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Original Article

Acupuncture at "Baihui" and "Xuanli" attenuates intracerebral hemorrhage-induced brain injury by modulating autophagy and the PI3K/AKT signaling pathway in rats

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Abstract: Objective: To investigate the neuroprotective effects of acupuncture at the "Baihui" and "Xuanli" acupoints on rats with intracerebral hemorrhage (ICH). Methods: An ICH rat model was established. For the 3-MA group, the autophagy inhibitor 3-MA (400 nM) was injected into the lateral ventricle 15 min before ICH induction. Histopathologic changes were observed, and expressions of autophagy-related proteins and inflammatory cytokines in hippocampal tissues were measured. Additionally, neuronal apoptosis, oxidative stress markers, and PI3K/AKT pathway-related protein expression were assessed. Results: Compared to the sham group, the model group exhibited increased levels of Beclin-1, ATG5, and pro-inflammatory cytokines in the hippocampus, along with enhanced neuronal apoptosis and oxidative stress, as well as downregulated phosphorylation of PI3K and AKT. Compared to the model group, the acupuncture group showed further upregulation of Beclin-1 and ATG5, reduced levels of pro-inflammatory cytokines, alleviated neuronal apoptosis and oxidative stress, and increased phosphorylation of PI3K and AKT. Compared to the acupuncture group, the 3-MA group displayed loss of hippocampal structural integrity, decreased expression of Beclin-1 and ATG5, elevated pro-inflammatory cytokine levels, exacerbated apoptosis and oxidative stress, and suppressed PI3K signaling activity. In contrast to the 3-MA group, the 3-MA + acupuncture group, the rapamycin group, and the rapamycin + acupuncture group all demonstrated protective effects, including attenuated neuronal injury. Conclusion: Acupuncture at the "Baihui" and "Xuanli" acupoints significantly alleviated inflammation, apoptosis, and oxidative stress in the hippocampal tissue of ICH rats. This effect was associated with concurrent upregulation of autophagy markers (Beclin-1, ATG5) and activation of the PI3K/AKT signaling pathway. However, given the pleiotropic actions of the pharmacological agents used, the precise mechanistic relationship between PI3K/AKT signaling and autophagy remains correlative. Our findings suggest that functional autophagy is essential for acupuncture's neuroprotective effects, and that its therapeutic benefit may arise from coordinated modulation of the PI3K/AKT/mTOR/autophagy network rather than a simple linear cascade.

Keywords: Intracerebral hemorrhage, acupuncture, the PI3K/AKT pathway, autophagy

Introduction

Intracerebral hemorrhage (ICH) is a severe acute non-traumatic bleeding within the brain. The prevalence of ICH has increased in recent years, accounting for 18.8%-47.6% of all strokes, and is associated with high disability and mortality rates [1, 2]. Neuronal injury after ICH is primarily caused by hematoma occupation and secondary ischemia. Although surgical hematoma evacuation is the preferred method to achieve hemostasis and relieve intracranial

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pressure, early surgical hematoma evacuation does not consistently improve clinical outcomes. Therefore, elucidating the internal pathogenesis of ICH and identifying effective therapeutic strategies are essential for reducing its morbidity and mortality.

Traditional Chinese medicine (TCM) believes that ICH belongs to the category of "stroke" based on its etiology, pathogenesis, and clinical manifestations. It is manifested mainly as sudden loss of consciousness, facial deviation, tongue rigidity, and hemiplegia or partial paralysis. Acupuncture is one of the main therapies for treating stroke in TCM. Baihui (DU20), located at the vertex of the head, is the meeting point of all yang meridians and a principal acupoint of apoplexy, with effects of uplifting yang and restoring consciousness. Xuanli (GB6), located at the intersection of the anterior oblique line of parietal and temporal, is commonly used to improve facial motor dysfunction, motor aphasia, and peripheral facial paralysis [3].

Studies have shown that a series of cellular biological processes such as immune inflammation, apoptosis activation, and oxidative stress in surrounding tissues are critical contributors to secondary brain injury after ICH [4, 5]. Autophagy has been shown to be activated in perihematoma tissue as early as 6 h after ICH, peaking at 24-72 h, declining after day 3, and exhibiting marked aggregation of autophagic vesicles in neurons by day 7 [6]. The activation of autophagy is considered a self-protective mechanism of cells to eliminate damaged organelles and harmful cytoplasmic components [7]. The PI3K/AKT signaling pathway can regulate various biological processes, including macrophage autophagy and inflammatory suppression, and plays a key regulatory role in autophagy [8, 9]. Mammalian Beclin-1 interacts with PI3K/AKT to promote PI3K synthesis, thereby promoting lipid membrane extension, cargo recruitment, and autophagosome maturation [10, 11]. In addition, inflammation represents a fundamental response of brain tissue to stimulation. Following ICH, multiple stimuli activate inflammatory cells, promoting their release of inflammatory mediators, among which TNF-α can induce brain edema and participate in ICH progression [12].

Although acupuncture at Baihui or Baihui-penetrating-Qubin has been reported to alleviate brain injury after ICH through anti-inflammatory and anti-apoptotic mechanisms, the optimal acupoint combination for regulating the PI3K/AKT/autophagy axis remains unclear. Xuanli, located along the Gallbladder Meridian and adjacent to Baihui, is a key acupoint for treating post-stroke motor dysfunction, facial paralysis, and cognitive impairment-symptoms commonly observed in ICH patients. The penetrating needling technique from Baihui to Xuanli simultaneously stimulates the Governor Vessel (Du Mai) and Gallbladder Meridian, which is clinically preferred for severe stroke syndromes. However, its effects have rarely been investigated in preclinical ICH models. Moreover, whether this acupuncture paradigm exerts specific regulatory effects on the PI3K/AKT/autophagy signaling cascade, and whether its neuroprotective efficacy depends on functional autophagy, have not been systematically addressed.

Therefore, this study specifically aims to: (1) determine whether Baihui-penetrating-Xuanli acupuncture improves neurological outcomes in rat model of ICH; (2) investigate its effects on inflammation, apoptosis, oxidative stress, and the PI3K/AKT/autophagy pathway; and (3) evaluate whether its therapeutic effects are dependent on autophagy using pharmacologic modulators (3-MA and rapamycin). Our findings may provide support for this clinically relevant acupuncture protocol and clarify the role of autophagy in its neuroprotective effects.

Materials and methods

Reagents

The Beclin-1 antibody (rabbit monoclonal, Cat. No. 00080987, 1:1500), ATG5 antibody (rabbit polyclonal, Cat. No. 10008350, 1:1000), AKT antibody (serine/threonine kinase, rabbit monoclonal, Cat. No. 34d5362/4, 1:1000), p-AKT antibody (Cat. No. 3600001545, 1:800), PI3K antibody (Cat. No. M14JL5P, 1:1000), p-PI3K antibody (Cat. No. 3K58815, 1:500), IL-1β antibody (Cat. No. 66009-1-Ig, Chengdu Zhengneng Biotechnology Co., LTD., 1:2000) and β-actin (Wuhan Sanying Biotechnology Co., LTD., Cat. No. 10029187, 1:3000) were utilized in this study. Additionally, Horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG; Wuhan Sanying Biotechnology Co., LTD., Cat. No. 20001286/20001272, 1:3000) was employed. The autophagy agonist rapamycin (Cat. No. HY-10219) was purchased from MedChemExpress (MCE) Reagent Company.

Main instruments

The main instruments used in this study included a three-dimensional brain injection instrument (Rivold, H1829401-002), an ultrapure water machine (Sichuan Youpu, UPT-1-10T), a tissue embedding system (Wuhan Junjie, JB-P5), a tissue microtome (Leica, Germany, LEICARM22445), a slide scanning instrument (AperiodLV1, Leica, Germany), an ultramicro spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., K5800), a vertical electrophoresis instrument (Wicks Technology (Beijing) Co., Ltd., WIX-EP300), a protein transfer membrane system (Beijing Wicks, WIX-EP300), a chemiluminescent detector (Azure, C300, USA), a horizontal decolorizing shaker (Qilimbel instrument in Haimen City), and an adjustable mixing instrument (Dalong Xingchuang Laboratory Instruments (Beijing) Co., Ltd., D1008). Disposable acupuncture needles (Huatuo brand, model: 0.25 mm × 40 mm) were used for acupuncture treatment.

Animals

Twenty-three SPF-grade healthy male Sprague-Dawley (SD) rats, aged 8-12weeks, weighing 300-310 g, were purchased from Beijing Huafukang Biotechnology Co., Ltd. (Certificate No.: SCXK[Bing]2019-0008). All rats were raised in an SPF environment with a temperature of 20-26°C and a humidity of 40%-70%°C, under a standard light-dark cycle, with free access to food and water. To minimize animal suffering during and after surgery, perioperative analgesia was administered by subcutaneous injection of buprenorphine (0.05 mg/kg) immediately after ICH induction and every 12 h for the first 24 h if needed. Rats were kept on a warming pad until fully recovered from anesthesia, provided with soft food and easy access to water, and monitored twice daily for signs of distress (e.g., lethargy, inability to reach food/water, > 20% body weight loss). Rats showing severe neurological deficits or moribund status were humanely euthanized according to predefined humane endpoints approved by the ethics committee. At the end of the experiment, rats were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg). Death was confirmed by the absence of spontaneous breathing for more than 1 minute, loss of a corneal reflex, and cessation of heartbeat as assessed by palpation. All experimental procedures were approved by the Scientific Research Ethics Committee of Ningxia Hui Autonomous Region Hospital of Traditional Chinese Medicine (approval number: 2022-69).

Experimental design

A total of 23 rats were used in this study, with 3 randomly assigned to the sham group and the remaining 20 subjected to ICH modeling. Of these, 18 rats (90.0%) met the inclusion criteria for successful model establishment (defined as presence of hematoma in the caudate nucleus confirmed by gross inspection or histology, and baseline neurological deficit score ≥ 2 at 24 h post-surgery). The overall mortality rate was 10.0% (2/20), with one death each in the model group and the 3-MA group. The 18 successfully modeled rats were randomly divided into six groups: model group, acupuncture group, 3-MA group, 3-MA + acupuncture group, rapamycin group, and rapamycin + acupuncture group.

Sham operation group: Only surgical procedures were performed without other intervention. Model group: The ICH model was established according to the rat stereotaxic atlas [13] and the modified autologous blood injection method [14]. The rats were anesthetized and fixed on a stereotaxic instrument using ear rods, with the skull adjusted to the horizontal level. A longitudinal incision of about 2 cm was made along the midline of the rat head, the periosteum was removed using 3% hydrogen peroxide solution, and the bregma and lambda were fully exposed. Subsequently, ICH was induced by injecting arterial blood into the caudate nucleus. After injection, the needle was retained in place for 10 minutes followed by layered wound closure. Model + acupuncture group: On the basis of ICH modeling, acupuncture intervention (Baihui and Xuanli on the affected side, penetration depth: 1.5 cm) was initiated on the second day after operation. Baihui and Xuanli points on the affected side were selected according to the *Atlas of Acupoints of Experimental Animals* [15]. The depth of the needle insertion was 0.8 inches. The needle was manually twisted for 5 min and retained for 30 min. 3-MA group: The autophagy inhibitor 3-MA (400 nM) was injected into the lateral ventricle 15 min before ICH induction. 3-MA + acupuncture group: In addition to 3-MA treatment, rats received the same acu-

Table 1. Sequences of primers for RT-qPCR

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
Beclin-1	ATGAGGGCGACAGTGAACAG	CTGGATCAGCCTCTCCTCCT
ATG5	CACCCCTGAAATGGCATTATCC	TGGACAGTGTAGAAGGTCCTTT
TNF-α	GTGATCGGTCCCAACAAGGA	CTTGGTGGTTTGCTACGACG
IL-1β	ACAGAACATAAGCCAACAAG	ACACAGGACAGGTATAGATTC
β-actin	CCAATTGAACACGGCATTG	ACGCAGCTCATTGTAGAA

puncture intervention as that of the acupuncture group. Rapamycin group: Rapamycin (0.8 ng) was injected into lateral ventricle 15 min before ICH induction. Rapamycin + acupuncture group: Acupuncture intervention consistent with the acupuncture group was applied on the basis of rapamycin pretreatment.

Pathologic histology staining

H&E staining: After brain tissue collection, samples were immediately fixed in 4% paraformaldehyde for 24 h, followed by dehydration, paraffin embedding, and sectioning in sequence. Sections were stained with hematoxylin solution for 10 min, then rinsed under running water for 3 min. Differentiation was performed using 1% hydrochloric acid-ethanol for 30 s, followed by rinsing with running water for 1 minute. Sections were then counterstained with eosin solution for 1 min, rinsed with running water for 1 minute, and then rinsed twice with 95% ethanol (3 s each), dehydrated with 100% ethanol three times (2 min each), and cleared sequentially in xylene I for 10 min and xylene II for 5 min. After natural drying, the slices were sealed with neutral gum and images were collected.

Nissl staining: After deparaffinization, the sections were rinsed with distilled water and then incubated with Nissl staining solution at 50-60°C for 20-40 min, followed by gentle rinsing with distilled water. Then, 95% ethanol was used for rapid differentiation, followed by dehydration with absolute ethanol and clearing with xylene. Finally, sections were mounted with neutral gum for microscopic observation.

Immunohistochemical staining: Paraffin sections were dewaxed and rehydrated, then subjected to antigen retrieval in 0.1 mol/L citric acid solution. After antigen retrieval, sections were washed with PBS three times to remove endogenous enzyme. Sections were blocked with blocking solution for 1 h, followed by incubation with antibodies against Beclin-1, ATG5, IL-1β, and TNF-α overnight at 4°C. After washing with PBS, sections were incubated with secondary antibodies at room temperature. Immunoreactivity was visualized using a DAB chromogenic solution. Sections were then dehydrated through gradient ethanol, cleared with xylene, mounted with neutral gum, and examined under a light microscope.

RT-qPCR

Total RNA was extracted from brain tissues using TRIzol regent, and RNA concentration and purity were assessed spectrophotometrically. Reverse transcription was performed using a cDNA kit (Cat. No. RR037A, Takara Bio, Japan) with total RNA (1 μg) under the following conditions: 42°C for 60 min and 70°C for 5 min. The synthesized cDNA was stored at 4°C until further use. All experiments were repeated 3 times, with β-actin as internal reference, and the relative fold change of mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ method. Genes and primers were synthesized by Shaanxi Qingke Biotechnology Co., Ltd., and primer sequences are shown in **Table 1**.

ELISA

IL-1β and TNF-α levels were quantified using commercial ELISA kits (MultiSciences Biotech Co., Ltd., China). All procedures were performed strictly according to the instructions of the reagent kit. Absorbance was measured at 450 nm using a microplate reader, and cytokine concentrations were calculated based on the corresponding standard curves.

Western blotting

Brain tissues were lysed under the protection of liquid nitrogen and centrifuged at 12,000 r/min for 15 min at 4°C. The supernatants were collected, and protein concentrations were determined using the BCA method. Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with corresponding secondary antibodies at room temperature. Protein bands were visualized using

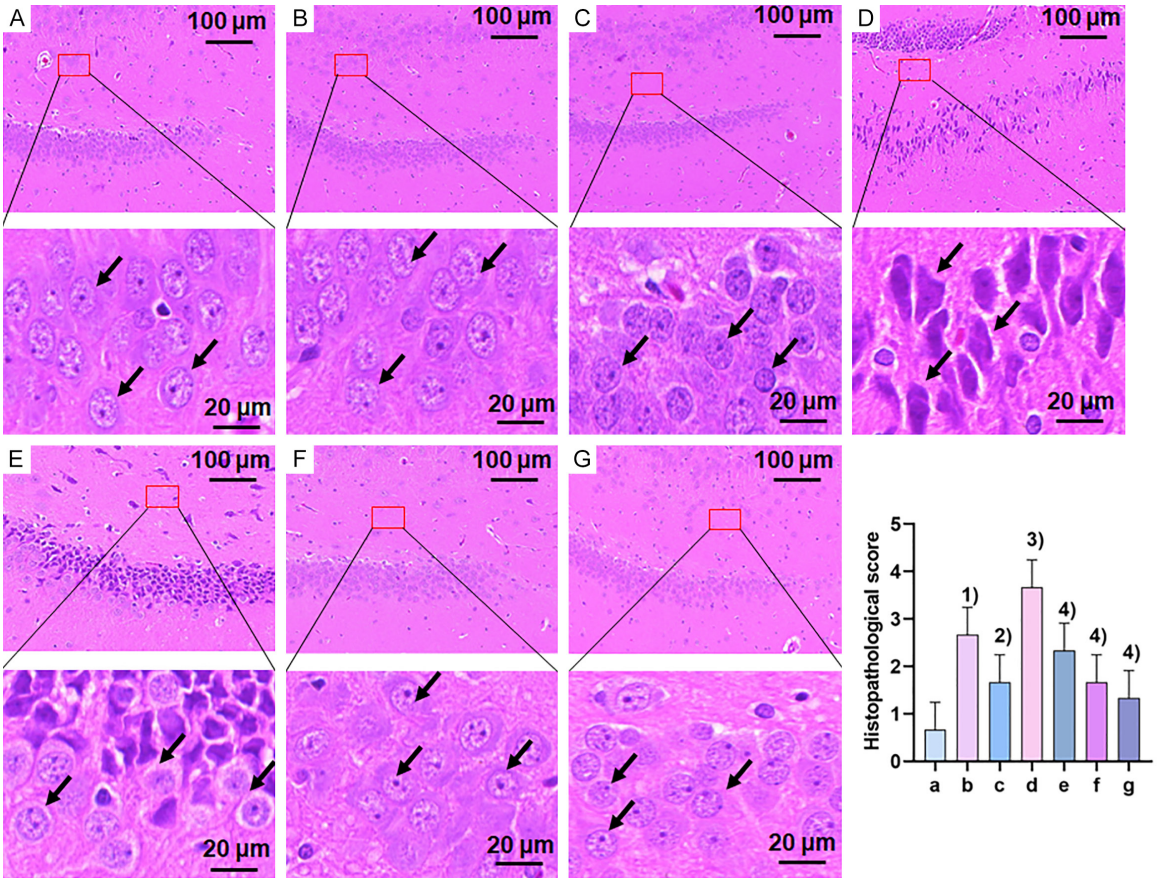


Figure 1. H&E staining of hippocampal neurons in rats. (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. Scale bars: 100 μm (low magnification) and 20 μm (high magnification). [1)]$P < 0.05$, compared to the sham group; [2)]$P < 0.05$, compared to the model group; [3)]$P < 0.05$, compared to the acupuncture group; [4)]$P < 0.05$, compared to the 3-MA group.

an enhanced chemiluminescence (ECL) detection kit and imaged with a chemiluminescent detector (Azure C300, USA). Band intensities were quantified using ImageJ software (NIH, USA). For each target protein, the optical density of the band was normalized to that of β-actin in the same sample to correct for loading variations. The relative expression level was expressed as the ratio of target protein/β-actin.

Statistical analysis

All statistical analyses were reviewed and verified by a biostatistician. Data from at least three independent experiments were presented as mean ± SD. Normality and homogeneity of variances were confirmed prior to parametric testing. Comparisons between the two groups were analyzed using the t-test. For the comparison of multiple groups, one-way ANOVA followed by Tukey's post hoc test was applied. A p value of < 0.05 indicated statistical significance.

Results

Acupuncture treatment alleviated inflammatory injury in hippocampal neurons of rats

As shown in **Figure 1**, hippocampal neurons in the sham group exhibited normal morphology, showing round or oval with regular arrangement. No inflammatory cell infiltration or microglial accumulation was observed in the hippocampus. However, the model groups demonstrated marked inflammatory cell infiltration and evident neuronal swelling, accompanied by disordered cellular arrangement.

Compared to the model group, the acupuncture group showed alleviated inflammatory cell infiltration and neuronal swelling. Neuronal morphology was relatively preserved.

In contrast, the 3-MA group exhibited increased microglial cell accumulation, severe cellular disorganization, and prominent nuclear membrane damage when compared to the acupuncture group.

Compared to the 3-MA group, the 3-MA + acupuncture group, rapamycin group and rapamycin + acupuncture group demonstrated varying degrees of attenuation of inflammatory cell infiltration and alleviation of neuronal injury, along with regular cell arrangement.

Acupuncture treatment enhanced hippocampal neuronal integrity in rats

As shown in **Figure 2**, Nissl bodies in the brain tissues of rats in the sham group were clearly visible without significant reduction or disappearance. However, in the model group, the number of Nissl bodies significantly decreased, accompanied by neuronal shrinkage, nuclear condensation, and deeply stained cytoplasm.

Compared to the model group, Nissl bodies were notably preserved, appearing dense and with regularly arranged morphology in the acupuncture group. In contrast, Nissl bodies in the hippocampus of rats in the 3-MA group were markedly reduced or absent. Compared to the 3-MA group, the 3-MA + acupuncture group showed partial restoration of Nissl bodies. In the rapamycin group and rapamycin + acupuncture group, Nissl bodies were clearly visible and increased in number.

Acupuncture treatment enhanced autophagy and attenuated the inflammatory response in hippocampal neurons of rats

As shown in **Figure 3A**, strong positive expression of ATG5, Beclin-1, IL-1β, and TNF-α was observed in the hippocampus of rats in model group. Compared to the model group, the positive expression of ATG5 and Beclin-1 was further enhanced in the acupuncture group, while the positive signals of IL-1β and TNF-α were weakened. In the 3-MA group, hippocampal tissue structure was severely disrupted, and immunoreactive signals were markedly reduced. Compared to the model group, 3-MA treatment significantly reduced the expression levels of Beclin-1 and ATG5, indicating effective suppression of autophagy. In contrast, the 3-MA + acupuncture group, rapamycin group, and rapamycin + acupuncture group showed partial restoration or enhancement of ATG5 and Beclin-1 expression, together with attenuation of IL-1β and TNF-α immunoreactivity.

Consistent with the immunohistochemical findings, the mRNA expression (**Figure 3B-E**) and secretion levels (**Figure 3F-I**) of Beclin-1, ATG5, IL-1β, and TNF-α were significantly increased in the model group. Compared to the model group, acupuncture further increased the levels of Beclin-1 and ATG5 expression, while reducing IL-1β and TNF-α levels. In contrast, compared to the model group, the 3-MA group demonstrated decreased Beclin-1 and ATG5 levels, along with elevated IL-1β and TNF-α. Furthermore, compared to the 3-MA group, the 3-MA + acupuncture group, the rapamycin group, and the rapamycin + acupuncture group all exhibited increased Beclin-1 and ATG5 levels, accompanied by reduced IL-1β and TNF-α. These results were further supported by western blot analysis (**Figure 3J-M**).

Acupuncture treatment attenuated apoptosis in the hippocampus of rats

To evaluate hippocampal apoptosis, TUNEL staining was performed. The results revealed that the model group exhibited a higher number of TUNEL-positive cells (**Figure 4A**), elevated expression of cleaved caspase-3 (**Figure 4B**), upregulated pro-apoptotic factor Bax (**Figure 4C**), and downregulated anti-apoptotic factor Bcl-2 (**Figure 4D**). Compared to the the model group, the acupuncture group showed a marked reduction in apoptotic activity. Conversely, the 3-MA group displayed an increase in both apoptotic cell counts and pro-apoptotic protein expression compared to the acupuncture group. However, when compared to the 3-MA group, the 3-MA + acupuncture, rapamycin, and rapamycin + acupuncture groups all demonstrated attenuated apoptosis.

Our analysis showed that the Bcl-2/Bax ratio was significantly reduced in the model group, consistent with enhanced apoptotic signaling. In the acupuncture group, the Bcl-2/Bax ratio was markedly increased, reflecting suppressed apoptosis. Conversely, the 3-MA group exhibited a further decline in the Bcl-2/Bax ratio relative to the acupuncture group, whereas the 3-MA + acupuncture, rapamycin, and rapamycin + acupuncture groups all showed an elevated Bcl-2/Bax ratio (**Figure 4E**).

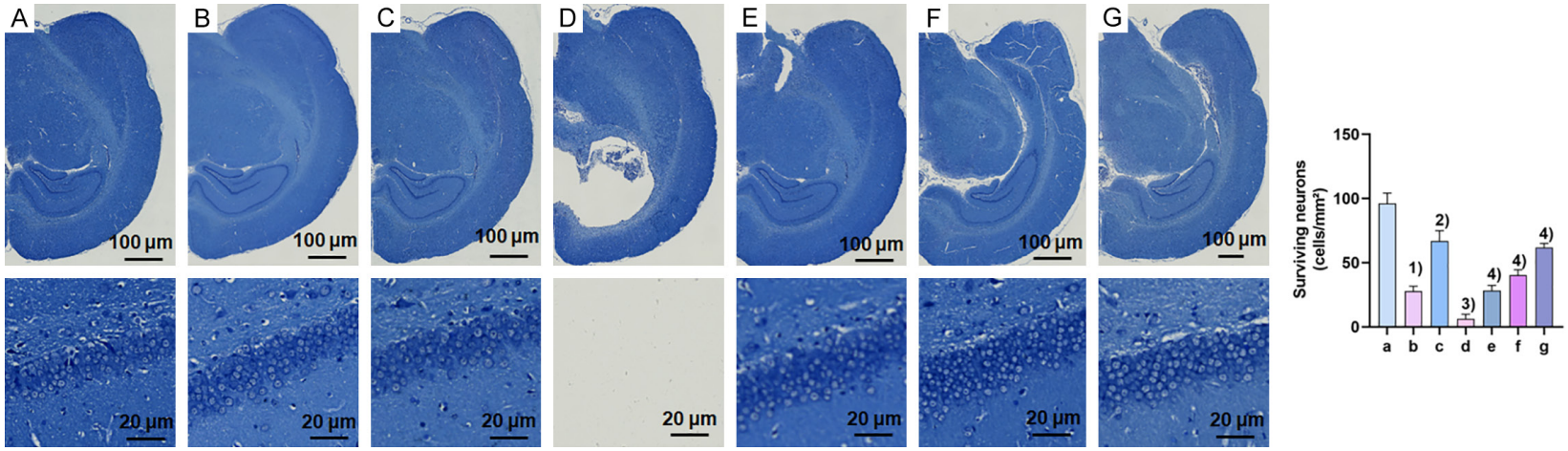
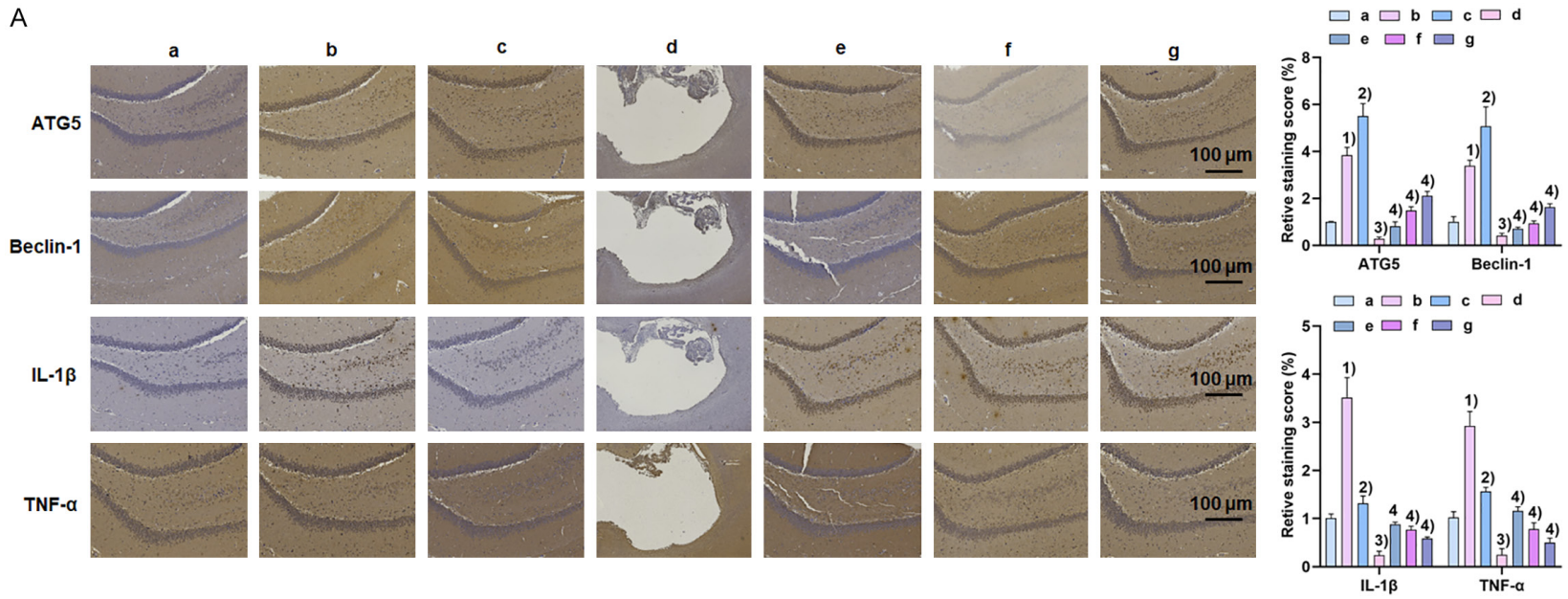


Figure 2. Nissl staining of hippocampal neurons in rats. Representative images and quantification of surviving neurons (cells/mm^2). (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. Scale bars: 100 μm (low magnification) and 20 μm (high magnification). $^{1)}P < 0.05$, compared tothe sham group; $^{2)}P < 0.05$, compared to the model group; $^{3)}P < 0.05$, compared to the acupuncture group; $^{4)}P < 0.05$, compared to the 3-MA group.



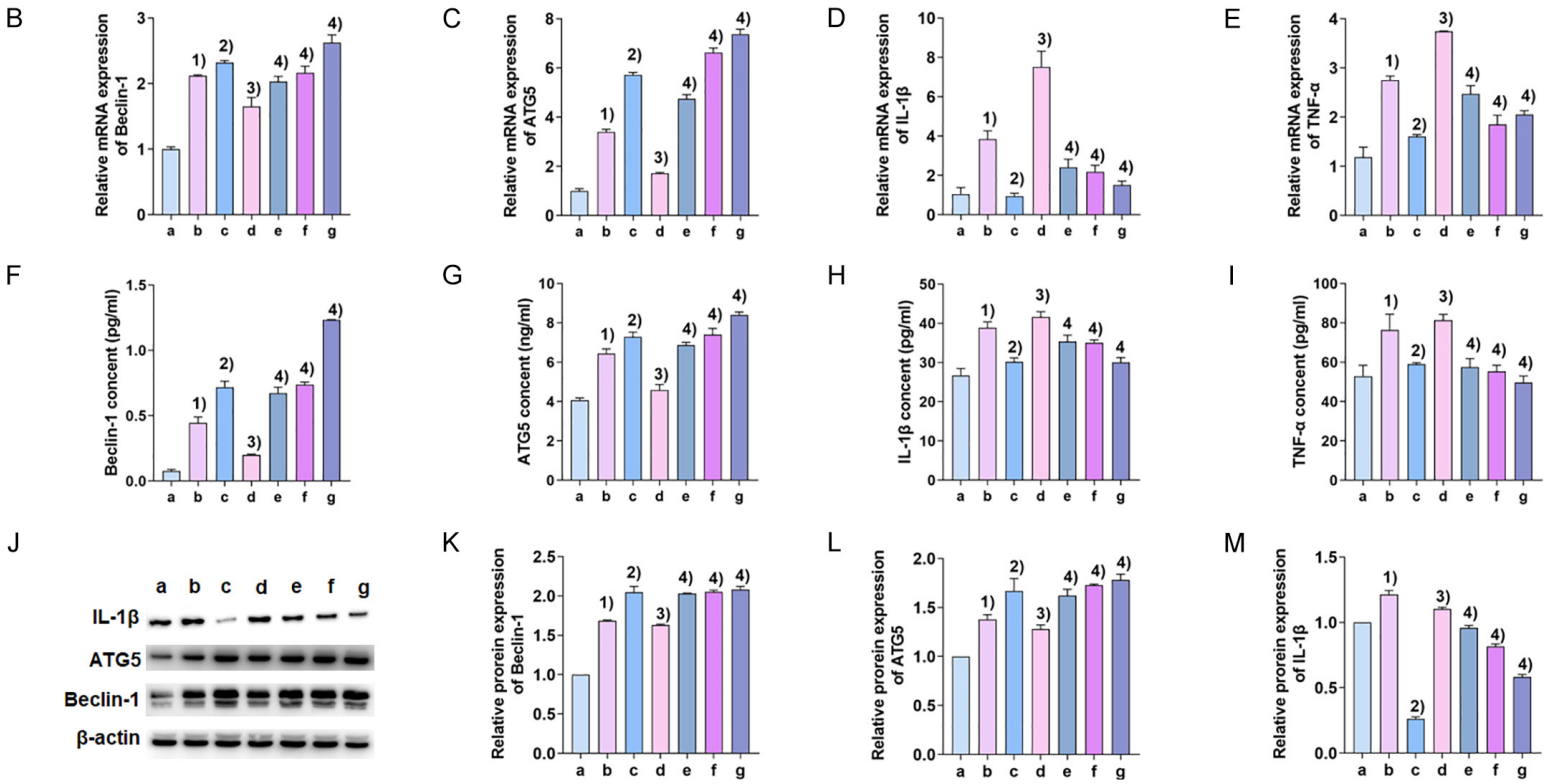


Figure 3. The levels of Beclin-1, ATG5, IL-1β and TNF-α in rat hippocampus and serum. A. Immunohistochemical staining images of autophagy related molecules in hippocampus. Scale bars = 100 μm. B-E. The mRNA expression of Beclin, ATG5, IL-1β and TNF-α in rat hippocampus. F-I. Secretion levels of Beclin, ATG5, IL-1β and TNF-α in rat serum. J-M. Protein expression of Beclin-1, ATG5, and IL-1β in the hippocampus. (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. [1)]$P < 0.05$, compared to the sham group; [2)]$P < 0.05$, compared to the model group; [3)]$P < 0.05$, compared to the acupuncture group; [4)]$P < 0.05$, compared to the 3-MA group.

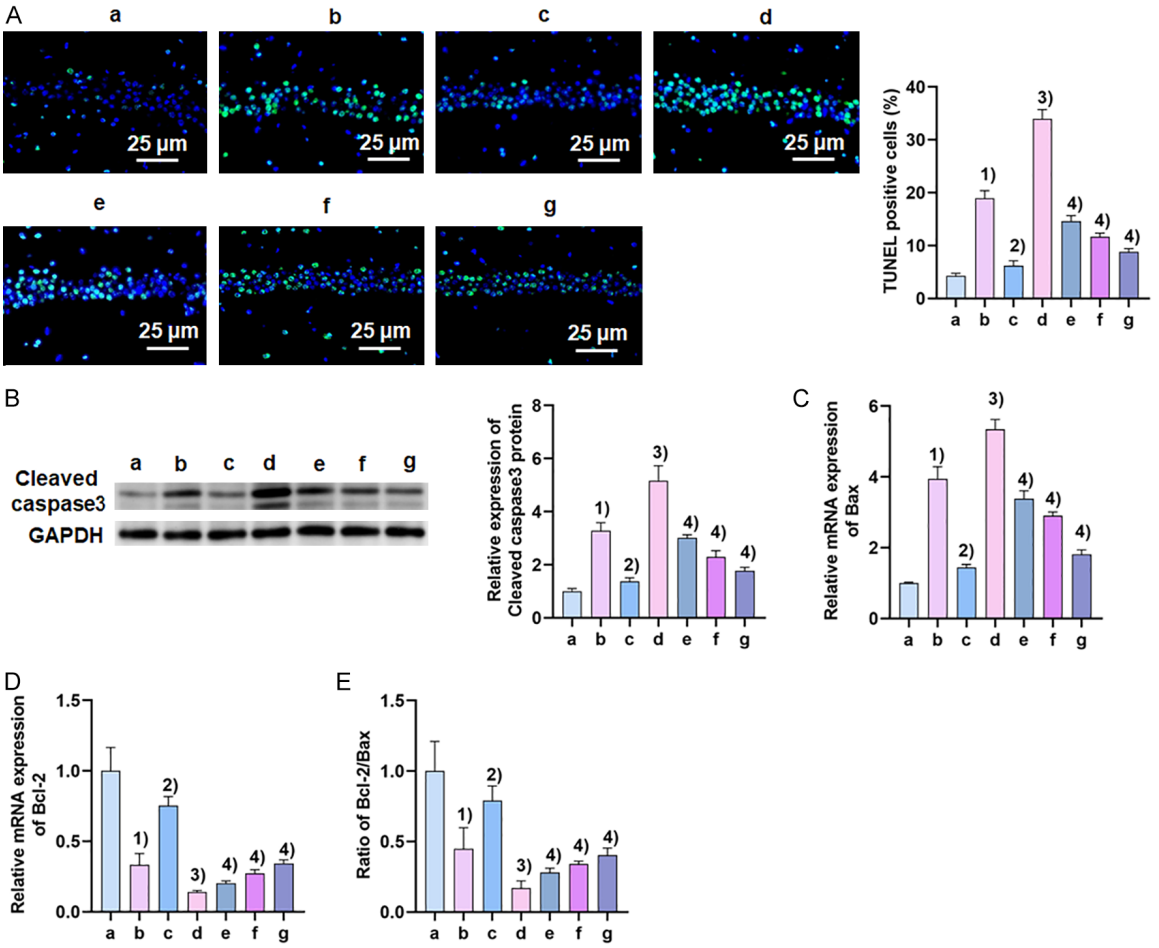


Figure 4. Apoptosis levels in rat hippocampus. A. TUNEL apoptosis staining. B. The protein expression of cleaved caspase-3. C, D. mRNA expression of Bax and Bcl-2. E. The ratio of Bcl-2/Bax: (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. [1)]$P < 0.05$, compared to the sham group; [2)]$P < 0.05$, compared to the model group; [3)]$P < 0.05$, compared to the acupuncture group; [4)]$P < 0.05$, compared to the 3-MA group.

Acupuncture treatment alleviated oxidative stress in the hippocampus of rats

During the course of ICH, the accumulation of blood-derived components within the brain parenchyma triggers a cascade of oxidative stress-related reactions. In the model group, the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) were markedly reduced, accompanied by elevated levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), indicating pronounced oxidative stress in the hippocampus.

Compared to the model group, the acupuncture group showed enhanced antioxidant activity, evidenced by enhanced SOD, CAT, and GSH-PX activities, along with reduced concentrations of H_2O_2 and MDA. In contrast, the 3-MA group displayed further decreases in SOD, CAT, and GSH-PX activities and elevated H_2O_2 (**Figure 5A-D**) and MDA levels (**Figure 5E**). Furthermore, compared to the 3-MA group, the 3-MA + acupuncture group, rapamycin group, and rapamycin + acupuncture groups all demonstrated improved antioxidant capacity and reduced oxidative stress.

Acupuncture treatment activated the PI3K-AKT pathway in the hippocampus of rats

As shown in **Figure 6A-E**, phosphorylation levels of PI3K and AKT proteins were significantly reduced in the hippocampus of rats in the model group. Compared to the model group,

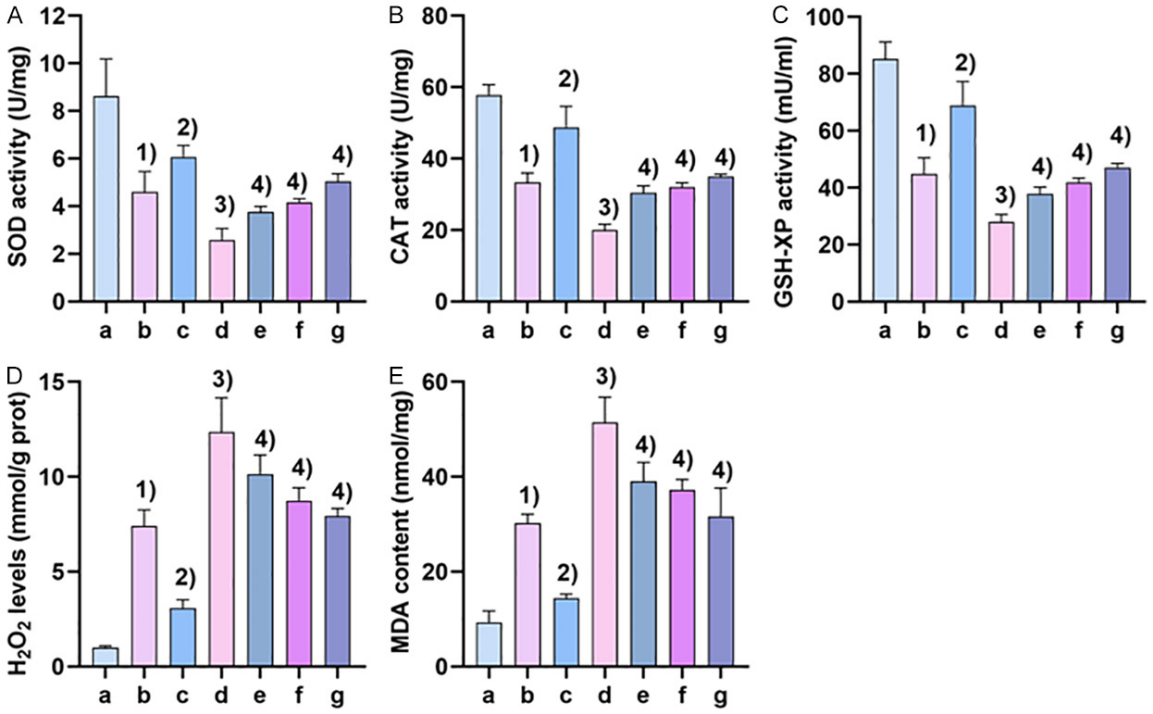


Figure 5. Oxidative stress levels in rat hippocampus. A. Superoxide dismutase (SOD) activity. B. Catalase (CAT) activity. C. Glutathione peroxidase (GSH-PX) activity. D. Hydrogen peroxide (H_2O_2) levels. E. Malondialdehyde (MDA) content. (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. [1)]$P < 0.05$, compared to the sham group; [2)]$P < 0.05$, compared to the model group; [3)]$P < 0.05$, compared to the acupuncture group; [4)]$P < 0.05$, compared to the3-MA group.

acupuncture significantly increased the expression of p-AKT and p-PI3K proteins. These changes occurred in parallel with upregulation of autophagy markers.

Compared to the acupuncture group, the phosphorylation levels of AKT and PI3K proteins were reduced in the 3-MA group; In addition, compared to the 3-MA group, the 3-MA + acupuncture group, rapamycin group, and rapamycin + acupuncture group all showed notable increases in p-AKT and p-PI3K levels (**Figure 6A-E**).

Discussion

It has reported that acupuncture and moxibustion can attenuate neuronal necrosis and delay neuronal damage in experimental models [16]. Acupuncture therapy, as an important therapeutic modality of TCM, has been widely used in clinical treatment of various post-ICH sequelae, and its efficacy has been widely recognized [17]. From the perspective of TCM theory, ICH is categorized as "stroke" with core pathogenesis characterized by disruption of qi and blood flow, obstruction of cerebral vessels, or extravasation of blood beyond the vessels.

The "Baihui" acupoint is regarded as the "meeting point of all yang meridians" capable of lifting yang qi, restoring consciousness, and opening the orifices. The "Xuanli" acupoint is located in the temporal region, and is thought to regulate qi along the Shaoyang meridian and harmonize qi and blood. Stimulation of these two acupoints is traditionally considered to promote "unblocking of brain meridians, restoration of consciousness, and regulation of qi and blood". From a modern medicine perspective, these acupoints are located in regions corresponding to cerebral areas supplied by major intracranial arteries. Acupuncture stimulation may improve local cerebral blood flow and regulate cerebrovascular tone and vasodilation functions, providing a favorable microenvironment for PI3K/AKT pathway activation and autophagy regulation [18, 19]. This "meridian-anatomical-molecular" multi-level interaction highlights the unique value of exploring acupuncture mechanisms guided by TCM theory.

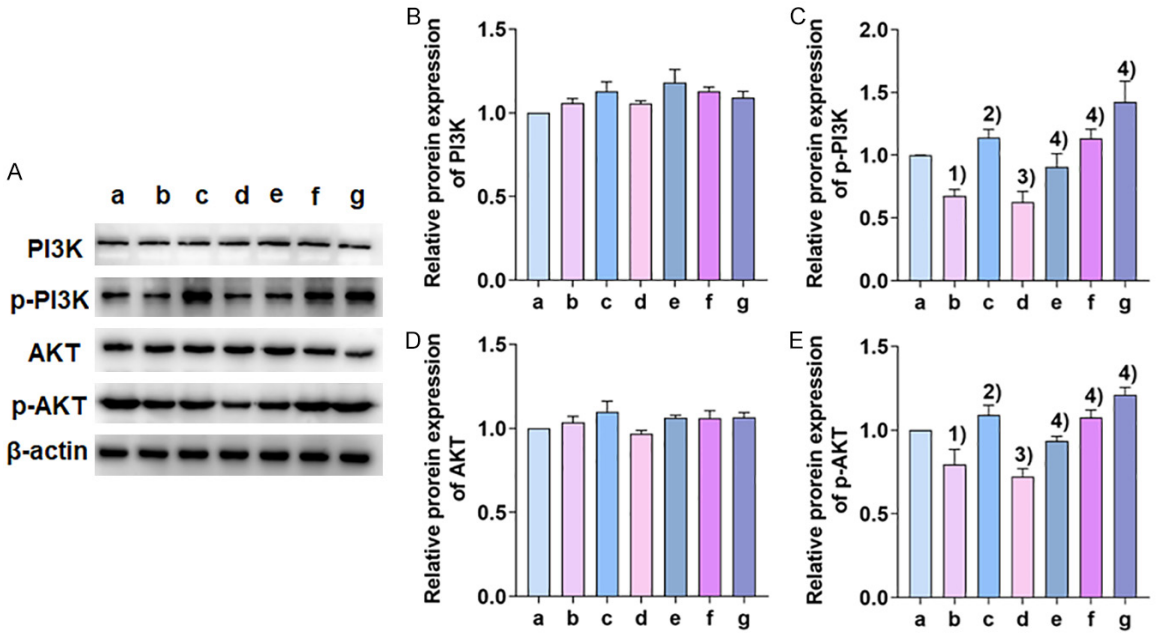


Figure 6. Expression levels of autophagy and PI3K pathway proteins in rat hippocampus. A-E. Phosphorylation levels of PI3K and AKT. (a) sham group (b) model group (c) acupuncture group (d) 3-MA group (e) 3-MA + acupuncture group (f) rapamycin group (g) rapamycin + acupuncture group. [1)]$P < 0.05$, compared to the sham group; [2)]$P < 0.05$, compared to the model group; [3)]$P < 0.05$, compared to the acupuncture group; [4)]$P < 0.05$, compared to the 3-MA group.

However, the effectiveness and mechanism of acupuncture in treating ICH remain to be further elucidated. Previous studies had reported that acupuncture at the "Baihui" and "Qubin" can effectively inhibit M1 polarization of microglia, which is beneficial for alleviating ICH-induced brain tissue injury [20, 21]. Liu Hao et al. showed that acupuncture intervention significantly downregulated IL-1β expression in brain tissue of ICH rats, which was associated with improved neurological deficit scores and enhanced neurological recovery [22]. In addition to its anti-inflammatory effects, evidence suggests that modulation of autophagy-related protein expression may also contribute to neuroprotection after ICH. Autophagy is a self-degradation process to maintain cellular homeostasis. Following ICH, autophagy is initiated and participates in endogenous neuroprotective responses by eliminating damaged organelles [23]. Consistent with this notion, previous studies had shown that acupuncture can upregulate Beclin-1 expression in the brain tissue of ICH rats [24]. While autophagy is generally considered neuroprotective in the early phase after ICH, its role is highly context-dependent. Excessive or dysregulated autophagy may contribute to autophagic cell death under severe stress. In the present study, all assessments were performed at 72 h post-ICH, a time window widely reported as the peak of adaptive autophagy. The observation that 3-MA (which reduced Beclin-1 and ATG5) exacerbated brain injury and inflammation, whereas rapamycin (an autophagy inducer) mimicked acupuncture's benefits, supports the notion that moderate upregulation of autophagy at this stage is protective. However, the net impact of autophagy depends on its intensity, duration, and cellular context-factors that warrant further investigation.

We evaluated the effects of acupuncture on multiple pathologic processes following ICH by measuring inflammatory cytokines, autophagy-related proteins, neuronal apoptosis, and oxidative stress. Compared to the model group, rats receiving acupuncture at "Baihui" penetrating toward "Xuanli" exhibited reduced levels of IL-1β and TNF-α, decreased neuronal apoptosis, and attenuated oxidative stress. Concurrently, the expression of Beclin-1 and ATG5 was upregulated in the acupuncture group. Pharmacological modulation further revealed that 3-MA not only suppressed Beclin-1 and ATG5 expression but also exacerbated inflam-

mation, apoptosis, and oxidative damage, whereas rapamycin produced effects similar to those of acupuncture. These findings suggest a close association between enhanced autophagic activity and the mitigation of neuroinflammation, apoptosis, and oxidative stress following ICH. While a direct causal relationship cannot be established due to the pleiotropic actions of the pharmacologic agents used, the data collectively indicate that functional autophagy - marked by elevated Beclin-1 and ATG5 - coincides with broad neuroprotective effects, including downregulation of IL-1β and TNF-α, suppression of apoptotic pathways, and restoration of redox balance. Thus, acupuncture at "Baihui"-"Xuanli" appears to confer protection against ICH-induced secondary brain injury through a multifaceted modulation of these interconnected pathologic processes.

Recent studies have reported that acupuncture may exert neuroprotective effects by modulating the PI3K/AKT signaling pathway [25, 26]. Studies have shown that acupuncture can modulate this pathway through two mechanisms. First, acupoint specificity: the "Baihui" acupoint is a key acupoint on the Governor Vessel, which is closely connected with brain function, whereas "Xuanli" acupoint belongs to the Gallbladder Meridian of the Foot-Shaoyang, participating in the circulation of Shaoyang meridian qi circulation. Given the anatomic and meridian connections between the Governor Vessel and Gallbladder Meridian in the head region, penetrating acupuncture between Baihui and Xuanli may enhance central regulatory effects through synergistic effects [27]. Second, neuro-humoral regulation: acupuncture may stimulate peripheral nerves in the scalp, transmit signals through the spinal cord to the central nervous system, activate endogenous neuroprotective mechanisms such as the release of brain-derived neurotrophic factor, thereby activating the PI3K/AKT pathway [28]. In this study, acupuncture at Baihui-penetrating-Xuanli was associated with concurrent upregulation of phosphorylated PI3K/AKT and autophagy-related markers (Beclin-1, ATG5), alongside reduced inflammation and improved histologic outcomes. However, it is important to acknowledge that the relationship between PI3K/AKT activation and autophagy induction in this context remains correlative rather than causally established. Notably, the pharmacologic agents (3-MA and rapamycin) used are not selective modulators of autophagy. 3-MA inhibits both Class I and Class III PI3K, thereby potentially suppressing AKT phosphorylation, while rapamycin acts primarily on mTOR, which may trigger feedback activation of upstream kinases, including AKT. Therefore, the observed effects of these agents cannot be interpreted as selective validation of a linear 'PI3K/AKT → autophagy' cascade. Instead, our data suggest that the therapeutic efficacy of acupuncture may depend on a coordinated modulation of the broader PI3K/AKT/mTOR/autophagy network, where multiple nodes are simultaneously influenced. The observation that 3-MA attenuated both the neuroprotective effects and the molecular signatures of acupuncture, whereas rapamycin mimicked them, supports the notion that functional autophagic activity is required for the full therapeutic benefit of acupuncture, even though the precise upstream regulators remain incompletely defined. Future studies employing genetic models (e.g., ATG5 knockout) or more specific pathway inhibitors would help dissect the individual contributions of each pathway component.

Conclusion

Acupuncture at Baihui-penetrating-Xuanli exerts significant neuroprotective effects in a rat model of ICH, as evidenced by improved neurological function, reduced neuroinflammation (decreased IL-1β and TNF-α levels), and enhanced expression of autophagy-related proteins Beclin-1 and ATG5. These protective effects are accompanied by activation of the PI3K/AKT signaling pathway, although the precise mechanistic interplay among these components remains correlative rather than causally defined. Critically, the attenuation of acupuncture-induced effects by autophagy inhibitor 3-MA and their replication by autophagy activator rapamycin underscore the functional importance of intact autophagic activity in mediating its therapeutic outcomes. Despite limitations related to pharmacologic specificity and the absence of direct assessment of mTOR/p-mTOR data preclude definitive pathway mapping, our findings provide compelling evidence that Baihui-penetrating-Xuanli acupuncture may protect against ICH-induced brain injury through coordinated modulation of inflammation and autophagy within a broader PI3K/AKT/mTOR signaling context. This work not only supports the clinical application of this specific acupuncture proto-

col but also lays a foundation for future studies employing more precise molecular tools to dissect the complex pathways underlying acupuncture's neuroprotective actions.

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Disclosure of conflict of interest

None.

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