

# Alda-1, an Activator of ALDH2, Protects Cardiac and Neurological Function Postresuscitation by Inhibiting Pyroptosis in Swine

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## Research Article

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# Abstract

## Purpose

The enzyme aldehyde dehydrogenase 2 (ALDH2) has been shown to protect the heart and brain from oxidative stress injury, and this effect is related to the inhibition of pyroptosis. In the present study, we hypothesised that the ALDH2 activator *N*-(1,3-benzodioxol-5-ylmethyl)-2,6-dichloro-benzamide (Alda-1) would mitigate cardiac and neurological injury postresuscitation in a preclinical swine model of CA.

## Methods

Following 8 minutes of untreated ventricular fibrillation, and another 8 minutes of cardiopulmonary resuscitation (CPR), the swine randomly received either Alda-1 (0.88 mg/kg, n = 6) or saline (n=5) after restoration of spontaneous circulation (ROSC). Hemodynamic parameters and cardiac function were monitored, and serial blood samples were collected postresuscitation to detect biomarkers of cardiac and neurological injury. At 24 hours postresuscitation, first, neurological scores were evaluated, and then, the swine were sacrificed and pyroptosis-related proteins, proinflammatory cytokines, and oxidative stress were assessed in heart and brain samples.

## Results

Cardiac and neurological injury were significantly improved in the Alda-1 group compared with the CPR group postresuscitation. In addition, after treatment with Alda-1, the NLR family pyrin domain-containing 3 (NLRP3) inflammasome, Gasdermin D (GSDMD), and proinflammatory cytokine levels were markedly suppressed. Moreover, 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), indicators of oxidative stress, were also significantly inhibited after Alda-1 administration, which was accompanied by increased ALDH2 activity and protein expression.

## Conclusion

Alda-1 improves cardiac and neurological dysfunction postresuscitation in a swine model of CA, at least partly by inhibiting oxidative stress-mediated NLRP3 inflammasome activation and pyroptosis.

## Background

The high morbidity associated with cardiac arrest (CA) and the high mortality postresuscitation are of great concern worldwide[1, 2], although unremitting efforts have been made to advance the rate of successful resuscitation and improve organ function postresuscitation in recent years. According to recent research, cardiac and neurological dysfunction are the two predominant causes of death of CA patients[3] and are induced by global ischemia/reperfusion (I/R) insult. Therefore, the active protection of cardiac and neurological function is critical for the improvement of postresuscitation outcomes.

Many studies have shown that cardioprotective and neuroprotective effects can be achieved by regulating the apoptosis, necrosis, autophagy, and pyroptosis pathways, which are the most common pathways of programmed cell death (PCD) [4]. Pyroptosis is promoted by NLR family pyrin domain-containing 3 (NLRP3) inflammasome activation and crosstalks with apoptosis[5], necrosis[6], and autophagy[7]. A large amount of evidence has demonstrated that activation of pyroptosis is essential for cardiac[8] and neurological[9] deterioration postresuscitation. Our previous *in vivo*[10] and *in vitro*[11] research found that I/R injury exacerbated neuronal damage by activating NLRP3-mediated neuroinflammation, which indicated that the inhibition of pyroptosis might be a potential therapeutic strategy for the improvement of cardiac and neurological function postresuscitation.

N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichloro-benzamide (Alda-1), an activator of aldehyde dehydrogenase 2 (ALDH2), can attenuate I/R injury in most organs, such as the heart[12], brain[13], liver[14], and kidney[15]. ALDH2 is a mitochondrial enzyme that is abundantly expressed in the liver and is also found in the myocardium and brain tissue[16]. ALDH2 has recently been shown to be involved in I/R injury[17], and elimination of toxic endogenous aldehydes, including 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), is an important role of ALDH2. A rat model of CA demonstrated that enhanced activity or expression of ALDH2 attenuated myocardial dysfunction postresuscitation, and the protective effect of ALDH2 might occur through suppressing 4-HNE-mediated mitochondrial reactive oxygen species (ROS) production[18]. In addition, a local I/R rat model revealed that increasing ALDH2 activity-induced 4-HNE clearance can reduce neurological impairment[13]. ROS are the most crucial factors in the pathogenesis of I/R injury postresuscitation[18] and are activators of NLRP3 inflammasome-dependent pyroptotic cell death[19]. Recently, a series of animal studies confirmed that Alda-1 could protect both cardiac[12] and neurological[13] function against local I/R injury. However, the cardiac and neurological effects of Alda-1 in a clinically relevant swine model of CA as well as the underlying mechanism have not yet been investigated.

Therefore, the present study investigated the effect of Alda-1 on postresuscitation cardiac and neurological dysfunction in a clinically relevant swine model of CA. We hypothesized that Alda-1 improved cardiac and neurological function by activating ALDH2, partly by inhibiting ROS-mediated NLRP3 inflammasome-induced pyroptosis.

## Methods

### Animals

The experimental protocol and all the experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Affiliated Hospital, Zhejiang University School of Medicine. The experimental outline is summarized in Fig. 1. Twenty-two 6-month-old male white domestic swine, weighing  $38.6 \pm 2.4$  kg, were obtained from Shanghai Jiagan Biotechnology Inc. (Shanghai, China). All the swine were fasted overnight, but provided free access to water, before the experiment.

### Anesthesia and Surgeries

Following initial intramuscular injection of midazolam (0.4-0.5 mg/kg) and then intravenous injection of propofol (2 mg/kg), endotracheal tubes (7.0 mm) were inserted into the trachea. Anesthesia was maintained by continuous intravenous injection of propofol (4 mg/kg) by an intravenous pump. After the animals were intubated, mechanical ventilation was initiated with a volume-controlled ventilation model (Oxylog 3000 plus, Drager, Luebeck, Germany). The ventilator parameters were as follows: the tidal volume was 12 mL/kg, peak flow was 40 L/min, and fraction of inspired oxygen (FiO<sub>2</sub>) was 0.21. End-tidal carbon dioxide (EtCO<sub>2</sub>) was monitored with a M Series Monitor Defibrillator (ZOLL Medical Corporation, Chelmsford, MA) and maintained between 35 and 40 mmHg by adjusting the minute volume. Lead II ECG and pulse oxygen saturation were continuously recorded. To measure cardiac function at the indicated timepoints, a 7F central venous catheter (Abbott Critical Care # 41216, Chicago, IL) was placed in the left external jugular vein, and another 4F thermistor-tipped arterial catheter (Abbott Critical Care # 41216, Chicago, IL) was placed into the left femoral artery. Both catheters were connected to the (Pulse index Continuous Cardiac Output) PiCCO Monitor system (BeneVision N22, Mindray, Shenzhen, China). To continuously measure arterial pressure, an 8F catheter (Model 6523, C.R. Bard Inc., Salt Lake, UT) was inserted into the thoracic aorta through the right femoral artery. To measure right atrial pressure (RAP) during the resuscitation period, a 7F pentalum, thermidilution tipped catheter (Abbott Critical Care # 41216, Chicago, IL) was inserted into the right atrium through the right femoral vein. To induce ventricular fibrillation (VF), a 5F pacing catheter (EP Technologies Inc., Mountain View, CA) was placed into the right ventricle through the right external jugular vein. The positions of all the catheters were confirmed by characteristic pressure morphology. All the catheters were filled with heparin saline (5 IU/mL) and intermittently flushed. To record temperature, a thermal probe was inserted into the rectum, and the rectum temperature was maintained at 38.0 ± 0.5°C during the surgical preparation stage in all the animals.

## **Groups and Intervention**

The animals were randomized to one of three cohorts after the surgical procedures were completed: 1) the sham group ( $n = 6$ ) underwent anesthesia and surgery similar to the other groups but without induction of CA, cardiopulmonary resuscitation (CPR) or other interventions; 2) the CPR group ( $n = 8$ ) experienced CA and CPR after anesthesia and surgery as described above and 3) the Alda-1 group ( $n = 8$ ) was treated similar to the CPR group, except for the intravenous injection of Alda-1 (0.88 mg/kg) after the restoration of spontaneous circulation (ROSC), and the dose was converted from a mouse research and based on body surface area [13, 20].

## **VF induced CA**

This model of VF-induced CA in swine was described in our previous study[21]. Ten minutes before the induction of VF, baseline measurements were obtained. VF was induced by a 1-mA alternating current delivered to the right ventricular endocardium. Mechanical ventilation was discontinued after VF. Before CPR, the pacing catheter was removed. After 8 minutes of untreated VF, manual chest compression was initiated, accompanied by ventilation at a ratio of 30:2, which was performed by a bag respirator attached

to the endotracheal tube. The depth and rate of chest compression were monitored by an E Series Monitor Defibrillator (PlamCPR, Sunlife, Suzhou, China) to guarantee a depth of 50 to 60 mm and a rate of 100 to 120 per minute. After 2 minutes of CPR, a bolus of epinephrine (20 µg/kg) was injected. After 8 minutes of CPR, a single 150-J electrical shock was achieved for defibrillation by the E Series Monitor Defibrillator. ROSC was defined as the appearance of an organized rhythm with a mean arterial pressure (MAP) > 50 mmHg that persisted for more than 5 minutes. If ROSC was not observed, CPR and ventilation were continued for another 2 minutes before the next defibrillation. The protocol was repeated until ROSC occurred for a duration of up to 10 minutes. Repeating doses of epinephrine were administered at 4-minute intervals after the first injection. After ROSC, mechanical ventilation was continued with an FiO<sub>2</sub> of 1.0 for the first 0.5 hours and then 0.21 for the last 3.5 hours. At 4 hours postresuscitation, all the catheters were withdrawn, and the animals were transferred to closed cages for 20 hours of observation. At 24 hours postresuscitation, the animals were euthanized with an intravenous injection of propofol (3 mg/kg) and then intravenous injection of sodium pentobarbital (150 mg/kg).

### **Sample collecting**

Venous blood samples were collected at baseline and 1, 2, 4, and 24 hours after resuscitation, and then, the sera were preserved in [liquid nitrogen](#) after centrifugation. After sacrifice, the left ventricular myocardium, right hippocampus, and right frontal cortex were harvested in [liquid nitrogen](#) for the evaluation of proinflammatory cytokines, pyroptosis-related proteins, oxidative stress markers, and ALDH2 expression and activity.

### **Monitoring**

Hemodynamics and ECG were continuously recorded by a patient-monitoring system (BeneVision N22, Mindray, Shenzhen, China). Coronary perfusion pressure (CPP) was calculated as the difference between diastolic blood pressure and time-coincident RAP during the CPR period. E<sub>t</sub>CO<sub>2</sub>, dose of epinephrine, number of defibrillations, and duration of CPR were also monitored or recorded.

### **Cardiac function evaluation**

Global ejection fraction (GEF) and stroke volume (SV), as indicators of cardiac function, were measured with the PiCCO system at baseline and 1, 2, and 4 hours after resuscitation.

### **Neurological function evaluation**

Neurological function was evaluated using a validated scale of neurological deficit score (NDS) before anesthesia at 24 hours postresuscitation, as previously described[22]. Four aspects, namely, consciousness, respiratory pattern, motor and sensory function, and behavior, were evaluated. NDS ranged from 0 (normal) to 400 (brain death) and was scored by an independent researcher who was familiar with the scoring system but blinded to the study.

### **ELISA**

The levels of cardiac troponin I (TnI, MEIXUAN Biological Science and Technology LTD, Cat No. MEXN-P0615) and neuron-specific enolase (NSE, MEIXUAN Biological Science and Technology LTD, Cat No. MEXN-P0992) in the serum and the levels of interleukin-1 $\beta$  (IL-1 $\beta$ , MEIXUAN Biological Science and Technology LTD, Cat No. MEXN-P0002), interleukin-18 (IL-18, MEIXUAN Biological Science and Technology LTD, Cat No. MEXN-P0007) and 4-HNE (Abcam, Cat No. ab238538) in the supernatants of myocardium, hippocampus and cortex homogenates were detected by using swine enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

### **Western blot**

The supernatants of myocardium, hippocampus and cortex homogenates were also used for the western blot analysis of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), Cleaved Caspase-1, Gasdermin D (GSDMD) and ALDH2 protein concentrations. Primary antibodies against NLRP3 (Proteintech, Cat No. 19771-1-ap), ASC (Proteintech, Cat No. 10500-1-ap), Cleaved Caspase-1 (CST; Cat No. 4199), GSDMD (Proteintech, Cat No. 20770-1-ap), ALDH2 (Proteintech, Cat No. 15310-1-ap), and NAPDH (BBI, Cat No. D190090-0100) were first used, and then, anti-mouse (BBI, Cat No. D110087-0100) or anti-rabbit (BBI, Cat No. D110058-0100) antibodies were used as secondary antibodies. Finally, quantification of the band intensities was performed by using ImageJ (NIH, Bethesda, MD, USA), and the results of these analyses were all normalized as the ratio between the level of the protein of interest and the level of NAPDH.

### **MDA level**

Lipid peroxidation was determined by the reaction of its end product malondialdehyde (MDA) with thiobarbituric acid. The supernatants of myocardium, hippocampus and cortex homogenates were used for analysis according to the manufacturer's protocol provided with the MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Cat No. A003-1). The MDA levels are expressed as MDA nmol/mg of proteins.

### **ALDH2 activity**

The supernatants of myocardium, hippocampus and cortex homogenates were used to assess ALDH2 activity according to the manufacturer's protocol provided with the Mitochondrial ALDH2 Activity Assay kit (Abcam, Cat. No. ab115348). This experiment was conducted according to the manufacturer's protocol, and ALDH2 activity was expressed as mOD/min.

### **Statistical analysis**

Continuous variables were presented as the mean  $\pm$  standard deviation when they were normally distributed or as a median (25th, 75th percentiles) when nonnormally distributed. Normality tests were performed by a one-sample Kolmogorov-Smirnov Z test. For the comparison of resuscitation data, Student's t-test was used for statistical analysis. For normally distributed endpoint data, one-way analysis of variance was used for comparison. If there was a significant difference in the overall comparison of

groups, comparisons between individual groups were made by the LSD test. For nonnormally distributed endpoint data, the Kruskal-Wallis test was used, followed by the Mann-Whitney U test. Comparisons between time-based variables within each group were performed with repeated-measurement analysis of variance. For the comparison of categorical variables such as ROSC, Fisher's exact test was used. All the analyses were performed using R Studio (version 1.4.1103), with a value of  $P < 0.05$  considered statistically significant.

## Results

### Resuscitation and survival data

As mentioned in the previous section, a total of 22 swine were utilized in our study, 16 swine underwent CA and CPR (the CPR or Alda-1 group), 11 of which were successfully resuscitated. In addition, CPP (Fig. 2a) and EtCO<sub>2</sub> (Fig. 2b) were similar in the CPR and Alda-1 groups during the 8 minutes of CPR. No significant differences were observed in the number of defibrillations, dose of epinephrine or duration of CPR between the two groups. The Alda-1 group achieved a higher rate of ROSC than the CPR group (75.0% vs 62.5%), but these data did not reach a significant difference. Furthermore, the mortalities were both 0% in the CPR and Alda-1 groups at the end point of observation (24 hours postresuscitation).

### Hemodynamics, cardiac function, and biomarker of myocardial injury

After resuscitation, hemodynamic and cardiac function were markedly impaired in the CPR and Alda-1 groups, with an increase in HR (Fig. 3a) and a decrease in MAP (Fig. 3b), SV (Fig. 3c) and GEF (Fig. 3d) during 4 hours of observation. Alda-1 treatment had a beneficial effect on HR and MAP, as shown by the sharply decreased HR and increased MAP during the observation time. In addition, Alda-1 treatment significantly improved cardiac function compared with CPR. Furthermore, a time-course analysis of serum TnI (Fig. 3e) was also performed, and a higher level of TnI was observed in both the CPR and Alda-1 groups at 1, 2, 4, and 24 hours after resuscitation. Overall, a reduction in TnI was observed after treatment with Alda-1, but significant differences were only observed between the two groups at 4 hours after resuscitation.

### Neurological function, and biomarker of cerebral injury

NDS (Fig. 4a) was used to assess neurological deficits at 24 hours postresuscitation. Compared with those of the sham group, the swine in the CPR and Alda-1 groups exhibited significantly worse neurological performance, and the NDS of swine was significantly increased in the two groups. Alda-1 treatment notably improved neurological function, and the scores of the scale decreased significantly compared with those in the CPR group. In addition, serum NSE was increased in the CPR and Alda-1 groups postresuscitation, especially at 24 hours ( $49.38 \pm 8.87$  ng/ml in the CPR and  $35.41 \pm 9.06$  ng/ml Alda-1 groups, respectively, Fig. 4b). There was a tendency toward a lower level of NSE in the Alda-1 group than in the CPR group at 1 hour postresuscitation, and statistical significance was observed between the two groups at 2 and 4 hours after resuscitation.

## **NLRP3 inflammasome and GSDMD in the myocardium, hippocampus and cortex**

To confirm the role of NLRP3 inflammasome-mediated pyroptosis in cardiac and neurological dysfunction, the levels of the proteins in the NLRP3 inflammasome (NLRP3, ASC, and Cleaved-Caspase-1) and the downstream protein GSDMD in the myocardium, hippocampus and cortex were first measured at 24 hours postresuscitation. As expected, the protein levels were all upregulated in the myocardium (Fig. 5a-b), hippocampus (Fig. 5c-d) and cortex (Fig. 5e-f). Alda-1 treatment attenuated NLRP3 inflammasome activation in the myocardium. However, the expression of GSDMD showed no difference between the CPR and Alda-1 groups, although the expression of GSDMD showed a downward trend. In the hippocampus and cortex, Alda-1 notably inhibited NLRP3 inflammasome activation and GSDMD expression.

## **Biomarkers of proinflammatory cytokines in the myocardium, hippocampus and cortex**

To determine the association between activated NLRP3 inflammasomes and proinflammatory cytokine levels, the levels of IL-1 $\beta$  (Fig. 6a) and IL-18 (Fig. 6b) in the myocardium, hippocampus, and cortex were examined by ELISA. Similar to the change in the NLRP3 inflammasome, the levels of the two proinflammatory cytokines dramatically increased in the myocardium, hippocampus, and cortex. After the administration of Alda-1, the levels of IL-1 $\beta$  and IL-18 in the myocardium were significantly decreased (IL-1 $\beta$  decreased by 27.0%, and IL-18 decreased by 28.6%, respectively). A significant change in IL-1 $\beta$  was also observed in the hippocampus, and slight downward trends in IL-18 in the hippocampus and IL-1 $\beta$  and IL-18 in the cortex were observed, but the trends were not significant. Taken together, these data indicated that Alda-1 partly inhibited NLRP3 inflammasome activation and the release of proinflammatory cytokines in the myocardium, hippocampus and cortex at 24 hours postresuscitation.

## **4HNE activity and MDA levels in the myocardium, hippocampus and cortex**

4HNE activity and levels of MDA were also examined in supernatant of myocardium, hippocampus and cortex homogenates at 24 hours postresuscitation. The 4-HNE activity and MDA levels increased in all three tissues in the CPR and Alda-1 groups compared with the sham group. However, these effects on 4-HNE (Fig. 7a) and MDA (Fig. 7b) were both mitigated by Alda-1 treatment compared with the CPR group.

## **ALDH2 activity and expression of ALDH2 in the myocardium, hippocampus and cortex**

After resuscitation, ALDH2 activity was significantly decreased in the myocardium, hippocampus and cortex. Alda-1 treatment increased ALDH2 activity by 2.7-fold (Fig. 8a) in the myocardium, 3.0-fold (Fig. 8d) in the hippocampus, and 2.8-fold (Fig. 8g) in the cortex compared with CPR alone. Western blot consistently revealed significant decreases in the protein levels of ALDH2 in the myocardium (Fig. 8b-c), hippocampus (Fig. 8e-f) and cortex (Fig. 8h-i) postresuscitation. Furthermore, Alda-1 treatment apparently increased the expression of the protein.

# **Discussion**



In our present study, the results demonstrated that treatment with Alda-1, an ALDH2 activator, markedly alleviated not only the function or performance in the heart and brain but also oxidative stress injury and inflammatory responses at 24 hours postresuscitation. More importantly, Alda-1 treatment decreased the protein expression of NLRP3 inflammasome components (NLRP3, ASC, cleaved caspase 1) and GSDMD. In total, we inferred that ALDH2 activation by Alda-1 can mitigate cardiac and neurological injury postresuscitation in swine, possibly in part by inhibiting ROS-mediated NLRP3 inflammasome activation, eventually inhibiting pyroptosis.

Postresuscitation cardiac and neurological dysfunction are the main contributions to death from CA[3]. CA causes I/R insult to all organs, of which the heart and brain are the most vulnerable. Insufficient cardiac output and low perfusion pressure, characterized as hemodynamic instability and myocardial dysfunction in the early postresuscitation period, may worsen global I/R injuries. Increased support of the circulatory system during the early postresuscitation period can stabilize hemodynamics and improve cardiac function[23]. Alda-1, a selective ALDH2 activator, has demonstrated protective effects on hemodynamics and cardiac function in I/R myocardial injury[24]. In our study, we observed a significant increase in HR and a reduction in MAP, SV and GEF postresuscitation. However, treatment with Alda-1 stabilized hemodynamics and improved cardiac dysfunction. This result is consistent with the findings of previous studies showing that Alda-1 treatment effectively improved myocardial dysfunction in myocardial infarction rats[24] and in CA/CPR rats[18]. Improved recovery of stable hemodynamics and cerebral blood perfusion may contribute to improvement of neurological function[25], but the possibility that Alda-1 exerts direct neuroprotective effects cannot be excluded, given its roles in other neurological pathologies[13]. However, the effect of Alda-1 on postresuscitation neurological dysfunction has not been reported previously. In our study, we found that severe neurological disorders were detected in swine subjected to CA/CPR compared to sham-operated animals. However, it is worth noting that a single dose of Alda-1 improved neurological performance within 24 hours postresuscitation in addition to the abovementioned positive effects on myocardial hemodynamics in our swine model. Similarly, Li et al.[13] demonstrated a beneficial effect of Alda-1 on neurological impairment in local cerebral ischemia injury in rats. In addition, our time-course data showed that biomarkers of myocardial injury (Tnl) and cerebral injury (NSE) both decreased after Alda-1 treatment. These results revealed the protective effect of Alda-1 in myocardial and cerebral injury.

Myocardial and cerebral I/R injury are pathological processes leading to the death of cardiomyocytes and neurocytes. Accumulating evidence has emphasized the pivotal role of the NLRP3 inflammasome in mediating pyroptosis and promoting excessive release of proinflammatory cytokines after myocardial and cerebral I/R injury[26, 27]. Pyroptosis is a new form of PCD, and specific stimuli activate the NLRP3 inflammasome. Active caspase-1, an effector enzyme of the NLRP3 inflammasome, not only cleaves the cytokines pro-IL-1 $\beta$  and pro-IL-18 into their mature active forms (IL-1 $\beta$  and IL-18) but also cleaves GSDMD, which creates pores in the plasma membrane, thereby causing release of IL-1 $\beta$  and IL-18 from the cells[9]. Previous studies have found that global I/R injury can induce overactivation of the NLRP3 inflammasome, GSDMD and proinflammatory cytokines in the heart[8] and brain[28, 29]. In this study, we established a systemic I/R model and assessed the NLRP3 inflammasome, GSDMD and downstream

inflammatory factors in the heart and brain. We found that the expression levels of NLRP3, ASC, Cleaved Caspase-1, and GSDMD in myocardial and cerebral tissues were significantly increased, which was accompanied by elevated levels of IL-1 $\beta$  and IL-18 postresuscitation. These data are consistent with our previous neurological findings in vivo[10] and in vitro[11]. These findings suggest that the NLRP3 inflammasome complex is formed and activated in pyroptosis during global myocardial and cerebral I/R injury. Moreover, after treatment with Alda-1, the expression of these four pyroptosis-related proteins and the levels of two proinflammatory cytokines were markedly decreased. These results indicate that Alda-1 can inhibit NLRP3 inflammasome-induced pyroptosis.

Currently, a wide range of stimuli can activate the NLRP3 inflammasome and then promote pyroptosis. Oxidative stress injury and the subsequent generation of ROS may be common stimuli of NLRP3 inflammasome activation[30, 31]. The [integrated](#) process of I/R includes no perfusion in the CA period, low perfusion in the CPR period, and reperfusion after ROSC. Oxidative stress injury already exists in no perfusion and low perfusion and is then exacerbated after reperfusion[32], which causes persistent myocardial and cerebral injury in both the ischemia and reperfusion periods. A previous study suggested that mitigating ROS production by the administration of antioxidants following ROSC was able to reduce myocardial and cerebral injury, resulting in improved postresuscitation cardiac and neurological function[8]. There is growing evidence that Alda-1 exerts cardioprotective and neuroprotective effects through the inhibition of oxidative stress[12, 13], which prompted us to determine its antioxidant capacity in CA/CPR. In our study, we used MDA and 4-HNE as indicators of oxidative stress injury, and increases in the MDA levels and 4-HNE activity were observed postresuscitation. Treatment with Alda-1 resulted in decreases in both indicators, which were accompanied by a significant inhibition of pyroptosis-related proteins and inflammatory cytokine expression. All the results above confirmed that there was a relationship between ROS and the NLRP3 inflammasome, and the protective roles of Alda-1 in cardiac and cerebral function may be partly mediated by inhibiting the ROS-mediated NLRP3 inflammasome and pyroptosis.

Both the activity and expression of ALDH2, a receptor of Alda-1, were detected. ALDH2 is the crucial enzyme for metabolizing aldehydes. It converts acetaldehyde to acetic acid and thereby lowers toxicity. Cardioprotective effects were the first revelation of ALDH2 in I/R injury[33]. Since then, many studies have consistently demonstrated the protective effects of Alda-1 in cardiac injury and other I/R injury models, including the brain[18]. However, the effect of Alda-1 on myocardial and cerebral injury caused by systemic I/R remains unknown. Our present study found that both ALDH2 activity and protein expression increased postresuscitation, and Alda-1 treatment further enhanced this increasing trend.

The protective roles of Alda-1 in the heart and brain are illustrated in our present study, and several aspects should be considered. Due to the unexpected occurrence of cardiac events, the administration of Alda-1 before CA is impossible under routine circumstances. However, our results provide a new viewpoint that ALDH2-targeted treatment in CA could be initiated at an earlier time window, such as during basic life support. In addition, although our evidence is from preclinical studies, it is worth translating Alda-1 into clinical practice.

## Limitations

There could be several limitations to our present study. First, although three or four timepoints were observed regarding hemodynamics, organ function, and organ damage markers, we investigated only one timepoint for survival, neurological recovery, oxidative stress injury, pyroptosis-related protein levels, and downstream cytokines postresuscitation. Second, we did not conduct pilot experiments to choose the optimal dose and therapeutic time period of Alda-1. Administration of a higher dose, or administration at an even earlier time, such as the administration of epinephrine in the CPR period, may have a better therapeutic effect. Furthermore, we evaluated the benefits of Alda-1 in only the short term and only in the heart and brain, and these possible beneficial effects of Alda-1 in the long term and in other organs should be determined in future studies.

## Conclusion

In conclusion, we demonstrate that Alda-1 activates ALDH2, alleviates myocardial and cerebral injury, and improves cardiac and neurological dysfunction postresuscitation in a swine model of cardiac arrest. More importantly, the protective role of Alda-1 may be partly mediated through the suppression of ROS production, eventually inhibiting NLRP3 inflammasome-induced pyroptosis.

## Declarations

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### Availability of Data and Material

The data that support the findings of this study are included in electronic supplementary material.

**Code available** Not applicable.

### Authors' contributions

MD, JX, MZ, and WH conceived, designed and supervised the study. MD, JX, JW, MZ, FL, LS, ZK, CW, and YZ performed the experiments. MD, JX, and MZ analyzed the data. MD wrote the manuscript. All the authors read and approved the final manuscript.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

## **Ethics Approval**

All the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Second Affiliated Hospital, Zhejiang University School of Medicine.

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

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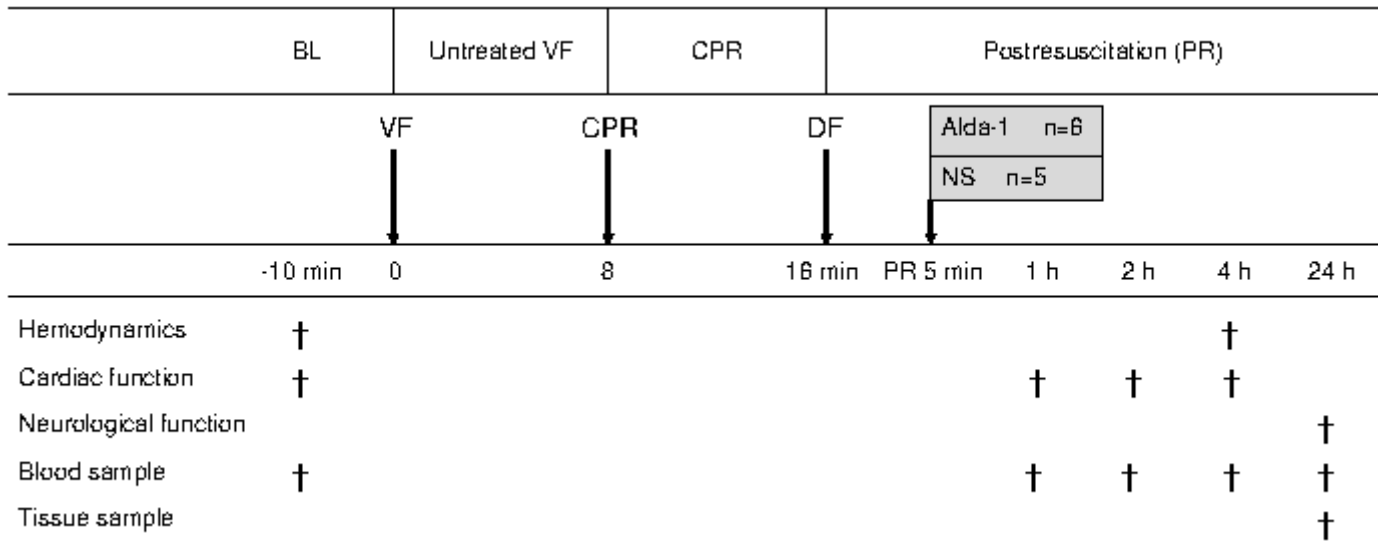
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## Figures



**Figure 1**

The flowchart of the study BL, baseline; VF, ventricular fibrillation; CPR, cardiopulmonary resuscitation; DF, defibrillation; and NS, normal saline.

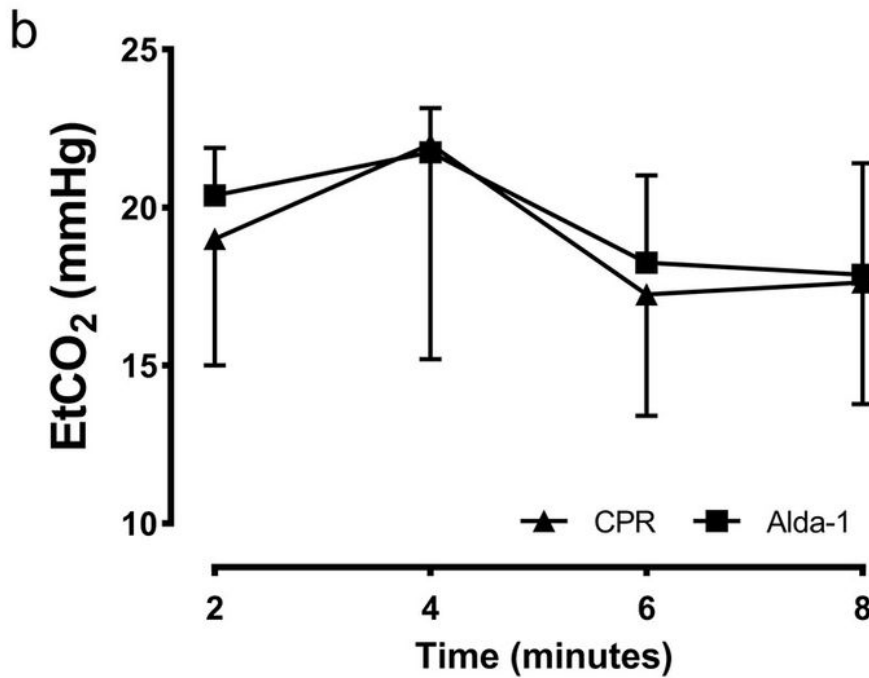
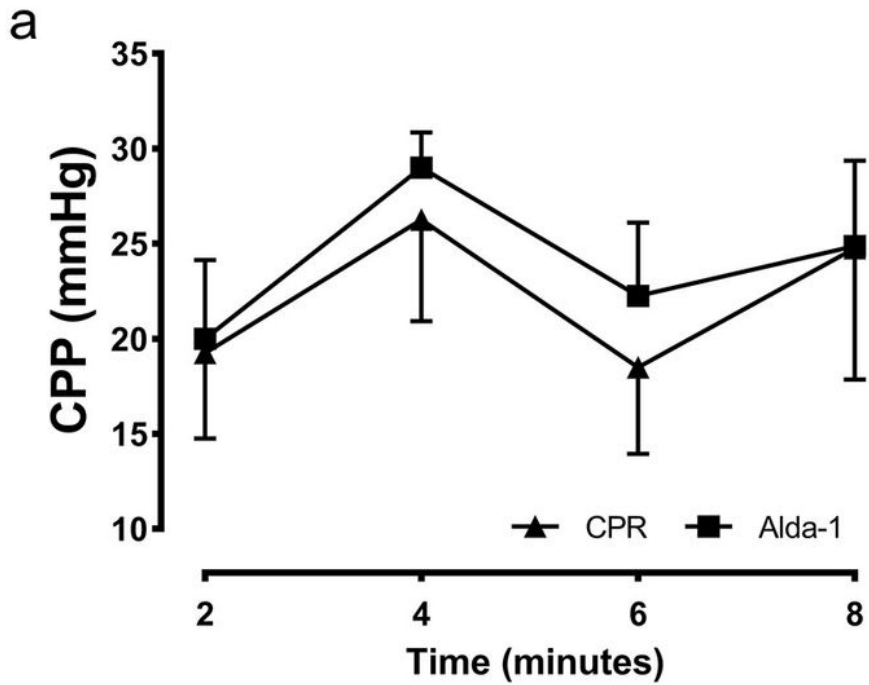
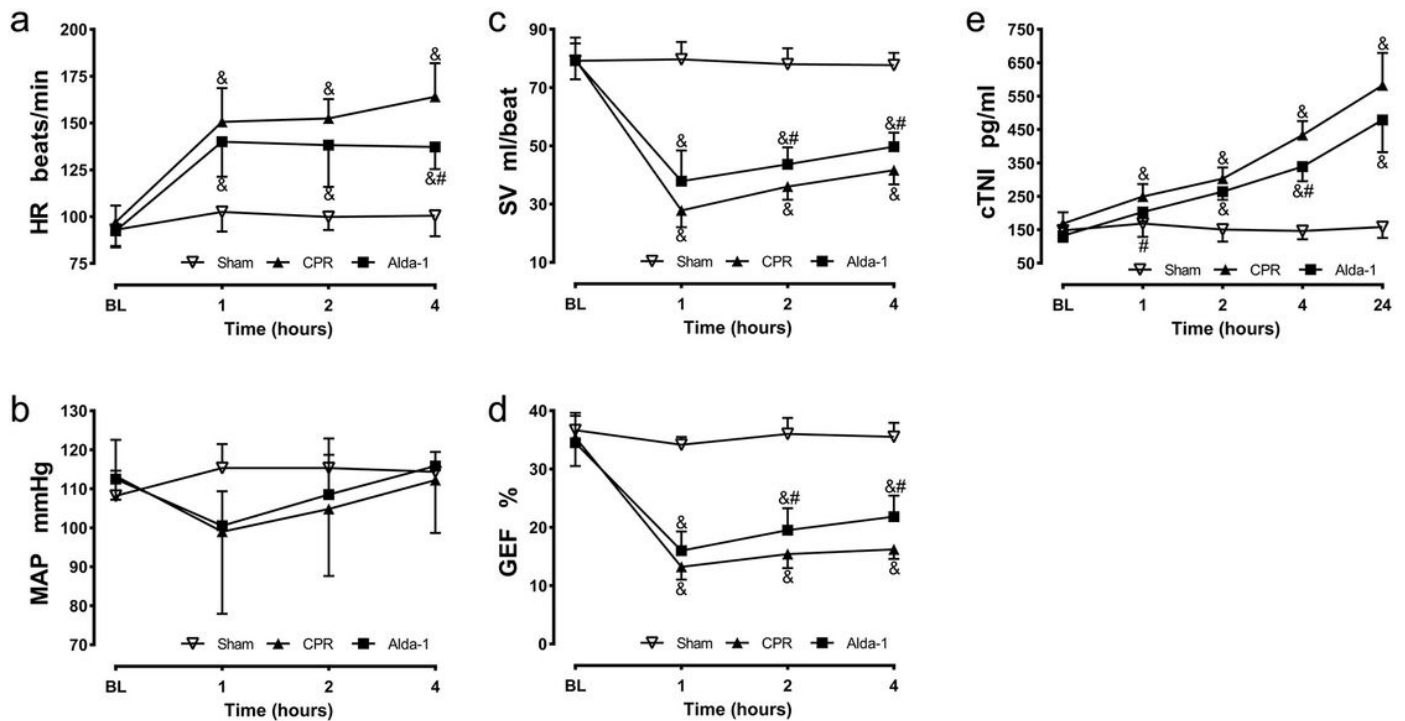


Figure 2

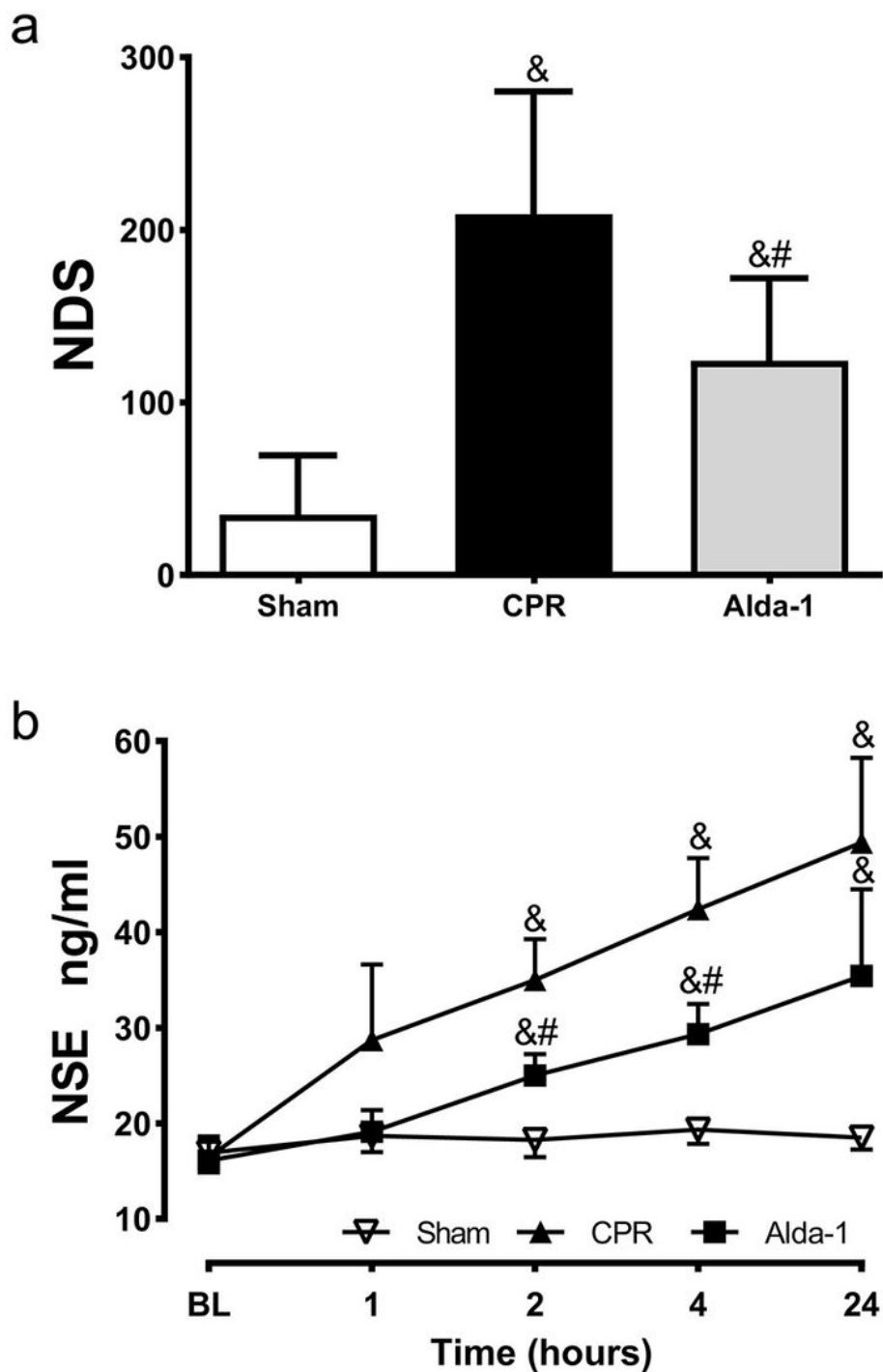
Resuscitation data in the CPR- and Alda-1-treated animals during the resuscitation period. a CPP at 2, 4, 6, and 8 minutes during the resuscitation period. b EtCO<sub>2</sub> at 2, 4, 6, and 8 minutes during the resuscitation period. CPP, Coronary perfusion pressure; and EtCO<sub>2</sub>, End-tidal carbon dioxide. The data are presented as the mean ± SD.





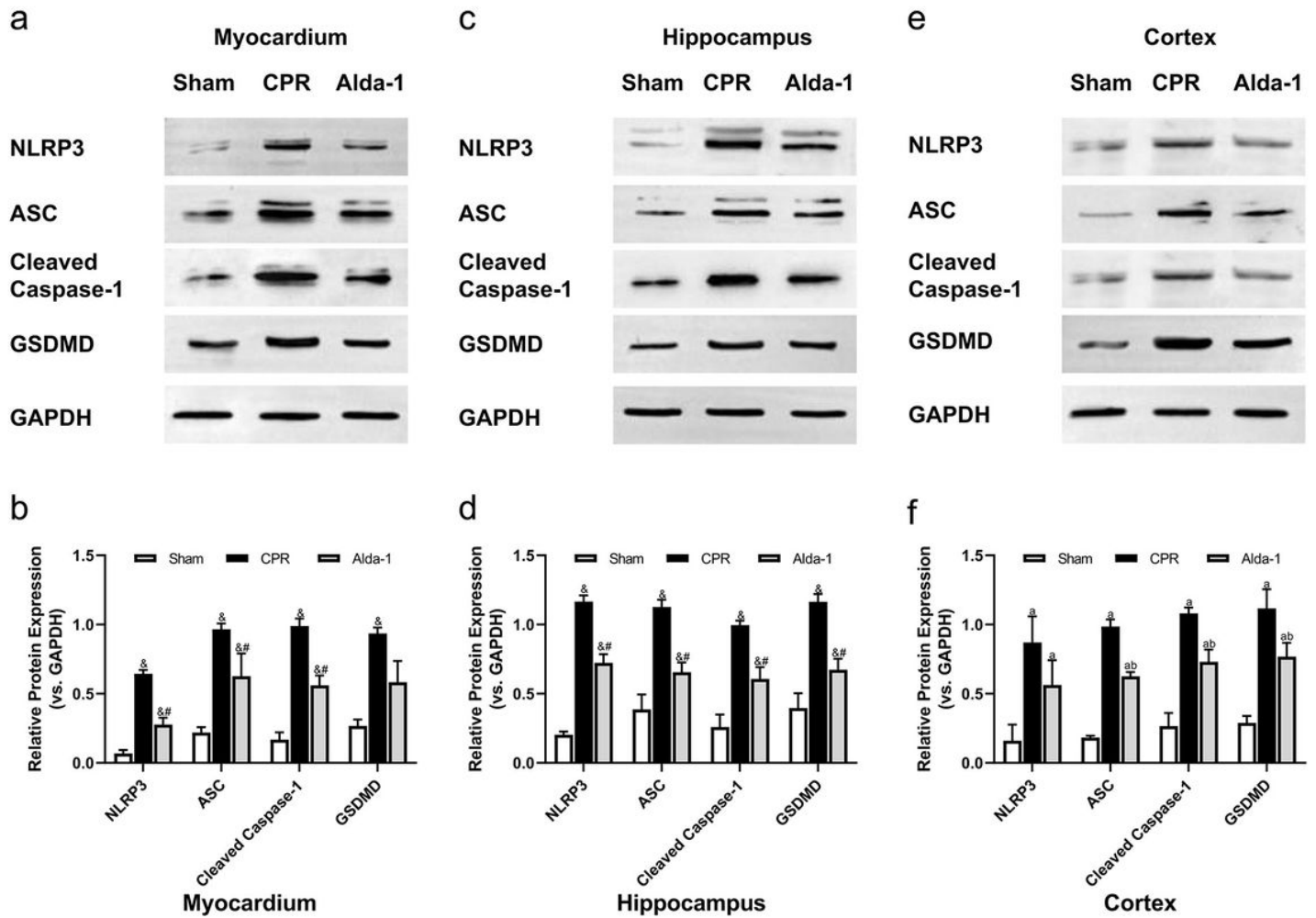
**Figure 3**

Hemodynamic, cardiac function, and biomarker of myocardial injury postresuscitation. a HR; b MAP; b SV, d GEF, and e cTNI in the sham-, CPR- and Alda-1-treated animals at baseline and during the 4-hour or 24-hour postresuscitation period. HR, heart rate; MAP, mean arterial pressure; SV, stroke volume; GEF, global ejection fraction; and cTNI, cardiac troponin I. The data are presented as the mean  $\pm$  SD, &  $P < 0.05$  vs the sham group, #  $P < 0.05$  vs the CPR group.



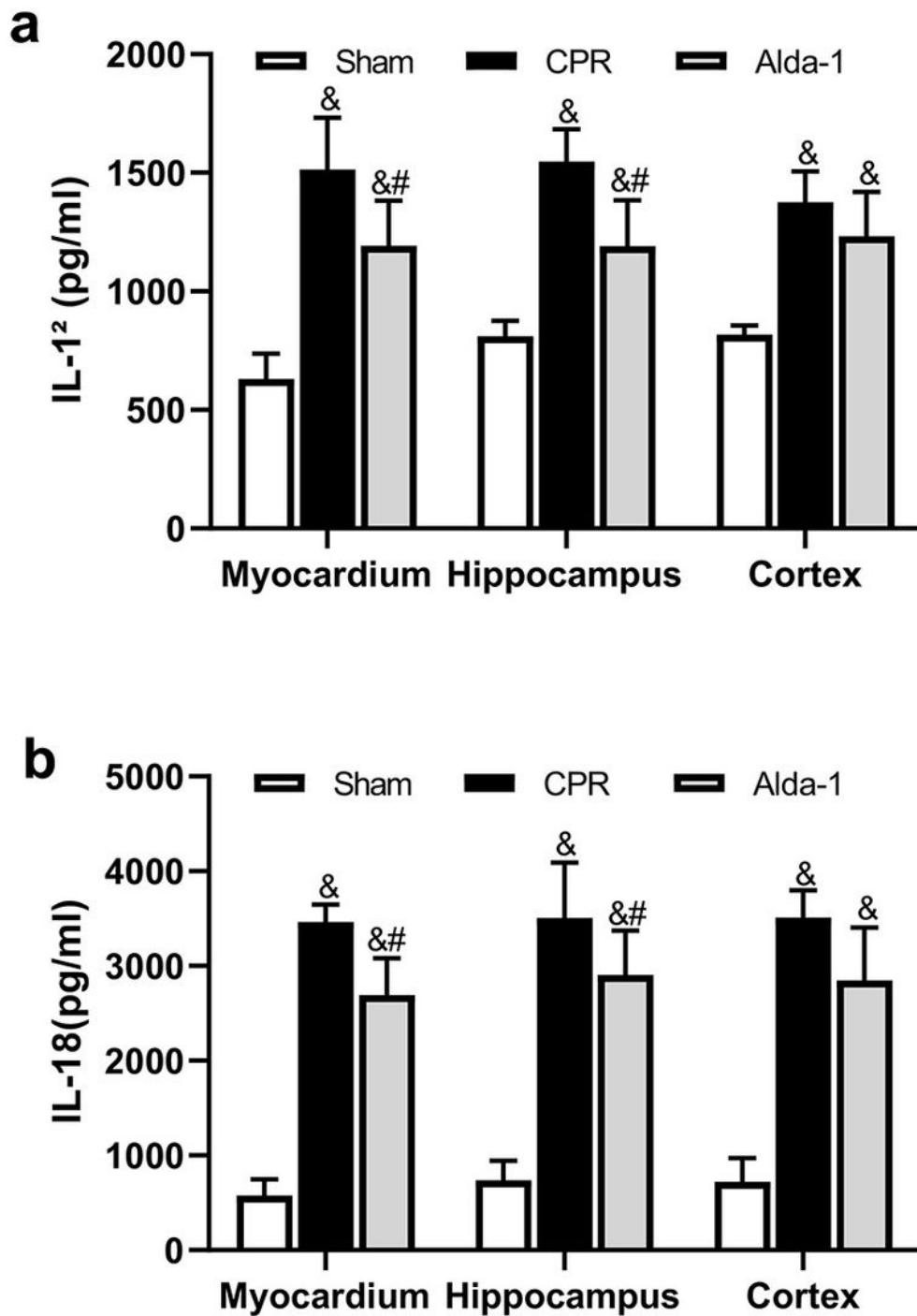
**Figure 4**

Neurological function and biomarker of cerebral injury postresuscitation. a NDS and b NSE in the sham-, CPR- and Alda-1-treated animals at baseline and during the 4-hour or 24-hour postresuscitation period. NDS, neurologic deficit scores; and NSE, neuron-specific enolase. The data are presented as the mean  $\pm$  SD, &  $P < 0.05$  vs the sham group, #  $P < 0.05$  vs the CPR group.



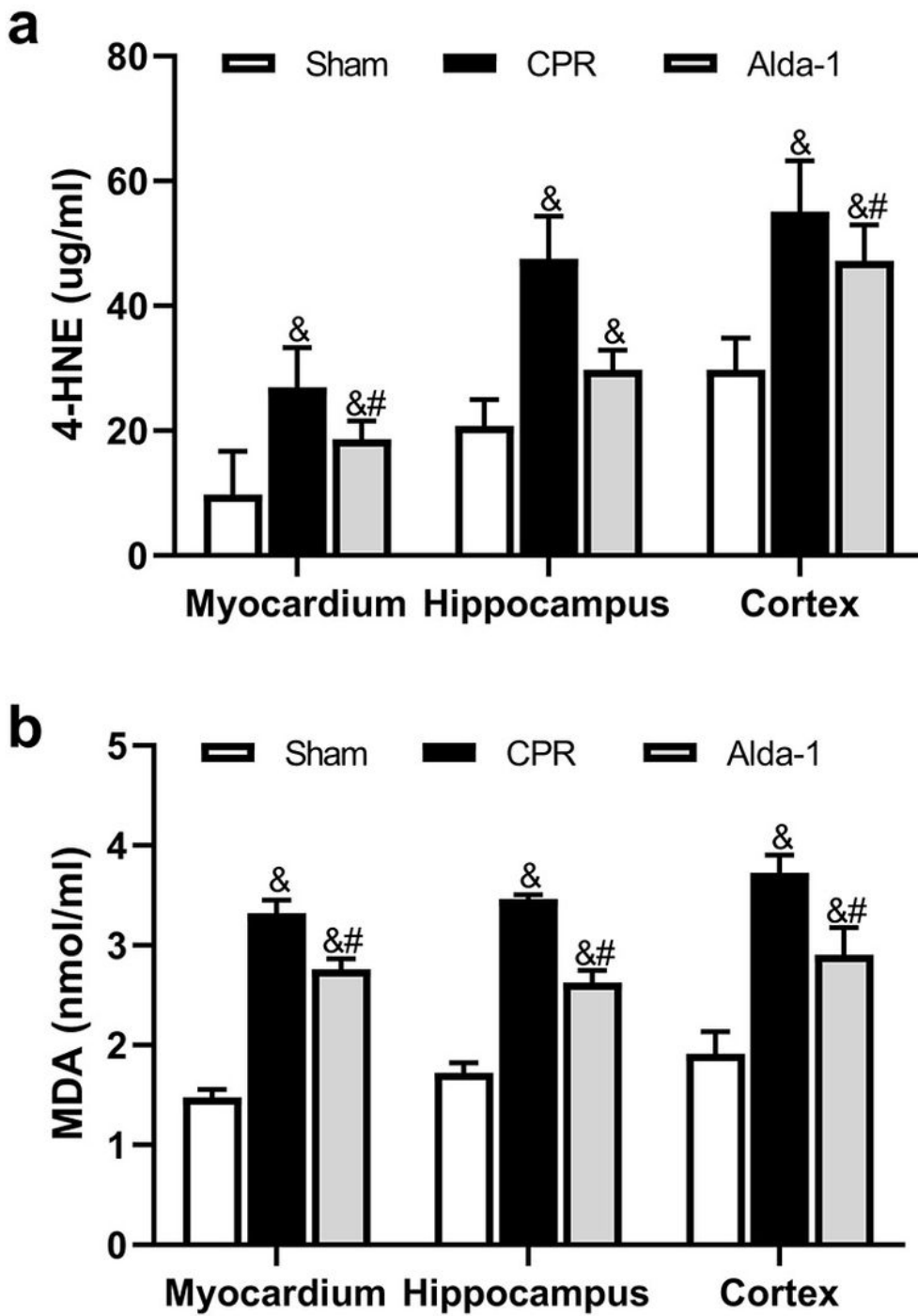
**Figure 5**

Expression of pyroptosis-related proteins in the myocardium, hippocampus and cortex at 24 hours postresuscitation in different groups. a Representative western blotting images of NLRP3, ASC, Cleaved Caspase-1, and GSDMD proteins in the myocardium. b Quantitative protein analysis of NLRP3, ASC, and Cleaved Caspase-1, and GSDMD expression in the myocardium. c Representative western blotting images of NLRP3, ASC, Cleaved Caspase-1, and GSDMD proteins in the hippocampus. d Quantitative protein analysis of NLRP3, ASC, and Cleaved Caspase-1, and GSDMD expression in the hippocampus. e Representative western blotting images of NLRP3, ASC, Cleaved Caspase-1, and GSDMD proteins in the cortex. f Quantitative protein analysis of NLRP3, ASC, and Cleaved Caspase-1, and GSDMD expression in the cortex. The data are presented as the mean  $\pm$  SD, &#x2606;  $P < 0.05$  vs. Sham group, &#x2603;  $P < 0.05$  vs. CPR group.



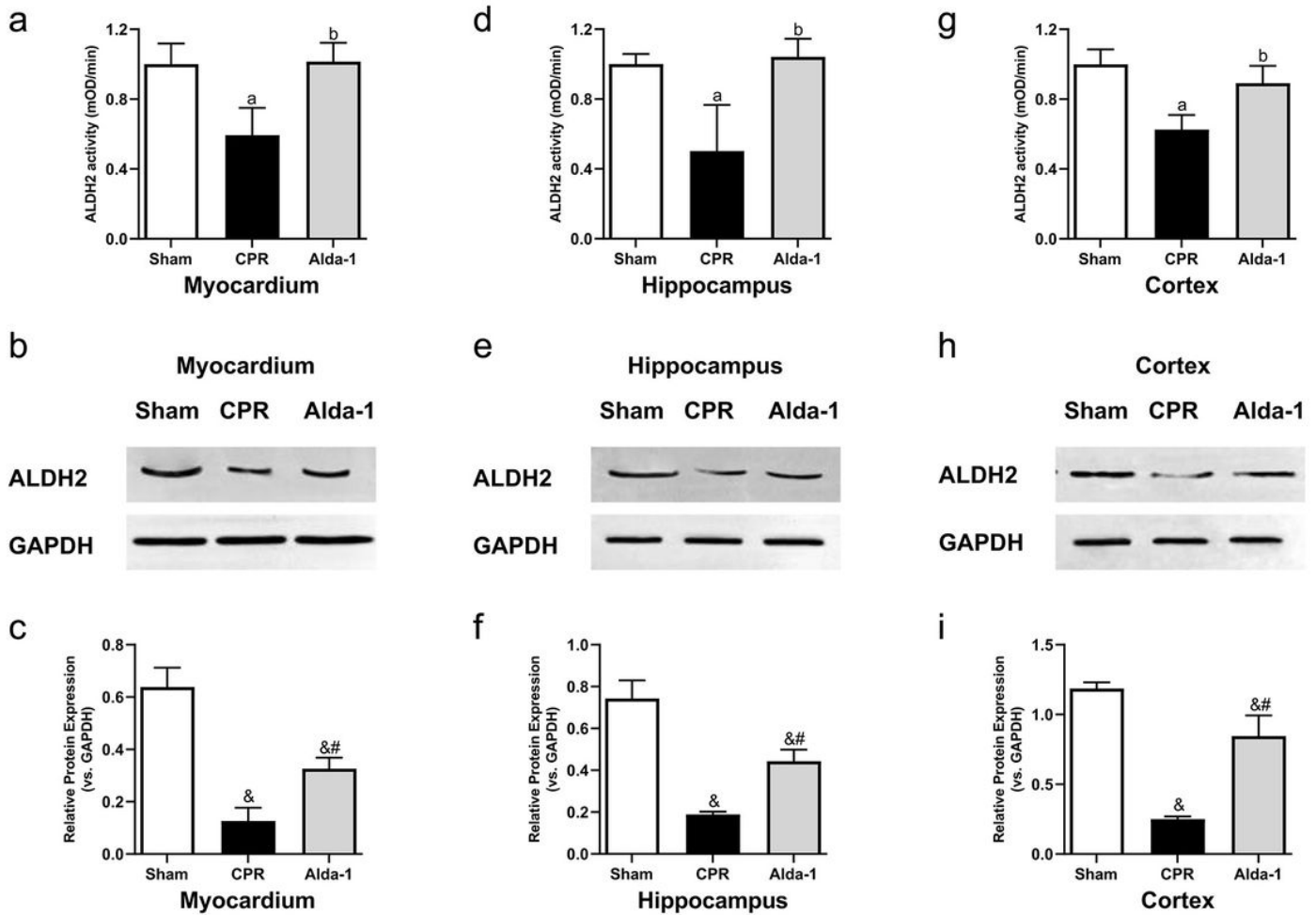
**Figure 6**

Levels of proinflammatory cytokines in the myocardium, hippocampus and cortex at 24 hours postresuscitation in different groups. a Interleukin-1 $\beta$  (IL-1 $\beta$ ), and b interleukin-18 (IL-18). The data are presented as the mean  $\pm$  SD, &  $P < 0.05$  vs. Sham group, #  $P < 0.05$  vs. CPR group.



**Figure 7**

Levels of 4-HNE and MDA in the myocardium, hippocampus and cortex at 24 hours postresuscitation in the different groups. a 4-HNE. b MDA. The data are presented as the mean  $\pm$  SD, &  $P < 0.05$  vs. Sham group, #  $P < 0.05$  vs. CPR group.



**Figure 8**

Protein levels of ALDH2 and ALDH2 activity in the myocardium, hippocampus and cortex at 24 hours postresuscitation in different groups. a ALDH2 activity in the myocardium. b Representative western blotting images of the ALDH2 protein in the myocardium. c Quantitative protein analysis of ALDH2 expression in the myocardium. d ALDH2 activity in the hippocampus. e Representative western blotting images of the ALDH2 protein in the hippocampus. f Quantitative protein analysis of ALDH2 expression in the hippocampus. g ALDH2 activity in the cortex. h Representative western blotting images of the ALDH2 protein in the cortex. i Quantitative protein analysis of ALDH2 expression in the cortex. The data are presented as the mean  $\pm$  SD, &  $P < 0.05$  vs. Sham group, #  $P < 0.05$  vs. CPR group.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Data.rar](#)