Underlying mechanisms of Electroacupuncture against Cerebral Ischemia-reperfusion Injury: Reduces Cell Apoptosisand Pyroptosis by Regulating Caspase-3 and Caspase-1

Li Cai
Southern Medical University https://orcid.org/0000-0002-3698-3817
Zeng-Yu Yao
Southern Medical University
Lu Yang
Southern Medical University
Xiu-Hong Xu
Southern Medical University
Meng Luo
Southern Medical University
Miao-Miao Dong
Southern Medical University
Guo-Ping Zhou (doctorzgp@sina.com)
Southern Medical University https://orcid.org/0000-0002-4613-4384

Research

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Abstract

Background

Cell apoptosis and pyroptosis are the primary forms of cerebral ischemia/reperfusion injury, and these two cell death methods are both regulated by the caspase gene family. Electroacupuncture can reduce the neuronal damage caused by cerebral ischemia/reperfusion. We hypothesized that inhibition of Caspase-1/Caspase-3 could be the mechanism of electroacupuncture against pyroptosis and apoptosis after cerebral ischemia/reperfusion injury.

Methods

The cerebral ischemia-reperfusion injury model of C57 and Caspase-1/Caspase-3 gene knockout mice was established by longa' s method. Electroacupuncture was conducted at acupoints Chize (LU5), Hegu (LI4), Sanyinjiao (SP6), and Zusanli (ST36) 1.5 hours after ischemia/reperfusion injury for 20 minutes, and observation was carried out after 24h. Neurological deficit scores evaluated the neurological function, cerebral infarction volume was observed by TTC staining, neuronal apoptosis index was measured by TUNEL, and the protein expression of Caspase-1 and Caspase-3 was detected by Western blot assay.

Results

Compared with I/R group, EA group showed lower neurological deficit score, smaller cerebral infarction volume and lower degree of nerve cell injury (P<0.05). In EA group, the protein expression of Caspase-3 in C57 and caspase-1 knockout mice and Caspase-3 in C57 and caspase-3 knockout mice were lower than that in I/R group (P<0.05).

Conclusions

Electroacupuncture plays a neuroprotective role by inhibiting the protein expression of Caspase-1 and Caspase-3, and reducing the activation of cell apoptosis and pyroptosis.

Background

Ischemic stroke is one of the most severe diseases that affect human health and death in the world. It has the characteristics of high morbidity, high disability rate, high mortality, and high recurrence rate[1–2]. After a certain period of cerebral ischemia, blood flow is recanalized. However, a large amount of blood oxygen supply can further cause more severe nerve damage and accelerate the death of nerve cells. This phenomenon is called cerebral ischemia/reperfusion (I/R) injury[3]. Apoptosis is the main form of cerebral I/R injury. Reducing apoptosis is helpful to the recovery of brain function[4]. Inflammatory response promotes secondary damage in cerebral I/R injury, which is another important cause of neuronal injury after cerebral I/R injury[5]. Pyrocytosis is a new form of cell death discovered and confirmed in recent

years. As a way of cell death accompanied by inflammatory reaction, it also has an important influence[6]. These two cell death patterns are mainly regulated by key regulatory genes of Caspase gene family.

Caspase is a family of cysteine proteases, and there are 14 kinds of caspases[7]. Caspase-3 is pivotal in the apoptosis pathway, and the current research suggests that Caspase-3 mediates apoptosis mainly through mitochondria, death receptors, and endoplasmic reticulum pathways[8]. Each pathway indicates that Caspase-3 is the key protease in cell apoptosis, mediates the common downstream effect of multiple apoptosis pathways, and plays the final pivotal role in the apoptosis process[9]. Due to the importance of Caspase-3 in apoptosis, Caspase-3 is often used as a marker of apoptosis in experimental studies[10].

Not all caspases are involved in the apoptosis response. There is another type of caspase that is inflammation-related, including Caspase-1, Caspase-4, Caspase-11, etc., which are mainly involved in cytokine-mediated inflammatory response but not in the apoptosis pathway[11]. It was confirmed that caspase-1 knockout mice did not affect apoptosis[12]. Other studies showed that the classic Caspase-1 signaling pathway, which mediates cell death, is activated in stroke and aggravates brain injury, inhibition of Caspase-1 activation can reduce stroke injury and play a protective role[13–14]. As the biological characteristics of cell pyroptosis depend on the way of cell death of Caspase-1, Caspase-1 plays a decisive role in the formation of cell pyroptosis.

Acupuncture is an effective method for treating ischemic stroke, which can activate the relevant brain regions of stroke patients, regulate cerebral blood flow and related molecules, and regulate multiple molecules and signal pathways leading to inflammation and neuronal death[15–16]. “Chize (LU5), Hegu (LI4)”, “Sanyinjiao (SP6), and Zusanli (ST36)” served as common acupoints in the clinical treatment of stroke[17–18]. Our previous studies have found that EA can reduce apoptosis and improve neurological injury after cerebral I/R, but its mechanism needs further investigation[19–21]. We speculate that the effect of EA against cerebral I/R neuronal apoptosis and pyroptosis is realized by regulating the Caspase gene family. In this study, the cerebral I/R injury models of C57, Caspase-1 knockout, and Caspase-3 gene knockout mice were established, the neurological deficit scores, cerebral infarction area, apoptosis index, and the expression of Caspase-1 and Caspase-3 were analyzed to explore the mechanism of electroacupuncture on nerve cell protection after cerebral I/R.

Methods

Animals

A total of 48 healthy C57BL/6 mice (half male and half male), weighing 20-25 g. A total of 96 Caspase gene knockout mice (48 Caspase-1, 48 Caspase-3, half male and half female), weighing 20-25 g, were purchased from Cyagen Biosciences Co. Ltd. (Guangzhou, China; license number: SCXK (Su) 2018-0003). The experimental procedure followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1986). The mice were fed standard rodent chow

and allowed free access to water. It was approved by the Animal Ethics and Welfare Committee of Southern Medical University of China (approval No. 2021-005). The temperature of the animal house was maintained at a room temperature between 20℃ and 22℃ and relative humidity of 65–70%. The C57 mice and the Caspase knockout mice were randomized into sham (sham control) group, I/R (model) group, and EA (I/R + EA) group (n = 16 per group).

Establishing cerebral I/R injury mouse model

The middle cerebral artery of the mice was blocked according to the modified Zea-Longa' s method to induce cerebral I/R model[22]. After anesthetizing the mice with 10% chloral hydrate (1 ml/kg) by intraperitoneal injection, the left common carotid artery, left internal carotid artery, external carotid artery, and vagus nerve were carefully exposed through the midline incision under the surgical microscope. A nylon wire of about 11 ± 0.5 mm was introduced into the internal carotid artery to occlude the middle cerebral artery until slight resistance was observed during insertion. After 30 minutes of occlusion, the blood supply of the ischemic area was restored by slowly drawing the thread, and the reperfusion was achieved. Mice in the sham group underwent the same procedures as described previously but without arterial occlusion. At 2 hours after the operation, the 5-point Zea-Longa' s criteria were used for evaluation[22]: 1–3 points indicated successful model establishment, and the corresponding mice were included in the study; the mice with scores 0 or 4 were excluded.

Electroacupuncture intervention

The mice in the EA group underwent EA stimulation 1.5 hours after I/R injury. Stainless steel acupuncture needles (diameter: 0.16×13 mm2; Suzhou Universal Acupuncture Medical Devices Co., Ltd., Suzhou, China) were inserted 2–3 mm into LU5, LI4, ST36, and SP6 acupoints of the paralyzed limb. Selection of acupoints and EA stimulation were made according to "experimental acupuncture" edited by Li[23]. The location of the selected acupoints was as follows: as for LU5, in the depression of the outer end of the transverse cubital crease, an acupuncture needle was inserted perpendicularly to a depth of 3 mm. As for LI4, located between 1st metacarpal bone and 2nd metacarpal bone, an acupuncture needle was inserted perpendicularly to a depth of 1 mm; as for ST36, located at 5 mm below fibular head at outer lateral posterior knee and puncture, an acupuncture needle was inserted perpendicularly to a depth of 7 mm; as for SP6, located at the tip of the inner ankle of the posterior limb, a needle was inserted upward 10 mm and perpendicularly to a depth of 5 mm; The acupoints were stimulated for 20 minutes with a dilatational wave of frequency 5/10 Hz and intensity 2 mA using an EA instrument (Model KWD-808I, Suzhou Universal Acupuncture Medical Devices Co., Ltd.).

Neurological deficit scores

At 24 hours after the operation (before tissue sampling), the neurological deficit score of mice was evaluated by the 5-point Zea-Longa' s method[22]. The neurological deficit scores were defined as follows: score 0, no obvious defect; score 1, failure to fully extend the right forepaw; score 2, circling to the contralateral side; score 3, falling to the opposite side; and score 4, not spontaneously walking or loss of consciousness.

Tissue sampling

The samples were collected 24 hours after I/R injury. After excessive anesthesia (10% chloral hydrate, 10 ml/kg), rapid perfusion with 0.9% normal saline (NaCl) and 4% paraformaldehyde in phosphate-buffered saline for 3–5 minutes eliminated the influence of blood factors. Then the whole brain was dissected out of the cranial cavity immediately. In each group, 4 fresh brain tissues were taken for TTC staining, 6 were placed in 4% paraformaldehyde solution for TUNEL staining, and 6 were stored in - 80℃ refrigerator for Western blot analysis.

Triphenyl tetrazolium chloride (TTC) Staining

Fresh brain tissue was stored at - 20℃ for 20 min and then sectioned every 2 mm in the coronal plane. The slices were placed in 2% TTC phosphate buffer, incubated in a 37℃ water bath for 30 minutes in the dark. Turn the slices evenly every 10 minutes to make the slices even in contact with the TTC staining solution. After staining, the infarct was stained white, and the normal brain tissue was stained red. Use the AlphaEaseFC analyzer software (AlphaInnotech, San Leandro, CA, USA) to measure the infarct size and calculate the brain infarct volume percentage (BIVP).

TdT-mediated dUTP Nick-End Labeling (TUNEL) assay

Brain tissues were routinely dehydrated, paraffin-embedded, and sliced. The sections were incubated with protein K at RT for 15 min, washed in phosphate-buffered saline (PBS) (Ruigu Biotechnology Co., Ltd., Shanghai, China) for 3 times (5 min/per time). Membrane lysis solution was added, incubated at room temperature for 10 min, and washed in PBS 3 times (5 min/per time). Reagent 1 (TdT) and 2 (dUTP) from the TUNEL kit (Kaiji Biotechnology Co., Ltd., Nanjing, China) were obtained, mixed at a ratio of 1:9, added to cover the tissues, and then incubated in a 37℃ water bath (Kaiwei Biotechnology Co., Ltd., Guangzhou, China) for 60 minutes. The sections were washed in PBS 3 times (5 min/per time), DAPI was used for staining the dried section. The section was incubated for 10 min at RT in the dark and was rinsed in PBS 3 times (5 min/per time). Morphological changes in the cerebral tissues were observed under a microscope (Jiangnan Optical Instrument Group, Nanjing, China), and the images were collected for analysis (AlphaEaseFC). Two 20x fields of view in the hippocampal CA1 area were randomly selected for photography. Apoptosis index (AI) was calculated according to the following formula: number of apoptotic cells/ total cells × 100%.

Western blot assay

Hippocampus tissues were homogenized in Radio Immunoprecipitation Assay (RIPA) lysis buffer and centrifuged at 12,000 ×g for 5 min, then determined protein concentration in supernatants. Protein lysates were separated by 10% SDS-PAGE gels and then electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Boston, USA). The membranes were blocked with 5% nonfat dry milk for 1 hour and incubated with primary antibodies: Caspase-1 (1:1000; Servicebio, Wuhan, CNH; GB11383), Caspase-3 (1:1000; Servicebio, Wuhan, CNH; GB11767C) overnight at 4°C. The membranes were incubated with a corresponding secondary antibody (1:3000; goat anti-rabbit/mouse IgG; Servicebio,

Wuhan, CNH; GB23303) in TBST for 30 minutes. The blots were developed using enhanced chemiluminescence, and the intensity of the bands was measured using the AlphaEaseFC analyzer software (AlphaInnotech, San Leandro, CA, USA). The optical density value ratio of the target band to the internal reference served as the relative expression of the target protein.

Statistical analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Quantitative data are expressed as the mean ± SD. If the data meet the normal distribution, one-way ANOVA is used for inter-group comparison, and LSD test is used for pairwise comparison within the group. If the data does not meet the normal distribution, Mann-Whitney nonparametric test is used for pairwise comparison, and Kruskal-Wallis test is used for multiple comparisons. A P value less than 0.05 was considered statistically significant.

Results

Effect of Electroacupuncture on the neurological function after I/R injury

The neurological deficit scores of three genotypes of mice were evaluated in the Sham, I/R, and EA groups. All mice in the sham group did not manifest any signs of cerebral damage, but there were obvious neurological deficits in the I/R group and EA group. There was no significant difference in neurological deficit scores among different genotypes of mice in the same treatment group (P>0.05). Figure 1 shows that the neurological deficit scores of the same genotype mice in the I/R group and EA group were significantly higher than those in the sham group (P<0.05), while the scores in the EA group were significantly lower than those in the I/R group (P<0.05).

Data are presented as the mean±SD (n = 16; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype : *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. ●P < 0.05, vs. C57 mice.

Effect of Electroacupuncture on cerebral infarction volume ratio after I/R injury

After TTC staining, the brain sections of the sham group were bright red, and no pale infarcts were found. In the I/R group and EA group, there were different sizes of pale infarcts. The volume of cerebral infarction in the I/R group and EA group was significantly higher than that in the sham group (P<0.05), while the EA group was lower than the I/R group (P<0.05). The cerebral infarction volume of Caspase-1 and Caspase-3 knockout mice in the I/R group was lower than that of C57 mice (P<0.05). However, there was no significant difference in cerebral infarction volume among different genotypes of mice in the EA group (P>0.05). (Figure 2).

Sham I/R EA

Data are presented as the mean±SD (n = 4; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype: *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ●P < 0.05, vs. C57 mice.

Effect of Electroacupuncture on apoptosis and pyroptosis of neural cells

TUNEL-immunoreactive showed that the normal cell nucleus was blue, and the apoptotic or pyroptotic cell nuclei were green. Few apoptotic or pyroptotic cells were seen in mice of each genotype in the Sham group. After successful model establishment, the number of apoptotic or pyroptotic cells was significantly increased(P<0.05). Compared with the I/R group, the apoptotic or pyroptotic cells in the EA group were significantly reduced(P<0.05). In the I/R group, the positive rates of Caspase-1 knockout and Caspase-3 knockout mice were significantly less than that of C57 mice(P<0.05). Similarly, in the EA group, the positive rates of Caspase-1 knockout and Caspase-3 knockout mice were also significantly less than that of C57 mice(P<0.05). (Figure 3).

C57

Cas-1 ko

Cas-3 ko

Sham I/R EA

Data are presented as the mean±SD (n = 4; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype: *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ●P < 0.05, vs. C57 mice. Comparison within the EA group: ●P < 0.05, vs. C57 mice.

Effect of Electroacupuncture on the protein expression of caspase-3 and caspase-1 in mice after I/R injury

Western blot assay results showed that the protein expression of caspase-1 in C57 and Caspase-3 knockout mice increased significantly after I/R (P<0.05). Compared with the I/R group, the protein expression of Caspase-1 in the EA group decreased (P<0.05), but there was no significant difference between C57 and Caspase-3 knockout mice (P>0.05). Similarly, the protein expression of Caspase-3 in C57 and Caspase-1 knockout mice increased significantly after I/R (P<0.05). Compared with the I/R group, the protein expression of Caspase-3 in the EA group decreased (P<0.05), and there was no significant difference between C57 and Caspase-1 knockout mice neither(P>0.05). (Figure 4).

Data are presented as the mean±SD (n = 6; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype : *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ▲P > 0.05, vs. C57 mice. Comparison within the EA group: △P > 0.05, vs. C57 mice.

Discussion

Electroacupuncture therapy in the early stage after cerebral I/R could reduce the degree of cerebral I/R injury and expand the therapeutic window[24–25]. Therefore, we chose electroacupuncture treatment 1.5 hours after cerebral I/R injury and 24 hours as the observation time to observe the effect of electroacupuncture acupoint combination of Chize, Hegu, Zusanli, and Sanyinjiao on cerebral I/R injury. Neurological deficit scores are an essential method to evaluate behavioral changes after cerebral I/R injury, and it is also an effective index to judge the recovery of neural function. The results showed that electroacupuncture could significantly improve the neurological deficit symptoms after cerebral I/R injury. In the I/R group, the neurological deficit scores of Caspase-1 and Caspase-3 mice were lower than those of C57 mice, which may be achieved by inhibiting cell apoptosis and pyroptosis. However, there was no difference in the EA group. It is suggested that electroacupuncture plays a similar role in inhibiting the expression of Caspase-1 and Caspase-3. Experimental studies have demonstrated that electroacupuncture could improve limb motor function and provide some neuroprotective effects against I/R injury[26].

We observed the volume of cerebral infarction in each group after cerebral I/R injury and obtained results similar to neurological deficit scores. The infarction volume of C57 mice in the I/R group was significantly higher than that of Caspase-1 and Caspase-3 knockout mice, and the EA group was significantly lower than that of the I/R group, but there was no significant difference among different genotypes. It is also suggested that electroacupuncture may inhibited the expression of Caspase-1 and Caspase-3, and reduced the infarction volume after cerebral I/R. Previous studies have also found that electroacupuncture could inhibit neuronal apoptosis and reduce the volume of cerebral infarction in JNK knockout mice with cerebral I/R injury[27–30].

Cell pyroptosis has both necrosis and apoptosis characteristics in morphology. The cells with pyroptosis also have nuclear concentration, chromatin DNA strand breakage, and TUNEL positive staining. Normal cells have no broken DNA fragments and will not react with markers, so the normal nucleus is blue[31]. In this study, the hippocampal CA1 region sensitive to cerebral I/R injury was observed. Compared with C57 mice, the positive rate of TUNEL staining in caspase-1 and caspase-3 knockout mice was lower. It was verified that apoptosis and pyroptosis were mainly regulated by caspase-1 and caspase-3. After electroacupuncture therapy, the positive rate of TUNEL staining in mice of different genotypes decreased significantly. It shows that electroacupuncture could reduce apoptosis and pyroptosis in the hippocampal CA1 region after cerebral I/R injury and may be achieved by inhibiting the expression of caspase-1 and

caspase-3. Comparative research also showed that acupuncture could inhibit the apoptosis of hippocampal neurons and promote the recovery of neurons after cerebral I/R injury[32].

Western blot assay further verified the results of TUNEL findings. After cerebral I/R injury, the expression of Caspase-1 and Caspase-3 in C57 mice increased significantly. While after electroacupuncture therapy, the expression of Caspase-1 and Caspase-3 decreased significantly. Related studies have shown that the expression of Caspase-3 mRNA in neurons in the cortex and hippocampal CA1 region of rats after cerebral I/R will increase and gradually decrease after 24 hours, with the same trend as that of apoptosis. Injection of Caspase-3 inhibitor Z-DEVD-FMK could effectively inhibit cell apoptosis and delay cell necrosis in rats with cerebral I/R injury[33–34]. Other studies have shown that in the cerebral stroke model, the expression of Caspase-1 increases significantly in neurons, astrocytes, and microglia after ischemia. Inhibiting the expression of Caspase-1 could reduce the damage of cerebral cells[35–36]. Previous studies and the results of this study confirmed the previous hypothesis that electroacupuncture has a neuroprotective effect on cerebral I/R injury and could effectively reduce neuronal apoptosis and pyroptosis. This effect is achieved by inhibiting the expression of Caspase-1 and Caspase-3.

However, this study still has limitations. After cerebral ischemia, neural function, inflammatory reaction of brain tissue, and expression levels of Caspase-1 and Caspase-3 are in dynamic changes. We only selected the time point 24 hours after cerebral I/R for observation. In the follow-up study, we need to add different time subgroups for systematic discussion. In addition, TUNEL positive staining could not distinguish apoptosis or pyroptosis. Therefore, future research needs a detection method that could determine apoptosis and pyroptosis.

Conclusion

In summary, electroacupuncture at acupoints“LU5,” “LI4,” “ST36,” and “SP6” could alleviate the neurological deficit symptoms and reduce cell apoptosis and pyroptosis in C57 and Caspase-1/Caspase-3 gene knockout mice with I/R injury. Inhibiting the expression of Caspase-1 and Caspase-3 may be the critical mechanisms involved in the effects of electroacupuncture on I/R injury.

Declarations

Ethics approval and consent to participate: All experimental procedures were approved by the Animal Ethics and Welfare Committee of Southern Medical University of China (approval No. 2021-005). The experimental procedure followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1985).

Consent for publication: All authors agreed to publish this article.

Availability of data and material: The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: We declare that we have no conflict of interest.

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Authors' contributions: GPZ obtained funding, participated in study concept and design, and paper authorization. XHXu and ML purchased animals and material instruments. ZYY analyzed data. LC wrote the paper. MMDong operated the acupuncture. LY ensured the integrity of the data. All authors approved the final version of the paper.

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Figures

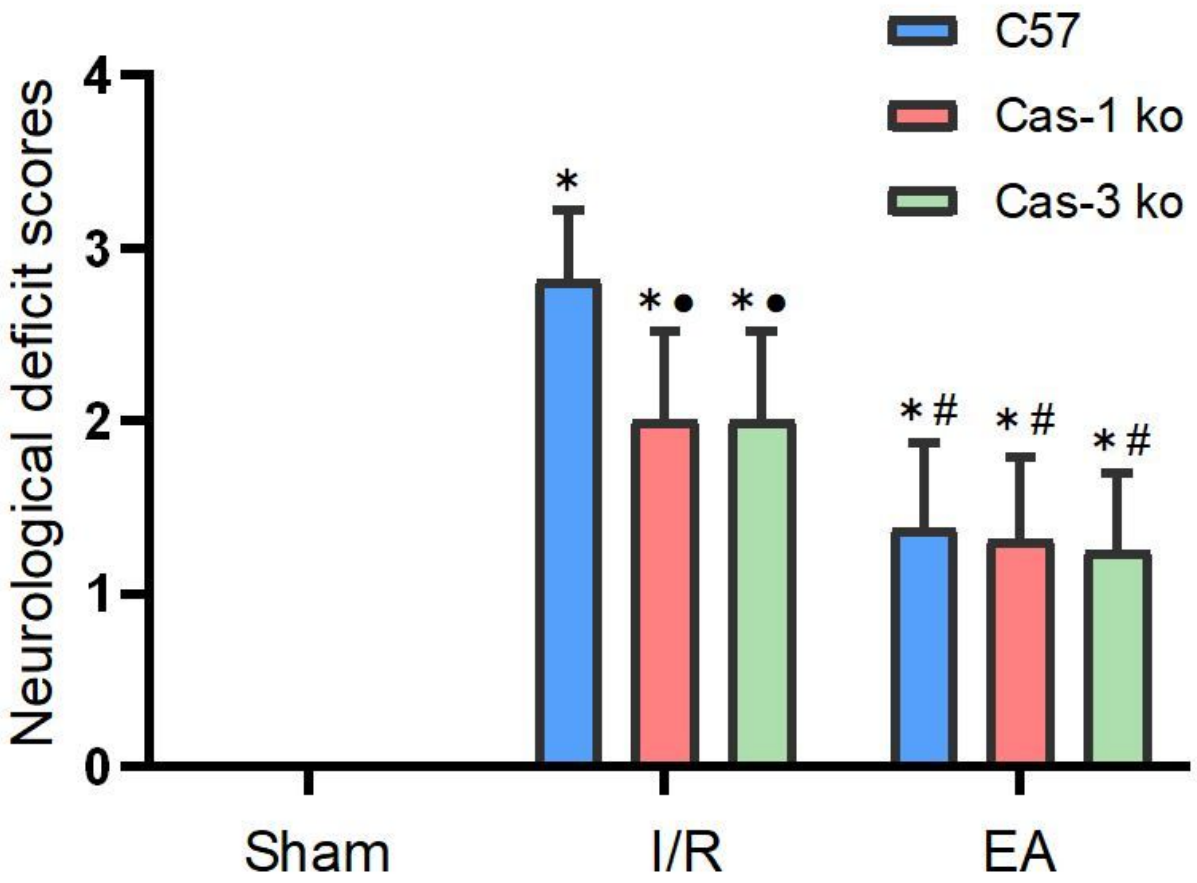

Figure 1

Neurological deficit scores Data are presented as the mean±SD (n = 16; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype : *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. ●P < 0.05, vs. C57 mice.

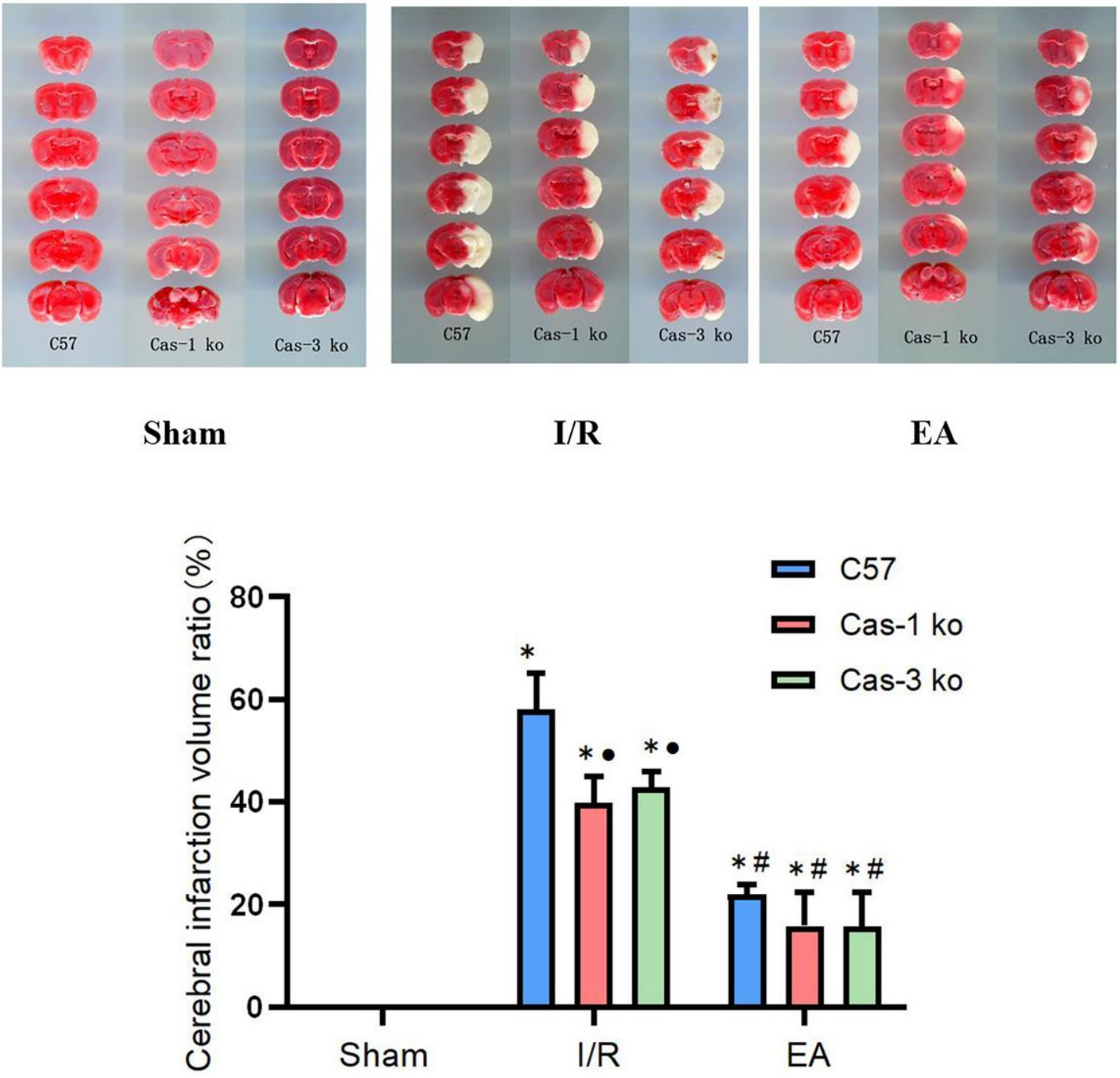

Figure 2

Brain tissue with TTC staining Data are presented as the mean±SD (n = 4; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype: *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ●P < 0.05, vs. C57 mice.

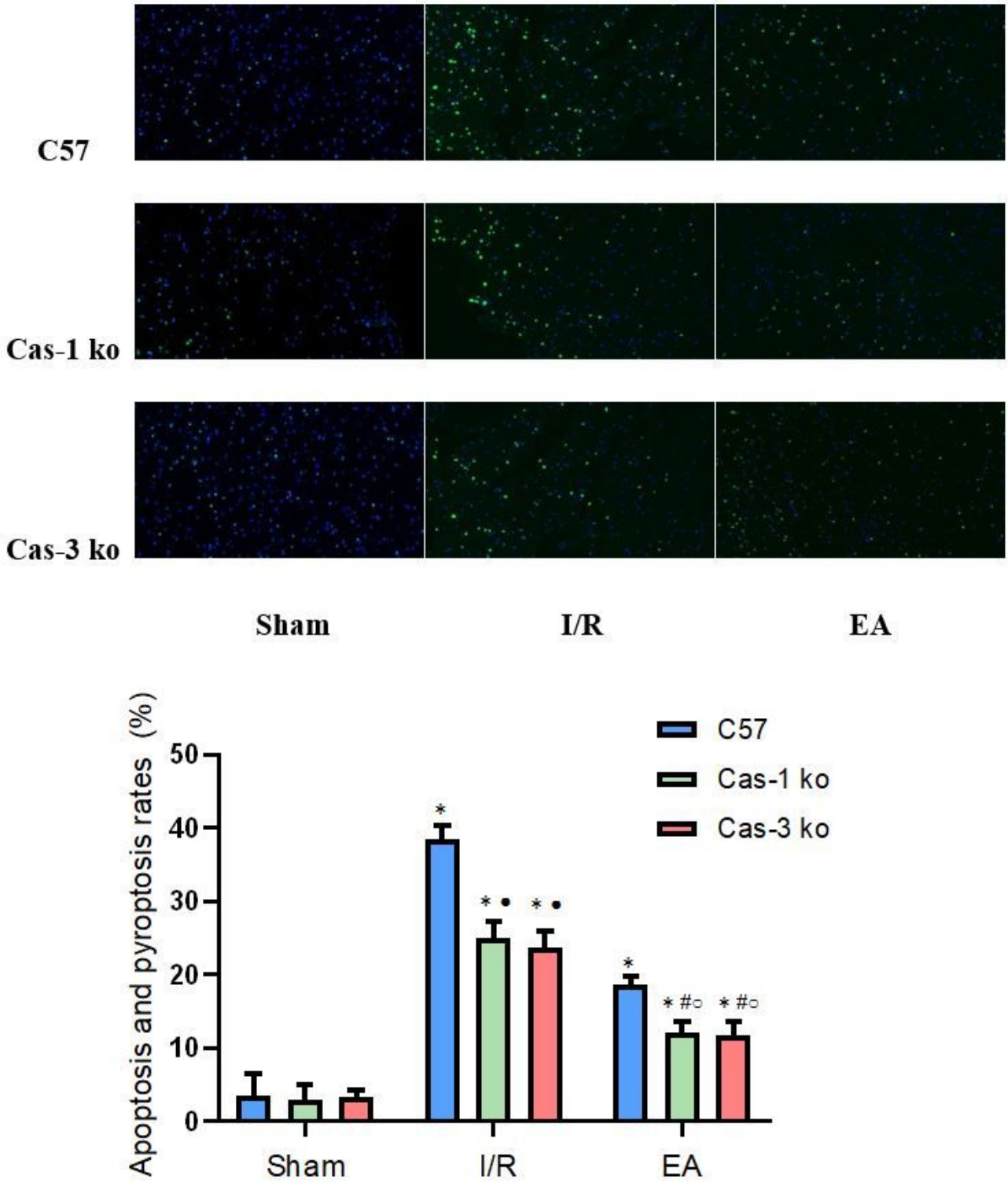

Figure 3

Apoptosis and pyroptosis rates of neural cells Data are presented as the mean±SD (n = 4; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype: *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ●P < 0.05, vs. C57 mice. Comparison within the EA group: ●P < 0.05, vs. C57 mice.

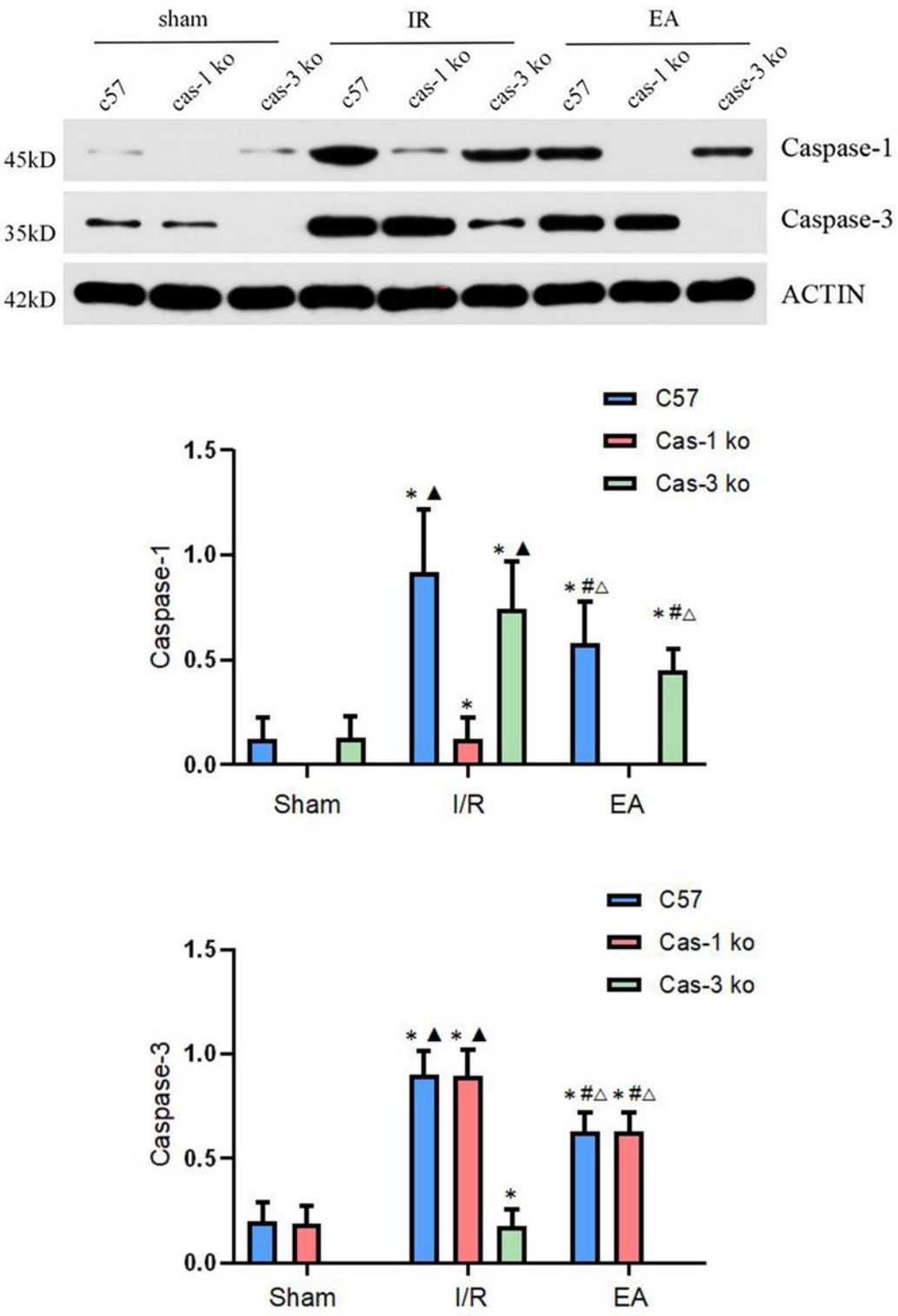

Figure 4

Protein expression of Caspase-1 / Caspase-3 Data are presented as the mean±SD (n = 6; one-way analysis of variance and leastsignificant difference test). Comparison within the same genotype : *P < 0.05, vs. Sham group; #P < 0.05, vs. I/R group. Comparison within the I/R group: ▲P > 0.05, vs. C57 mice. Comparison within the EA group: △P > 0.05, vs. C57 mice.