Electroacupuncture protects against cerebral ischemia-reperfusion injury via regulating P2X7R expression

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Abstract

Clinically, ischemic stroke is a serious disease hard to cure, therefore it is of great importance to slow down the depletion of ATP to enhance the tolerance of ischemic tissue through aggressive preconditioning. Electroacupuncture (EA) preconditioning has been reported to produce a tolerance for cerebral ischemia, with the underlying mechanism remaining unclear. Given that the P2X7 receptor (P2X7R) mediates the stimulation of microglial cells and is involved in the developmental process of cerebrum ischemia-reperfusion (I/R) damage, we hypothesized that the protective effect of Electroacupuncture preconditioning might be associated with the downregulated P2X7R. In this study, 2 '(3')-O-(4-benzoyl) adenosine triphosphate (BzATP) was utilized as a P2X7R agonist in rats with cerebral ischemia-reperfusion injury (MCAO), from which we found that EA preconditioning ameliorated neurologic scoring, decreased infarction volume, and necrotic neurons, and decreased the release of cytokines, while BzATP exacerbated cerebrum I/R damage and inflammation events compared with the favorable efficacy of EA. In addition, BzATP reversed the positive effects mediated by EA, which was consistent with increased expression of P2X7R. These findings suggest that EA induces cerebral ischemic tolerance to ischemia-reperfusion(I/R) damage by suppressing the expression of P2X7R and the release of inflammatory factors.

1. Background

Ischemic stroke is a general disorder of the nervous system associated with high rates of mortality and disability, a condition that can lead to physical disabilities and significant cognition-related impairment, often requiring urgent treatment. Cerebrovascular surgery, including intervention and aneurysm clipping, is associated with a risk of cerebrum ischemia attributed to vascular spasm(Boulouis et al. 2017), in which perfusion is greatly decreased, and cells begin to swell and die. Ischemia reperfusion is needed within a specified time window to get rid of the deterioration of neurological functions for patients' restoration(Rabinstein 2020), and existing clinical interventions are limited by the time window of treatment. Therefore, prolonging the tolerance time of nerve cells to ischemia is the starting point of our study.

Electro-acupuncture (EA) is a secure and valid therapeutic approach, a combination of acupuncture and electrical stimulation, in the therapies of different illnesses, which is extensively utilized in experiment researches and the treatment of ischemia stroke(Liu et al. 2019). As per our researches in the past, EA can ameliorate aberrant neurologic function, decrease infarction volumes, and reduce the quantity of ischemia damage(Xing et al. 2018). Moreover, EA therapy can produce neural regeneration efficacy in ischemia stroke, including facilitating cerebrum blood circulation, modulating oxidation stress, decreasing excitatory amino acids with neural toxicity, sustaining the completeness of the BBB, suppressing the programmed cell death of neurons, elevating neurotrophical factors, and generating cerebrum ischemia tolerance(Yang et al. 2021). Hence, timely treatment in the acute phase with EA is indicated. According to several studies, the inhibition of activated microglial cells and the release of

proinflammation are of great clinical significance to impact EA treatment for ischemic stroke-induced brain impairment (Liu et al. 2017; Xiao et al. 2020).

The P2X7 receptor (P2X7R) is a purinergic acceptor expressed on microglia and has been shown in numerous studies to be related to pain signal transduction, neurosensitisation, neuron stimulation, and neural inflammatory events(Honore et al. 2009; Apolloni et al. 2014; Yang et al. 2016; Jacobson et al. 2020). The expressing level of P2X7R is elevated in the spinal cord with a decrease in pain threshold in rats after chronic constriction injury (CCI)(Lin et al. 2018). Genetic knockdown or medicine blockade of P2X7R significantly reduced not only tenderness but also thermal hyperalgesia in neuropathic pain models (Chessell et al. 2005; Kobayashi et al. 2011; Shen et al. 2018), in which overexpressed P2X7R can induce the stimulation of microglial cells and overexpressed TNF-α and IL-1β(Vadivelu et al. 2015; Shen et al. 2018). Downregulated P2X7R with P2X7- specific siRNA avoided the onset of longterm potentiation (LTP) and elevated the liminal value of pain(Chu et al. 2010), and deeper researches revealed that the stimulation of P2X7R promoted the neural inflammatory reaction via the p38 MAPK signal path(Shiratori et al. 2010; Kim et al. 2015; Cheung-Flynn et al. 2019), whereas EA attenuated SNL-triggered microglia stimulation under the medication of p38 MAPK(Liang et al. 2016). Hence, our team assumed that the cerebral ischemic tolerance efficacy of EA might be realized via decreasing the expressing level of P2X7R, which inhibited the phosphonation of p38 MAPK.

Herein, it's observed that EA preconditioning facilitates the induction of cerebral ischemic tolerance. Our team utilized the specific P2X7R agonist 2’(3’)-O-(4-benzoyl)benzoyl ATP (BzATP) to determine the effects of P2X7R on the neuroprotective and antiinflammation efficacy with EA-stimulation and to determine the tight association between EA and P2X7R and their roles in cerebrum I/R impairment.

2. Materials And Methods

2.1Animal

The test scheme was supported by the Ethical Board of Lab Animals of our institution, and the experimental animals were offered by Shanghai Slack Lab Animal Company (no: SCXK (Shanghai) 2007-0005), including 40 healthy adult male rats of clean-grade weighing 220–250g, the rats of which were fed in a standard animal house for five days and fasted for 12 h before surgery.

2.2 Middle Cerebrum Artery Occlusion Model

Focal cerebrum ischemia was caused via the intraluminal filament method, as delineated previously(He et al. 2016). Rats were anesthetised via isoflurane and 2% pentobarbital sodium. The nylon thread (2636A4/2838A4, Beijing Cinontech Company) was utilized via insertion via the arteria carotis externa, while the blood circulation was subjected to blockade via adjusting the nylon thread from the arteria carotis communis to the carotis internal arterial to realize the occlusion of the middle cerebrum artery. Reinfusion was realized via the withdrawal of the thread posterior to the 1.5-h ischemia, with the wound sutured. Local cerebrum blood circulation was supervised via a transcranial laser Doppler flow measuring

device (PeriFlux 5000; Sweden), and if regional cerebrum blood circulation was reduced to 20% of the preischemia level, MCAO was considered sufficient; if not, the animals were excluded.

2.3. EA Pre-treatment.

The EA pre-treatment was completed as shown above(Zhou et al. 2013). According to the acupoint selection method of *Experimental Acupuncture and Moxibustion*, "Baihui" (GV20) and "Fengfu" (GV16) were selected in the middle of the parietal bone to form the stimulation loop for electroacupuncture. The corresponding acupoints were found, and then the needle was placed. The "Baihui"(GV20) and "Fengfu" (GV16) were stimulated using a Korean electroacupuncture stimulation instrument (HANS-100A, Nanjing Jisheng Medical Technology, China) with parameters set to 1mA current, 2/15 Hz wave frequency, and 30 min of stimulation duration. During the experiment, the rats were kept quiet, with their anal temperatures maintained at 37 ± 0.2°C until the rats regained consciousness.

2.4 Neurobehavioral Evaluation

Twenty-four hours after reperfusion, neurobehavioral deficit scores were measured and scored by blinded testers in the animal group(Hara et al. 1996). The specific scores were as follows, with 0 being no significant neurological symptoms of dysfunction; 1 being right forelimb flexion; 2 being right forelimb not fully extended with significantly reduced anti-lateral thrust; 3 being forelimb flexion, rotation, and crawling to the right; 4 being difficulty or inability to walk spontaneously. Rats with a score of 0 or 4 were considered to be modeling failures and were then excluded from the study.

2.5 Measuring of Infarct Size

Posterior to the 24-h reinfusion, the cerebral tissular sample was rapidly cleared with excessive isoflurane and 2% pentobarbital sodium, the remainder of which was made into 2mm thick coronal sections, which were later treated with 2% 2,3,5 - triphenyl four azole nitrogen (TTC) liquor for 20 min unerring 37°C, then fixed with 4% PFA for 24 h, and finally Image Pro Plus 6.0, software was utilized for shooting, scanning, and images analysis. Cerebrum infarction volume was presented as a proportion (%) of the infarction sample to the total cerebral tissular sample(Wexler et al. 2002).

2.6 Western Blotting (WB)

The total proteins were collected from cultured cells, and the protein contents were identified via a bicinchoninic acid protein analysis tool (Beyotime). The proteins (20 μg) from every specimen were isolated via SDS-PAGE and moved onto a PVDF film (Solarbio, PRC). Posterior to the blockade in 5% milk in Tris-buffered saline for 60 min under ambient temperature, films were cultivated with various antibodies under 4℃ nightlong, followed by incubation with the suitable HRP-conjugated second antibody(Beyotime). The bound antibodies were visualised on autoradiographic film via chemiluminescent identification, using anti-Bcl2(1:1000,CST), anti-Bax(1:1000,CST), anti-P38(1:1000,CST), anti-Phospho-P38 (1:1000, CST) antibodies. The bands were subjected to quantification via identifying the signal intensities via Image-Pro Plus 6.0, and normalising them to that of the Actin (1:8,000, Bioworld) antibody.

2.7 Immunofluorescence

In this study, paraffin slices (3.5 mm thickness) were dewaxed and soaked in 3% H_2O_2 for 600 s to realize the blockade of endogenic peroxidase activity, and slices were subjected to boiling using 10 mmol/L citric acid buffering solution to repair the antigen and naturally cooled at ambient temperature, which was sealed as 5% BSA in PBS under ambient temperature within 60 min, while rabbit anti-NeuN antibody was diluted with 1% BSA and incubated nightlong. Posterior to the cleaning in PBS for 3 times, the fluorescence second antibody was put for and the 4 '6-diamino-2-phenylindole (DAPI) plate was sealed. Fluorescent photos were collected with the aid of an Olympus BX51 fluorescence microscope with no variation in gain, threshold, and blackness levels in each experiment. The images were analyzed based on experiment-relevant conditions.

2.8 Enzyme-related Immunosorbent Assay (ELISA)

Blood was obtained for ELISA in the final stage of the experiment, and in this study, blood was subjected to centrifugation for 600 s at 3000 rpm to acquire serum. The serum contents of TNF-α, IL-1β, and IL-6 were acquired via commercial ELISA tools (R&D Apparatus, America) as per the supplier's specification.

2.9 qRT-PCR

Overall RNA was abstracted via the RNAeasyTM animal RNA separation tool via a spin column as per the supplier's specification, the separated RNA from which was afterwards converted to cDNA via reverse transcription via the PrimeScriptTM RT Master Mix (Perfect Real Time) as per the normal procedure, and eventually the qPCR test was completed via TB GreenTM Premix Ex TaqTM II (Tli RNaseH Plus) with the Applied Biosystems 7500 Real-Time PCR Apparatus (America)with the magnification variables of 95°C for 0.5 min, before 95°C for 5 s and 60°C for 34 s, 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s for 40 cycles. All specimens were studied for three times, with the comparative expression of mRNA computed posterior to the normalisation with β-actin. Every primer sequence is presented by Table 1, and the eventual outcomes were normalised, which were presented as a fold change vs the targeted gene/Actin.

Table 1
Primers for qPCR were used in this study

Gene	Sense(5′-3)	Anti-sense(3′-5)
IL-1β	ATCTCACAGCAGCATCTCGACAAG	CACACTAGCAGGTCGTCATCATCC
IL-6	ACTTCCAGCCAGTTGCCTTCTTG	TGGTCTGTTGTGGGTGGTATCCTC
TNF-α	AAAGGACACCATGAGCACGGAAAG	CGCCACGAGCAGGAATGAGAAG
Actin	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA

3.0 Statistics

The entire data, in addition to neurological scores, were displayed as average ± S.D. and were subjected to ANOVA for comparison and then examined with Tukey's multi-comparison. The neurological scoring was displayed as median (range) and contrasted via a non-parametrical approach (Kruskal–Wallis test) along with the Mann–Whitney U statistics with Bonferroni calibration. We utilized GraphPad Prism 7.0(America) to complete the statistic assay, and $P < 0.05$ had significance on statistics.

3. Results

3.1EA pretreatment was neuroprotective against cerebrum I/R damage, whereas BzATP suppressed the EA-induced enhancement of brain protection

To observe the neuronal cell death after reperfusion and whether P2X7R is vital for the progression of ischemic stroke in MCAO model rats, TTC staining, neurological deficit score, and Western blotting were performed 24 hours after reperfusion. Figures 1(A)to(C) showed infarction volume and neurologic function scoring after EA pretreatment were significantly improved vs MCAO (P<0.05). WB was utilized to identify the expressing levels of Bcl-2 and Bax proteins, as presented by Figure 1D, Bax protein contents were elevated while Bcl-2 protein contents were reduced in MCAO rats (P<0.05), while electroacupuncture can improve the abnormal expression of Bax and Bcl-2 protein in MCAO model rats (P<0.05). These results suggested that electroacupuncture ameliorated MCAO-induced brain nerve cell death, however, the electroacupuncture-induced cerebral protection was inhibited after administration of the P2X7R agonist BzATP.

3.2EA pretreatment reduced the expression of pro-inflammation factors and facilitated the expression of anti-inflammation mediators, but the contradictory effect was observed for BzATP

TNF-α, IL-6, and IL-1β are important inflammatory factors, the expression of which was identified via ELISA and quantitative PCR, and the outcomes revealed that the expressing levels of TNF-α, IL-6, and IL-1β were remarkably elevated in the model group vs the lower expressing levels of TNF-α, IL-6, and IL-1β in the sham operation group (Figure 2(A)to(F)), indicating higher levels of neuroinflammation at the site of damage posterior to cerebrum I/R. However, the effect of reducing inflammatory events in the EA group was inhibited after the administration of the P2X7R agonist BzATP (Figure 2(A)to(F)), suggesting that the up-regulation of P2X7R expression is involved in the modulation of neuroinflammation after cerebrum I/R damage.

3.3 EA reduced MCAO-induced overexpression of Iba1, the effect of which was prevented by BzATP

The expression level of IBA-1 in the ischemia penumbra was identified via Western blotting to ascertain the changes in microglia expression after cerebrum I/R damage, the outcomes of which showed that the expressing level of Iba1 was remarkably elevated in the model group (Figure 3(A)to(B)), indicating that microglia were activated in the cerebrum after cerebrum ischemia reinfusion. The expressing level of microglia decreased significantly after electroacupuncture pretreatment, while the level of Iba1 protein

was further increased after BzATP administration vs the electroacupuncture group. Those outcomes suggest that microglial activation after the cerebral ischemia-reperfusion injury is regulated by P2X7R.

3.4 EA realized the downregulation of the P2X7R and p-p38 expression after cerebral ischemia-reperfusion injury, whereas the opposite role was observed for BzATP

The p38 MAPK is an important pathway regulating neuroinflammation; therefore, the expression levels of P2X7R, p38, and p-p38 proteins were identified via WB (Figure 4(A)to(C)). There were no remarkable diversities in the p38 expressing levels in any group, with the MCAO group having greater contents of P2X7R and P-P38/P38 in contrast to the Sham group, and the EA group having smaller P2X7R and P-P38/P38 contents in contrast to the MCAO group, albeit with the EA+BzATP group having greater P2X7R and P-P38/P38 contents in contrast to the EA group.

4. Discussion

The current study indicates that EA facilitates the reduction of neuronal apoptosis and neurological impairment and the regulation of the expressing of inflammation factors through inhibiting the expressing level of P2X7R.

Given that the cerebrum is the most vulnerable organ, short periods of ischemia and hypoxia can cause irreversible functional and structural damage to the brain(Harutyunyan and Avitsian 2020), which is further exacerbated when blood flow is restored to the damaged area. Studies have shown that various excitatory neurotransmitters, a large amount of which can cause calcium overload and the generation of oxygen free radicals(Mattson 1996; Bak et al. 2018), including acetylcholine, aspartic acid, and glutamate, are closely related to cerebral ischemic injury(Liu et al. 2019; Caba et al. 2021). Therefore, if the release of these transmitters can be effectively controlled, the ischemic reperfusion injury to the brain tissue can be effectively alleviated.

Traditional Chinese Medicine has had a unique insight into “stroke” for millennia, while acupuncture and moxibustion are significant means of TCM treatment(Ifrim Chen et al. 2019). With the continuous development of science, the efficacy of acupuncture and moxibustion has been gradually recognized by the Western medical community, accompanied by clinical studies(Wang et al. 2021) which have confirmed that, through modern medicine, acupuncture and moxibustion treatments of patients with cerebral infarction have significantly improved the hemodynamic parameters and deposition indexes of red blood cells, and that acupuncture treatment also can contribute to the rehabilitation of stroke patients. 16 foreign scholars who acupunctured du meridian acupoints in rats in the cerebral infarction model found that neuron necrosis, cavitation, congestion, edema, and other ischemia-reperfusion injury were significantly reduced (Shiflett 2007) and functional magnetic resonance imaging (fMRI) was used to discover Zusanli point on the back in the brain's frontal activation(Li et al. 2008). In addition, it has been found that electroacupuncture stimulation of “Baihui point” in rats can induce cerebral ischemia tolerance(Kim and Bae 2010; Wu et al. 2015; Yao et al. 2018).

Hypoxic ischemia often leads to neuron death and inflammation which is mainly mediated by microglia(Hanisch and Kettenmann 2007). ATP, the precursor of adenosine, not only provides direct energy to cells but is also an important neurotransmitter in the central nervous system (CNS), while ATP(Giblett and Hoole 2017) and its related metabolites are involved in the purinergic signal transduction pathway by acting on the purinergic receptor (PR). There are two types of PR, P1 and P2, with the P1 receptor having the highest affinity for adenosine and the P2 receptor having the highest affinity for ATP. Studies have shown that electroacupuncture-induced cerebrum ischemia tolerance is closely related to the involvement of purinergic signals in neurons. P2X7R(Sluyter 2017), a purinergic receptor expressed on microglia, has been shown to be elevated in rats with peripheral nerval damage, whereas the antagonist of P2X7R (A804598) reduces phosphorylation of P38, glia cell stimulation, and the expressing level of IL-1B, coherent with less nerval impairment and improved neuron survival(Savio et al. 2017). Electroacupuncture inhibited SNL-triggered microglia cell stimulation under the medication of P38 MAPK(Liang et al. 2016; Liu et al. 2017). Assuming that the expressing levels of inflammation factors and the changes in neurobehavioral functions in post-stroke rats may be associated with the modulation of P2X7R and P38 MAPK. Herein, our team discovered that BzATP exacerbated neurological function and neuron injury in post-stroke rats, and facilitated the expressing levels of P2X7R, P-P38, IBA1, and pro-inflammation factors, which suggests that P2X7R may participate in the occurrence of cerebrum I/R damage.

This study also analyzed the relationship between EA and P2X7R, and the effect of electroacupuncture on cerebral ischemia tolerance as inhibited by the P2X7R agonist. The results of this study show that electroacupuncture can improve neuronal apoptosis and modulate the expressing levels of inflammation factors via suppressing the expressing level of P2X7R, whereas previously researches have revealed that the central causal link of electroacupuncture was anti-inflammatory may be associated with reduced expression of P-P38MAPK, thereby reducing microglial activation(Liang et al. 2016). Drawing on this, we hypothesized that electroacupuncture might repress the phosphonation of P38 via suppressing the activities of P2X7R in microglial cells, thereby reducing inflammatory events and producing ischemic tolerance.

In addition, this study has some limitations; firstly, the expressing level of P2X7R positive cells was identified without immune fluorescence staining and dual staining for P2X7R and IBA1 being performed. Secondly, we cannot reveal that the protective effect of electroacupuncture was achieved via suppressing the phosphorylation of P38 in microglial cells, a perspective that requires more investigation in the future. Thirdly, based on the current research, we can merely hypothesize that electroacupuncture is possible to be protective in the brain via decreasing the expressing level of P2X7R, a mechanism that should be further confirmed using P2X7R inhibitors or P2X7R siRNA.

In conclusion, this study shows that electroacupuncture may reduce the apoptosis and inflammatory response of nerve cells induced by cerebral ischemia reperfusion by down-regulating the expression of P2X7R, thus achieving cerebral ischemia tolerance, and therefore P2X7R can serve as a latent treatment target of electroacupuncture for brain protection. The results of this study reveal the mechanism of

electroacupuncture-induced focal ischemic tolerance, complementing the theoretical and scientific basis for such preconditioning.

Declarations

Authors' Contributions

Feihong Lin, Ye Zhu, and Haipeng Liu had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. Shan Li and Yunchang Mo designed the study and revised the manuscript. Feihong Lin, Haipeng Liu, and Xuliang Huang conducted the experiments and drafted the manuscript. Junlu Wang and Qinxue Dai gave their valuable advice about data analysis and interpretation. Haipeng Liu and Yunchang Mo provided the technique support for the experiment. All authors approved the final version of the paper.Feihong Lin, Ye Zhu, and Haipeng Liu contributed equally to the manuscript.

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Availability of data and materials

The data in our study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This experimental scheme was approved by the Ethics Committee of the Laboratory Animals of Wenzhou Medical University. Shanghai Slack Laboratory Animal Co., Ltd. (license number: SCXK (Shanghai) 2007-0005) offered the experimental animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

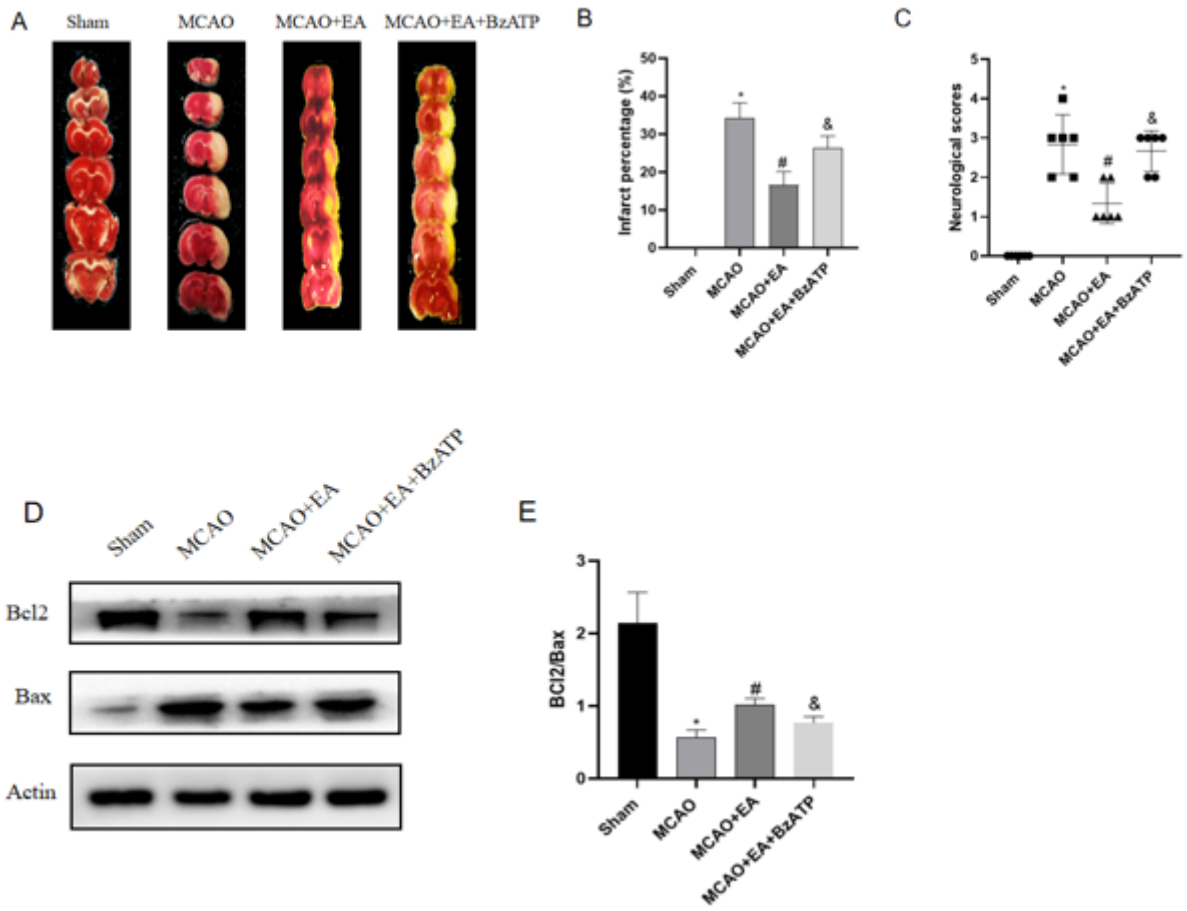

Figure 1

Effect of EA preconditioning on neuronal survival after I/R injury, as opposed to the effect of BzATP. (A) The detection of cerebral infarction in rats with TTC staining. (B) The proportion of cerebrum infarct volume in these 4 groups showed in the bar graph. (C) The assessment of neurological deficit posterior to I/R damage. (D) The protein expressing levels of Bcl2 and Bax in the ischemic penumbra and quantitative data on Bcl2/Bax expression in every group (*P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group)

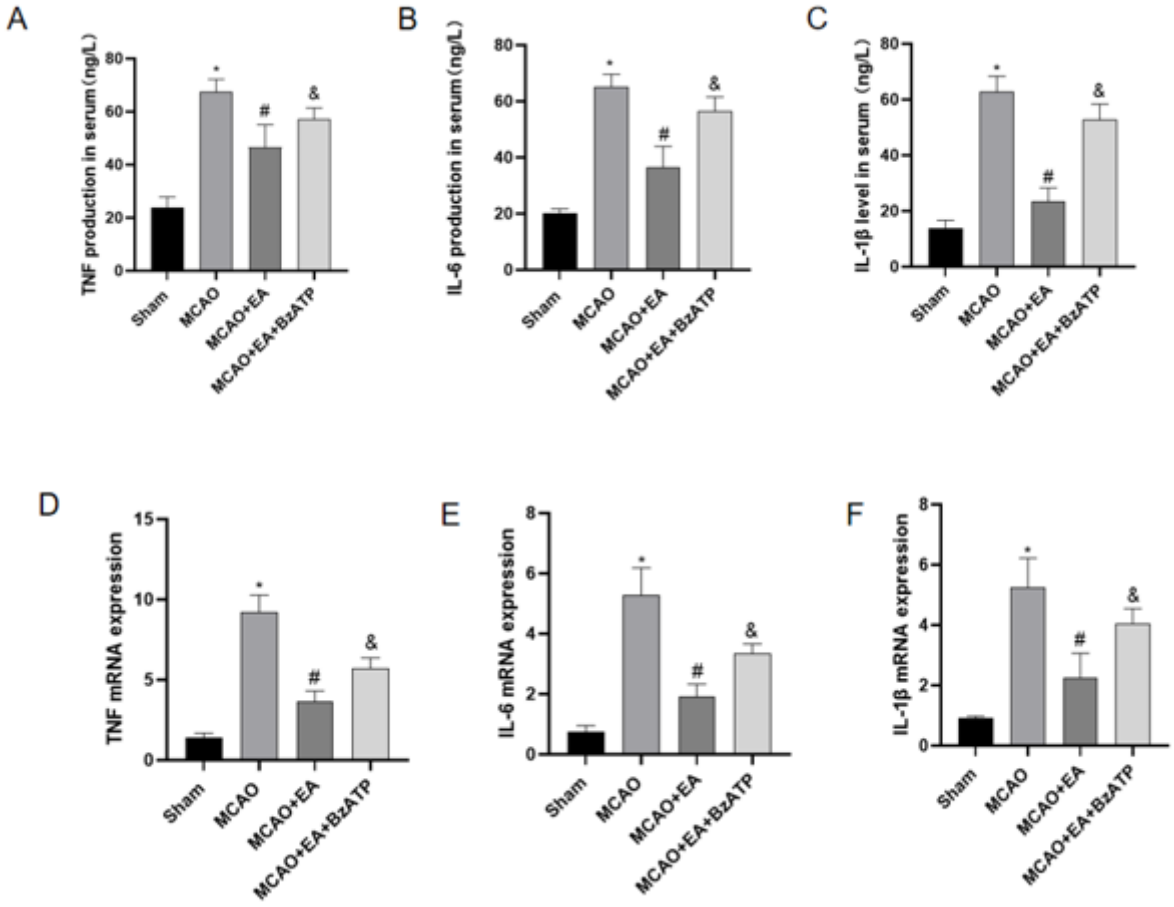

Figure 2

EA reduced the expressing levels of TNF-α, IL-6, and IL-1β after I/R, whereas the opposite effect was observed for BzATP. (A) to (C) showed that TNF-α, IL-6, and IL-1β levels in the rat serum identified via ELISA, while (D) to (F) showed that the expression of TNF-α, IL-6, and IL-1β mRNA in the ischemic penumbra by qPCR.(Columns represent the mean SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group)

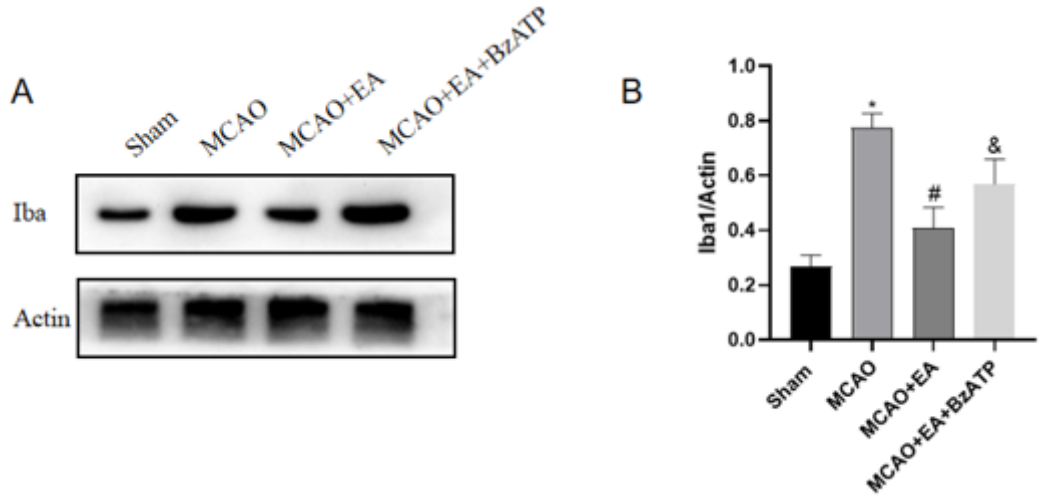

Figure 3

EA decreased the expression levels of Iba1, the effect of which was reversed by BzATP. (A) and (B) showed protein expressing levels of Iba1 in the ischemic penumbra and quantitative data for the expressing level of Iba1/Actin in every group.(Columns denote the average SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group)

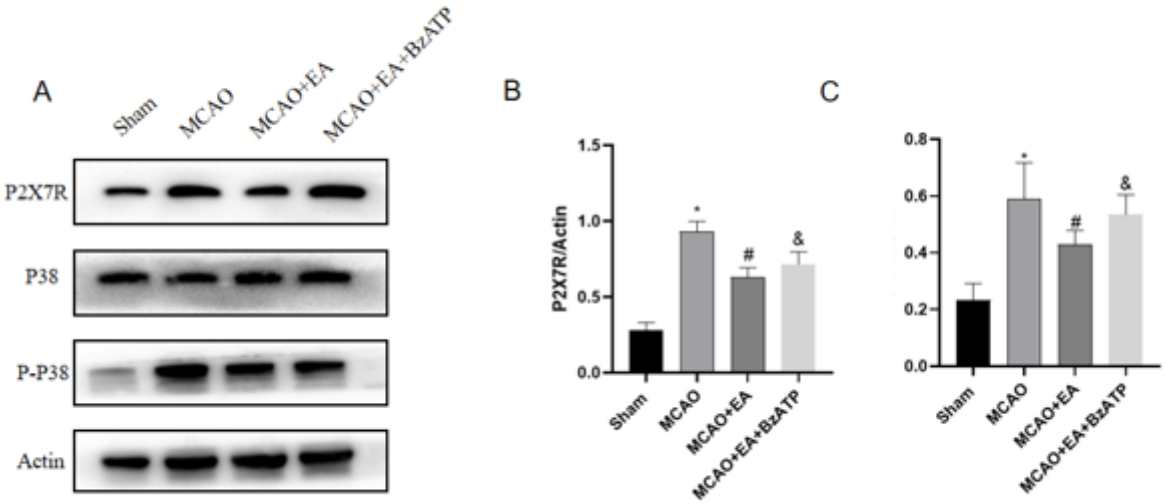

Figure 4

EA reduced the expressing levels of P2X7R and p-p38 after MCAO, the effect of which was reversed by BzATP. (A) to (C) showed typical WB results and quantitative data of the expressing levels of P2X7R/Actin, P-P38/P38 in every group.(Columns denote the average SD. *P < 0.05 vs sham group; #P < 0.05, vs MCAO group; &P < 0.05, vs MCAO+EA group