between the effect of the individual active components of ZYAN on IBI and that of the combined components has not been studied; a confirmatory study will be performed in the future.

In conclusion, ZYAN was found to reduce neuronal apoptosis and the area of cerebral infarction and improve CD in the rats with IBI. The underlying mechanism of action of the ZYAN may be related to the inhibition of neuronal apoptosis by ZYAN via the inhibition of the p38 MAPK signaling pathway and caspase-3 cascade reaction.

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Ethics approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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