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ORIGINAL PAPER

AdipoRon Protects Against Secondary Brain Injury After Intracerebral Hemorrhage via Alleviating Mitochondrial Dysfunction: Possible Involvement of AdipoR1–AMPK–PGC1α Pathway

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Abstract

Intracerebral hemorrhage (ICH) is a stroke subtype that is associated with high mortality and disability rate. Mitochondria plays a crucial role in neuronal survival after ICH. This study first showed that activation of adiponectin receptor 1 (AdipoR1) by AdipoRon could attenuate mitochondrial dysfunction after ICH. In vivo, experimental ICH model was established by autologous blood injection in mice. AdipoRon was injected intraperitoneally (50 mg/kg). Immunofluorescence staining were performed to explicit the location of AdipoR1, AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-γ coactivator-1a (PGC1α). The PI staining was used to quantify neuronal survival. The expression of AdipoR1 and its downstream signaling molecules were detected by Western blotting. In vitro, 10 μM oxyhemoglobin (OxyHb) was used to induce the neuronal injury in SH-SY5Y cells. Annexin V-FITC/PI staining was used to detect the neuronal apoptosis and necrosis. Mitochondrial membrane potential (Δψm) was measured by a JC-1 kit and mitochondrial mass was quantified by mitochondrial fluorescent probe. In vivo, PI staining showed that the administration of AdipoRon could reduce neuronal death at 72 h after ICH in mice. AdipoRon treatment enhanced ATP levels and reduced ROS levels in perihematoma tissues, and increased the protein expression of AdipoR1, P-AMPK, PGC1α, NRF1 and TFAM. In vitro, the JC-1 staining and Mito-tracker™ Green showed that AdipoRon significantly alleviated OxyHb-induced collapse of Δψm and enhanced mitochondrial mass. Moreover, flow cytometry analysis indicated that the neurons treated with AdipoRon showed low necrotic and apoptotic rate. AdipoRon alleviates mitochondrial dysfunction after intracerebral hemorrhage via the AdipoR1–AMPK–PGC1α pathway.

Keywords Mitochondrial dysfunction · ICH · AdipoRon · ROS

Abbreviations

ICH	Intracerebral hemorrhage
SBI	Secondary brain injury
ROS	Reactive oxygen species
AMPK	AMP-activated protein kinase
OxyHB	Oxyhemoglobin
FBS	Fetal bovine serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
MRI	Magnetic resonance imaging
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator-1a
Δψm	Mitochondrial membrane potential
NRF1	Nuclear respiratory factor 1
TFAM	Mitochondrial transcription factor A

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Introduction

Intracerebral hemorrhage (ICH) is a stroke subtype that is associated with high mortality and disability rate. It accounts for 2 million cases worldwide yearly and patients who survive typically have major neurological deficits [1]. At present, the mainstream view divides ICH injury into the primary damage and the secondary brain injury (SBI). The former is mainly caused by mechanical disruption after initial bleed, and the latter is caused by a series of mechanisms including: oxidative stress, inflammation, mitochondrial dysfunction, neuronal death (including apoptosis and necrosis) etc. [2, 3].

Neuronal damage after SBI has always been our focus. Mitochondria plays a crucial role in energy supply as well as generation of reactive oxygen species (ROS) which are key contributors to neuronal cell death [4]. According to the severity of mitochondrial dysfunction, the results of neuronal injury range from temporary metabolic suppression to cellular necrosis [3]. The hematoma after ICH results in glutamate releasing and further inducing mitochondrial dysfunction [5–7]. Then the deficiency of adenosine triphosphate (ATP) generation impairs cellular pumps causing cytotoxic edema and cell death. Notably, insufficient energy supply (induced by mitochondrial dysfunction) may be particularly injuring neurons which have a high energy demand compared to others cells [4]. Hence, mitochondrial dysfunction can be the potential therapeutic target for ICH-induced neuronal injury.

AdipoRon is the latest small-molecule agonist of adiponectin receptors (AdipoRs), which ameliorates insulin resistance and glucose intolerance through an AdipoR1 dependent manner [8]. Recent studies indicate that AdipoR1 are widely expressed in neurons and AdipoR1 activation exerts positive effects on neuron and neural stem cells after various nervous system injury [9–11]. AdipoR1 activation enhance the phosphorylation of AMP-activated protein kinase (AMPK) and consequently exert anti-apoptosis, anti-inflammatory effects after ischemic stroke [10, 12]. AMPK is initially known as a conserved serine/threonine kinase, which plays an important role in cellular energy homeostasis and metabolic pathways [13]. In brain, downregulated levels of cellular ATP can activate and phosphorylate AMPK, and consequently activate its downstream proteins [14]. As the crucial downstream signal molecule of AMPK, peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1a (PGC1α) is a transcriptional coactivator which played a key role in regulating mitochondrial biogenesis. PGC1α activation could improve mitochondrial DNA (mDNA) and ATP production via activating NRF1/TFAM axis (Nuclear Respiratory Factor 1, NRF1; mitochondrial transcription factor A, TFAM) in Alzheimer's disease and ICH [15, 16]. In addition, PGC1α activation can also attenuate the collapse of mitochondrial membrane potential (Δψm) and decrease mitochondrial ROS production via a sirt3 dependent manner [5, 17, 18]. The above-mentioned content reminds us that AdipoR1–AMPK–PGC1α axis may participate in the progress of mitochondrial damage after ICH.

A previous study demonstrated that AdipoRon increased the expression of phosphorylated AMPK (P-AMPK) and its downstream proteins. Then AdipoRs activation restored AMPK-mediated autophagic flux and attenuated myocardial ischemia/reperfusion injury in diabetic mice [19]. Hence, it arouses our interest that whether AdipoRon can ameliorate neuronal damage after ICH. Here, we hypothesize that AdipoRon activate AdipoR1–AMPK–PGC1α pathway and subsequently protect against mitochondrial dysfunction after experimental ICH.

Methods

Animals

All experimental protocols were warranted by the Institutional Animal Care and Use Committee of Zhejiang University. These protocols were in compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Adult male C57BL/6 mice (20–25 g) used for this study were purchased from Slac Laboratory Co., Ltd. (Shanghai, China). All mice were raised in triples in plastic cages with a 12-h day/night cycle (22 ± 1 °C; 60 ± 5% humidity).

Experimental Protocol (Fig. 6a)

Experiment 1

In order to assess the effectiveness of AdipoRon in improving neurological function. 18 mice were randomly (using random number table) divided into three groups: Sham group n = 6, ICH + vehicle n = 6, ICH + AdipoRon n = 6. Then these animals were be scheduled to receive behavioral testing at 24 h, 72 h, 5 days after ICH.

Experiment 2

In order to investigate the positive effects of AdipoRon at the cellular level, 54 mice were divided into three groups: Sham group n = 18, ICH 72 h + vehicle 72 h n = 18, ICH 72 h + AdipoRon n = 18. Then these animals were sacrificed for PI staining, ATP assay and ROS assay (n = 6 in each group).

Experiment 3

At the molecular level, in order to explore the potential signal pathway which participated in the biological function of AdipoRon. 21 mice were divided into 3 groups: Sham group n = 7, ICH 72 h + vehicle 72 h n = 7, ICH 72 h + AdipoRon n = 7. Then these animals were sacrificed for PI staining, ATP assay and ROS assay. Perihematoma tissues brains from six mice in each group were sampled for Western blot analysis. The rest three mice were sacrificed for immunofluorescence staining.

Experiment 4

The in vitro experiments were performed to visually observe the changes of mitochondria and accurately quantify neuronal damage. Neurons (SH-SY5Y cells) with different treatments were divided into three groups: control, oxyHb 48 h + vehicle, oxyHb 48 h + AdipoRon. Afterwards, neurons were applied to mitochondrial membrane potential ($\Delta\psi m$) test, mitochondrial biogenesis assay, MTT test and flow cytometry.

Experimental ICH Models

In Vivo

As previously described [5, 20], C57BL/6 mice were anesthetized with intraperitoneal pentobarbital (40 mg/kg) and fixed in a stereotaxic frame (Stoelting Stereotaxic instrument). A 1-mm-diameter burr hole was made in the skull (0.2 mm anterior to bregma, 2.5 mm right lateral to midline), then 30 µl of fresh autologous blood was injected into the right basal ganglia (3.5 mm depth below the skull) with a microperfusion pump within 5 min. The fresh arterial blood was collected from femoral artery catheterization with PE10 tube. In case of blood leakage, the needle was gradually removed at 10 min after complete injection, the burr hole was blocked with bone wax. In sham group, C57BL/6 mice received the same procedures but 30 µl saline was injected instead of the blood.

In Vitro

As previously described [5, 21], neurons (SH-SY5Y cells) were exposed to 10 µM oxyhemoglobin (OxyHb) in fresh medium. After incubation for 48 h, cells were used for different experiments.

Drug Administration

In vivo

AdipoRon was purchased from MedChemExpress (no. HY-15848) and injected intraperitoneally at a dose of 50 mg/kg immediately at 20 min after ICH (Administration of AdipoRon lasted for 72 h, once a day). The AdipoRon was dissolved in the mixture (DMSO: PBS = 1:1), then Tween80 (10% volume of the mixture) was added into the mixture. Finally the solution was diluted to a suitable concentration. In ICH + vehicle group, mice received intraperitoneal injections of the same volumes of DMSO, PBS and Tween80 mixture.

In Vitro

SH-SY5Y cells were incubated with 50 µM AdipoRon for 48 h. In oxyHb + vehicle group, cells received the same volumes of DMSO, PBS and Tween80 mixture.

Cell Culture

The human neuroblastoma SH-SY5Y cells cultured (37 °C, 5% CO_2) in DMEM/F12 (1:1) medium with15% fetal bovine serum (FBS) and 100 U/ml penicillin. All of the cell lines were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Flow Cytometry

SH-SY5Y cells were trypsinized by 0.25% trypsin (without EDTA) and centrifuged at 1000 rpm for 5 min, and then cells were resuspended in 500 µl buffer solution. Annexin V-FITC/PI staining Kit (BD Bioscience, Franklin Lakes, NJ) was used to detect the neuronal apoptosis and necrosis. SH-SY5Y cells incubated with 5 µl Annexin V and 5 µl PI for 20 min at 37 °C in the dark and then cell death was analyzed by a FACS flow cytometer C6 (BD Biosciences).

Cells in Q1 quadrant (Annexin V −/PI +) were necrotic cells. The Q2 quadrant (Annexin V +/PI +) represented late apoptotic cells; Q3 quadrant (Annexin V +/PI −) were early apoptotic cells and Q4 quadrant (Annexin V −/PI −) represented normal cells.

Immunofluorescence Staining

Mice were anesthetized with 4% pentobarbital at 72 h after ICH, and transcardial perfusion with 0.1 M PBS and 4% paraformaldehyde (PFA, pH 7.4) was subsequently performed. Then the cerebral hemispheres were removed and put into 4% PFA for post-fixation (4 °C, 24 h). After sucrose gradient dehydration (4 °C, 3 days), the cerebral hemispheres were coronally sliced into 10-mm sections. Then, Coronal cryosections were preprocessed with 10% donkey serum and 0.3% Triton X-100. Then the brain cryosections were incubated at 4 °C with AdipoR1 antibody (1:400, Abcam, ab126611), p-AMPK antibody (1:250, Abcam, ab23875), PGC1α antibody (1:250, Abcam, ab191838), NeuN antibody

(1:500, Abcam, ab177487). After the incubation overnight, cryosections were incubated with secondary antibody. Then, the fluorescence microscope (Olympus, Tokyo, Japan) was used to capture the images.

For PI staining, mice were injected with Propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally (10 mg/kg) at 1 h prior to sacrifice.

Western Blotting

Western blot was performed as previously described [5]. Briefly, mice were anesthetized with 4% pentobarbital at 72 h after ICH, and brain tissues were removed after transcardial perfusion with 0.1 M PBS. These perihematoma tissues (basal ganglia) were homogenized in RIPA lysis buffer (Beyotime). Then the suspension was centrifuged at 12,000×*g* for 15 min 4 °C. Ultimately, the supernatant was collected and used to prepare the protein sample. 40 μg protein from each sample was used for electrophoresis (80 V, 30 min; 120 V, 50 min). These protein samples were separated by 10% or 12% SDS-PAGE and transferred to the polyvinylidene fluoride membranes at 250 mA for 1 h. After that, the PVDF membranes were blocked with 5% milk for 1 h and incubated with the primary antibodies overnight, including: anti-AdipoR1 antibody (1:1000, Abcam, ab126611), anti-p-AMPK antibody (1:1000, Abcam, ab23875), anti-AMPK antibody (1:1000, Abcam, ab32047), anti-PGC1α antibody (1:500, Abcam, ab191838), anti-NRF1 antibody (1:2000, Abcam, ab175932), anti-TFAM (1:1000, Abcam, ab131607), β-actin (1:5000, Abcam, ab8226). Then, the PVDF membranes were disposed with relevant secondary antibodies (1:5000, Zhongshan Gold Bridge ZB-2301 or ZB-2305) for 1 h at normal temperature. The signals of protein bands were detected with Chemidoc detection system and quantified using Quantity One software (Bio-Rad).

ATP and ROS Assay

ATP levels were measured by using the luciferase-based ATP assay kit (Beyotime, Shanghai, China). As described in the instruction, the brain tissues were lysed in lysis buffer. After centrifugation (4 °C 12,000×*g*, 5 min), the supernatant was extracted for the ATP assay. The samples (20 μl) were incubated with ATP working reagents (100 μl; ATP detection reagent: ATP detection reagent diluent = 1: 9) in micro-well for 5 min at 37 °C, and then (2 s later at least) measured by Varioskan Flash (ThermoFisher Scientific).

Levels of ROS in brain tissues were examined using a ROS assay kit (JianCheng, China) according to the manufacturer's instructions. In brief, samples were lysed in 0.01 mol/l PBS. After centrifugation (4 °C 500×*g*, 10 min), the supernatant (190 μl) and DCFH-DA (10 μl, 1 mol/l) were mixed in a micro-well at room temperature for 30 min. Afterwards, the mixtures were measured by fluorophotometry.

Measurement of Mitochondrial Membrane Potential (Δψm) and Mitochondrial Mass

The Δψm was measured by a JC-1 kit (Beyotime, Shanghai, China) following the manufacturer's instructions. SH-SY5Y cells were planted in 96-well plates at a density range of 1×10^4/well–3×10^4/well. Cells were rinsed with PBS and incubated with JC-1 staining solution at 37 °C for 20 min in dark. The inverted fluorescence microscope (Olympus, Tokyo, Japan) was used to capture the pictures. Then these cells were harvested for flow cytometry analysis.

Mito-tracker™ Green (Beyotime, Shanghai, China) was used to quantify mitochondrial mass as described previously [22]. Briefly, cells were incubated in serum-free medium with 150 nM Mitotracker Green for 20 min in the dark. The inverted fluorescence microscope (Olympus, Tokyo, Japan) was used to capture the pictures. Then these cells were harvested for flow cytometry analysis.

Cell Viability: MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Beyotime, Shanghai, China) was used to measure cell viability. In brief, the cells were cultured in 96-well plates (4×10^4/well). These cells were treated with 10 μl of MTT solution (5 mg MTT in 1 ml 0.01 M PBS, pH 7.40; 10 μl in 100 μl culture medium/well) and incubated for 2 h at 37 °C. After incubation and discarding the medium, 100 μl of dimethyl sulfoxide (DMSO) was added in each well to dissolve insoluble formazan. The cell viability was determined by measuring the absorbance at 570 nm using a microplate reader.

Behavioral Testing

Accelerated Rotarod Test

Accelerated rotarod test was performed to test the motor coordination and limb strength of mice. As described previously [23], mice were placed on an on a six-lane accelerating rotarod (acceleration from 4 to 40 rpm within 5 min, increasing 4 rpm every 30 s and reaching the final speed at 300 s). The time the animal stayed on the rotarod was recorded. Before the formal test, all mice were trained for 3 days to adapt the new surroundings.

Adhesive Removal Test

As described previously [24, 25], the adhesive removal test was performed by a blinded researcher to detect the

sensorimotor deficits of mice. Before the formal test, mice were trained for 3 days to familiarize them with the test. Then an adhesive tape (3 mm × 3 mm) was pasted on left forepaw (affected side). The time to contact and remove the adhesive tape was measured.

Foot Fault Test

According to previous studies [26, 27], the foot fault test was performed to evaluate the motor functional recovery of the animals. Mice were placed on a metallic grid (mesh area 3 cm^2) for 2 min. The number of left forelimb foot fault was recorded, and then the percentage of foot faults of the left forelimb to total steps was calculated.

Statistics Analysis

All data are shown as mean ± standard deviation (SD). *T* test or one-way ANOVA followed by Tukey multiple comparisons test were used to compare the differences between each groups. The Kruskal–Wallis test was used to compare the difference of data in abnormal distribution. Then, Dunn–Bonferroni test were performed for post hoc comparison. Statistical Package for the Social Sciences (SPSS; version 22.0) and Prism (version 6.0) software were used for statistical analyses. The *P* value < 0.05 indicated statistical significance.

Results

AdipoRon Attenuates Neuronal Death, ATP Reduction and ROS Accumulation in Mice After ICH

PI staining were used to assess the neuronal death in mice. As shown in Fig. 1a, b, compared to the sham group, the ratio of PI-positive neurons were strongly increased in ICH + vehicle group at 72 h after ICH. The administration of AdipoRon could reduce the level of PI-positive neurons compared to ICH + vehicle group (P < 0.05, Fig. 1a, b).

ATP assay and ROS assay were performed to validate the assumptions that AdipoRon could alleviate mitochondrial dysfunction after ICH. In Fig. 1c, the results indicated that ATP reduction was found in ICH-induced brain injury, AdipoRon treatment enhanced ATP levels when compared with ICH + vehicle group.

In addition, mitochondrial dysfunction was the crucial sources of ROS. The results of ROS assay demonstrated that ROS levels were markedly increased after experimental ICH in mice (P < 0.05, Fig. 1d). The administration of AdipoRon could decrease the level ROS levels in perihematoma tissues compared to ICH + vehicle group (P < 0.05, Fig. 1d).

Location of AdipoR1, AMPK, PGC1α

In order to validate the hypothesis that AdipoRon could ameliorate neuronal mitochondrial dysfunction via activating AdipoR1–AMPK–PGC1α pathway. Primarily, immunofluorescence staining was performed to investigate the location of target proteins. As shown in Fig. 2, AdipoR1, p-AMPK and PGC-1α were markedly expressed in neurons at perihematoma region.

AdipoRon Improves Mitochondrial Biogenesis Through Activating AdipoR1–AMPK–PGC1α Pathway in Mice After ICH

As shown in Fig. 3a, the results of western blotting indicated that AdipoR1 levels were further enhanced by AdipoRon at 72 h after ICH (P < 0.05 vs ICH + vehicle group). Meanwhile, the expression of P-AMPK was increased at 72 h after ICH (Fig. 3b, P < 0.05 vs sham group), and AdipoRon treatment further increased the level of P-AMPK and its downstream PGC1α (Fig. 3b, c, P < 0.05 vs ICH + vehicle group).

Since PGC1α activation could improve mitochondrial DNA and ATP production via activating NRF1/TFAM axis [15, 16]. We also measured the expression of NRF1 and TFAM, and the markedly improvements were found in the protein levels of NRF1 and TFAM after AdipoRon treatment (Fig. 3d, e, P < 0.05 vs ICH + vehicle group).

The above-mentioned results indicated that AdipoRon may improve mitochondrial biogenesis through activating AdipoR1–AMPK–PGC1α pathway in mice after ICH.

AdipoRon Improves Mitochondrial Permeability Potential and Mitochondrial Mass In Vitro

The in vitro experiments were designed to visually observe the changes of mitochondria after ICH injury.

Mitochondrial membrane potential (Δψm) was detected by JC-1 staining. As shown in Fig. 4a, Normal membrane potential showed red fluorescence in control group. The green fluorescence intensity was increased in SH-SY5Y cells exposed to OxyHb. The JC-1 fluorescence intensity was ultimately quantified by flow cytometry (Fig. 4b). It demonstrated that the administration of AdipoRon significantly alleviated OxyHb-induced collapse of Δψm (P < 0.05 vs oxyHb + vehicle group).

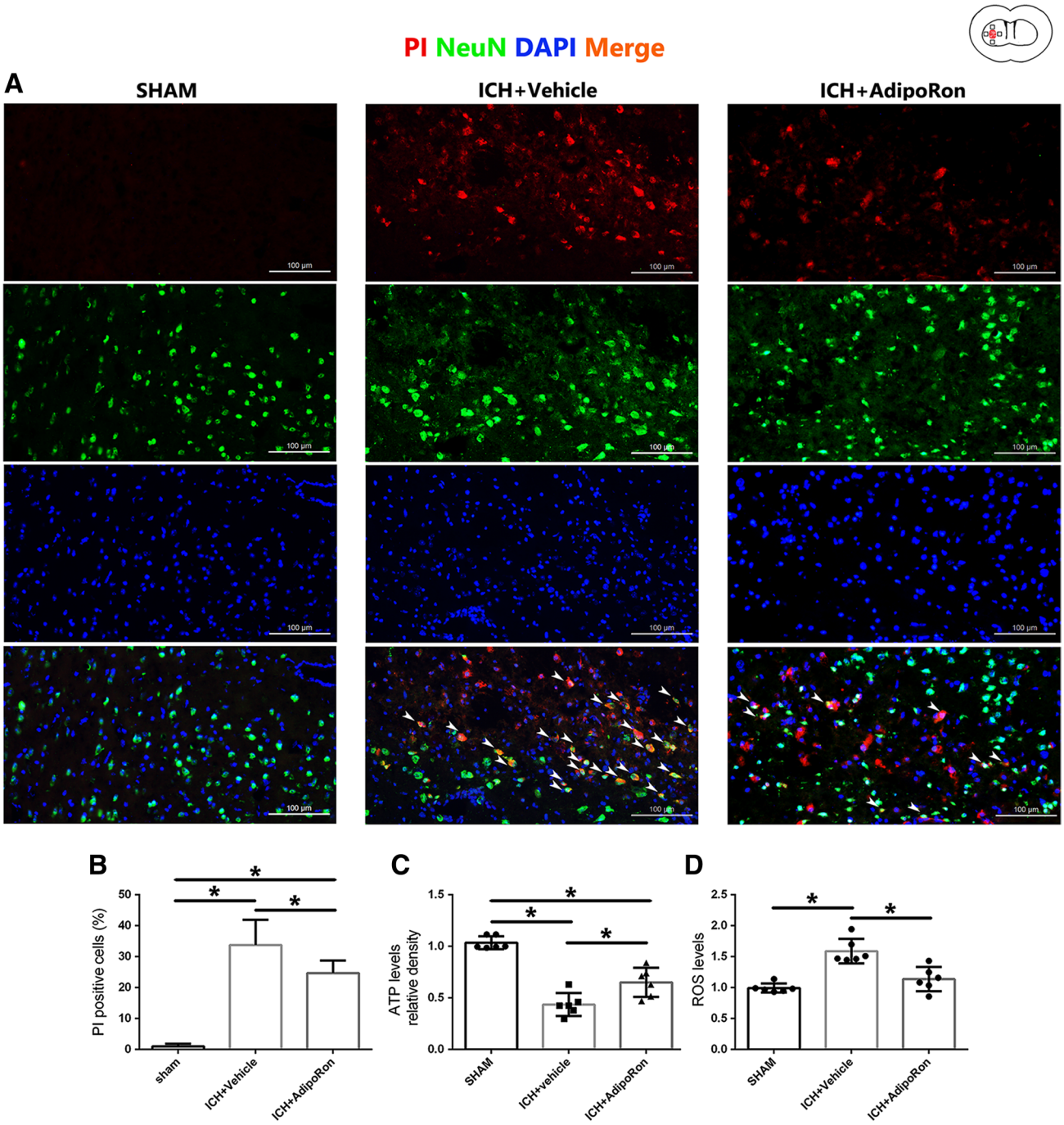

Fig. 1 AdipoRon attenuates neuronal death, ATP reduction and ROS accumulation in mice after ICH. **a** Representative co-labeling images for PI (red) and NeuN (green) at 72 h after ICH in ipsilateral basal ganglia (Scale bars: 100 μm). **b** Quantification of PI^+ $NeuN^+$ cells, *P < 0.05. **c** ATP levels, *P < 0.05. **d** ROS levels, *P < 0.05. n = 6 in each group. The black squares around the hematoma were the region for immunofluorescence (Color figure online)

The fluorescence probe (Mito-tracker™ Green) was used to observe and quantified mitochondrial mass in SH-SY5Y cells. As shown in Fig. 4c, d, we indeed found that OxyHb treatment strongly reduced mitochondrial mass (P < 0.05 vs control group). The administration of AdipoRon partly reversed the negative effects of OxyHb in SH-SY5Y cells (P < 0.05 vs oxyHb + vehicle group).

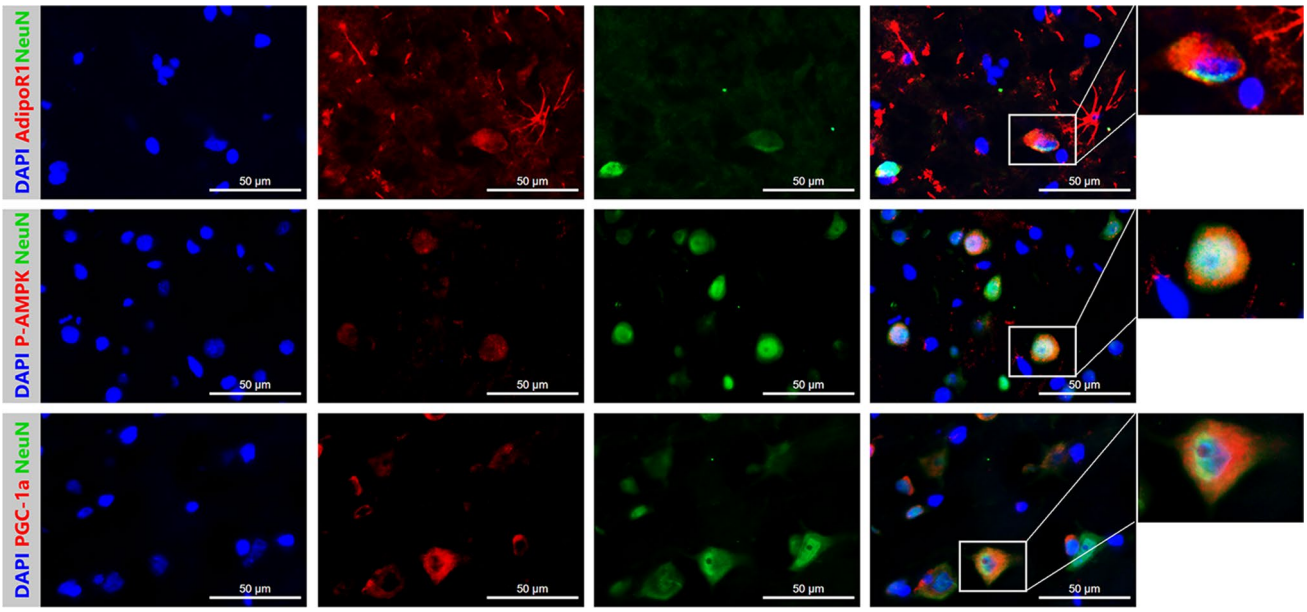

Fig. 2 Location of AdipoR1, AMPK, PGC1α. Scale bars: 50 μm

AdipoRon Attenuates Neuronal Apoptosis and Necrosis, Improves Cell Viability In Vitro

The representative images of flow cytometry were shown in Fig. 5a. The neurons (SH-SY5Y cells) treated with AdipoRon showed low necrotic and apoptotic rate compared to neurons treated with oxyHb only (Fig. 5b, c, $P < 0.05$). Likewise, neurons with AdipoRon treatment showed better cell viability when compared with other groups (Fig. 5d, $P < 0.05$).

AdipoRon Improves Neurological Function in Mice After Experimental ICH

Adhesive removal test was used to assess the sensorimotor deficits of mice at 24 h, 72 h and 5 days after ICH. The contact and removal time were significantly increased in mice with experimental ICH ($P < 0.05$, Fig. 6b, c). AdipoRon treatment significantly decreased the contact and removal time at 5 days after ICH compared to ICH + vehicle group ($P < 0.05$, Fig. 6b, c).

In order to evaluate the motor coordination and limb strength of mice in different groups, Accelerated rotarod test was performed and the results demonstrated that ICH significantly impaired the motor function at 24 h, 72 h and 5 days compared to sham group ($P < 0.05$, Fig. 6d). Meanwhile, AdipoRon could attenuate motor impairment at 72 h and 5 days after ICH when compared with ICH + vehicle group ($P < 0.05$, Fig. 6d).

The results of foot fault test showed that experimental ICH could induce markedly motor functional deficits ($P < 0.05$, Fig. 6e). AdipoRon treatment attenuated the foot fault of ICH mice when compared with ICH + vehicle group at day 5 ($P < 0.05$, Fig. 6e).

Discussion

The mitochondria is a crucial eukaryotic organelle which is responsible for intracellular ATP and oxygen free radical production [4, 28]. Mitochondrial dysfunction can result in a series of intracellular signaling cascades, oxidative stress, and eventually cause cellular apoptosis and necrosis [3, 29]. There is sufficient evidence that mitochondrial dysfunction plays an important role in SBI after ICH [5, 7, 30].

Since neuronal damage after SBI has always been our focus, mitochondrial dysfunction can cause neuronal apoptosis and necrosis in several ways. Firstly, dysfunctional mitochondria acts as an important source of free radicals in ICH, the ROS overproduction subsequently cause the expression of apoptosis-related protein including NF-kB, NLRP3, Bcl-2, etc. [4, 5, 31, 32]. Moreover, mitochondrial damage may cause mitochondrial permeability transition pore (MPTP) opening. Then several proteins (e.g., cytochrome c) release into the cytoplasm and consequently induce cell apoptosis [31, 33]. Furthermore, the deficiency of ATP generation impairs cellular pumps causing cytotoxic edema and cell death. Notably, insufficient energy supply may be particularly injuring neurons which have a high energy demand compared to others cells [4].

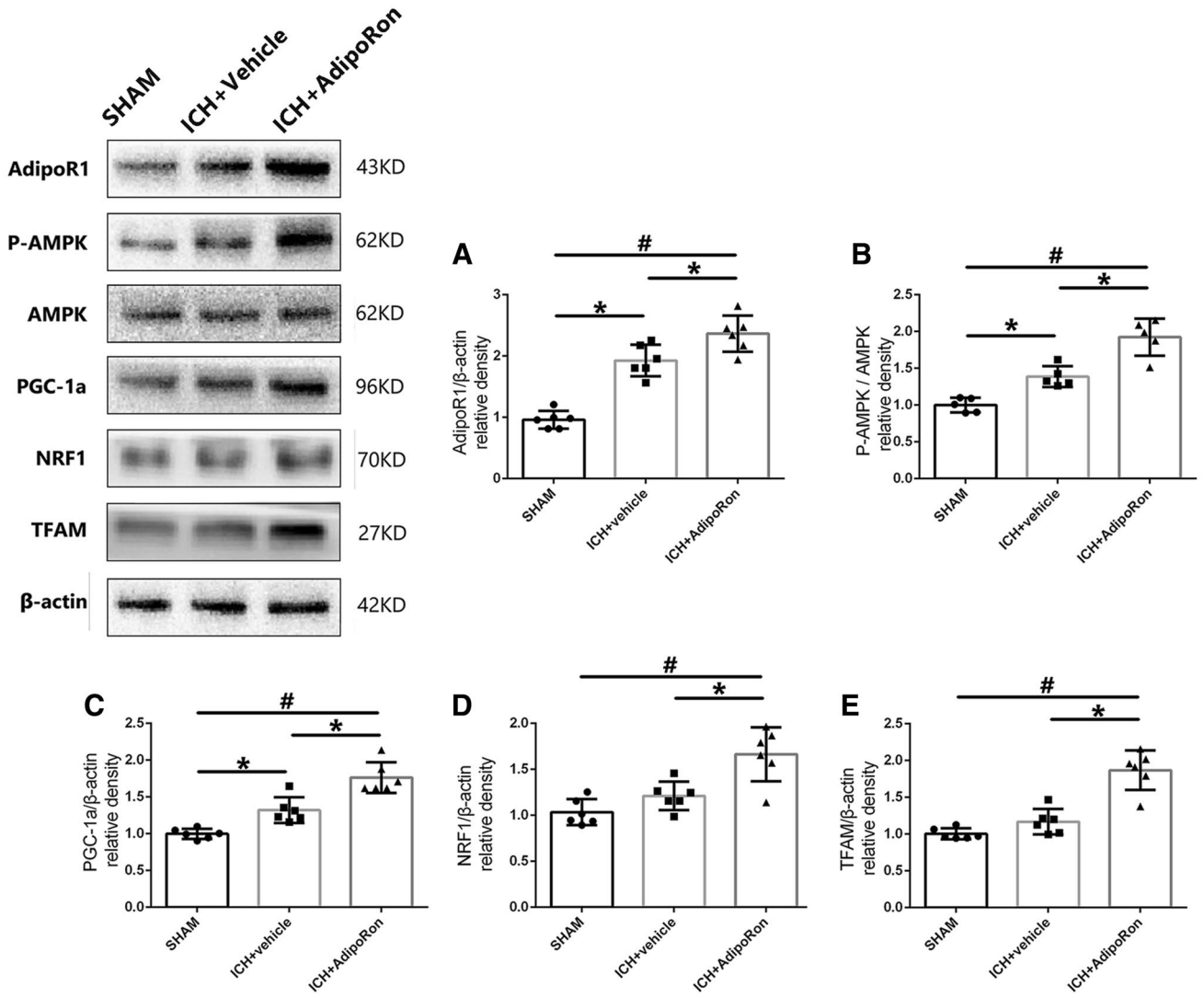

Fig. 3 AdipoRon activates the protein expression of AdipoR1, AMPK, PGC1α. Representative Western-blot bands showed the expression of **a** AdipoR1, **b** P-AMPK/AMPK, **c** PGC1α, **d** NRF1, **e** TFAM. *P < 0.05, #P < 0.01, n = 6 in each group

In the present study, we firstly demonstrated that AdipoRon alleviated mitochondrial dysfunction after ICH via activating AdipoR1–AMPK–PGC1α pathway. AdipoRon is the latest small-molecule agonist of adiponectin receptors (AdipoRs), which ameliorates insulin resistance and glucose intolerance via AdipoR1–AMPK–PGC1α pathway [8]. A previous study demonstrated that Chikusetsu Saponin IVa could prevent cellular apoptosis through an AdipoR1-mediated AMPK/GSK-3β pathway after cerebral Ischemia reperfusion injury [10]. Hence, the present study used PI staining and flow cytometry to assess the neuronal apoptosis and necrosis. The result of in vivo experiment showed that AdipoRon treatment could reduce the ratio of PI-positive neurons at 72 h after ICH. The in vitro experiment verified again that neurons (SH-SY5Y cells) treated with AdipoRon showed low necrotic and apoptotic rate compared to neurons treated with oxyHb only. Consistent with previous studies, such results preliminarily verified the positive effects of AdipoRon on neurons survival.

In order to testify whether such positive effects were associated with mitochondrial function. The in vivo experiment (ATP and ROS assay) and in vitro experiment were performed (Δψm and Mito-Tracker Green).The mitochondrial Electron Transport Chain (ETC) has been considered the crucial source of mitochondrial ROS. Because complexes I and III generate a mass of O^{2-} during oxidative phosphorylation and the tricarboxylic acid cycle [34]. Moreover, the ETC functions to maintain Δψm, which is required for ATP generation via proton flow through ATP synthase. Dysregulated ETC function consequently result in ROS overproduction and the collapse of Δψm

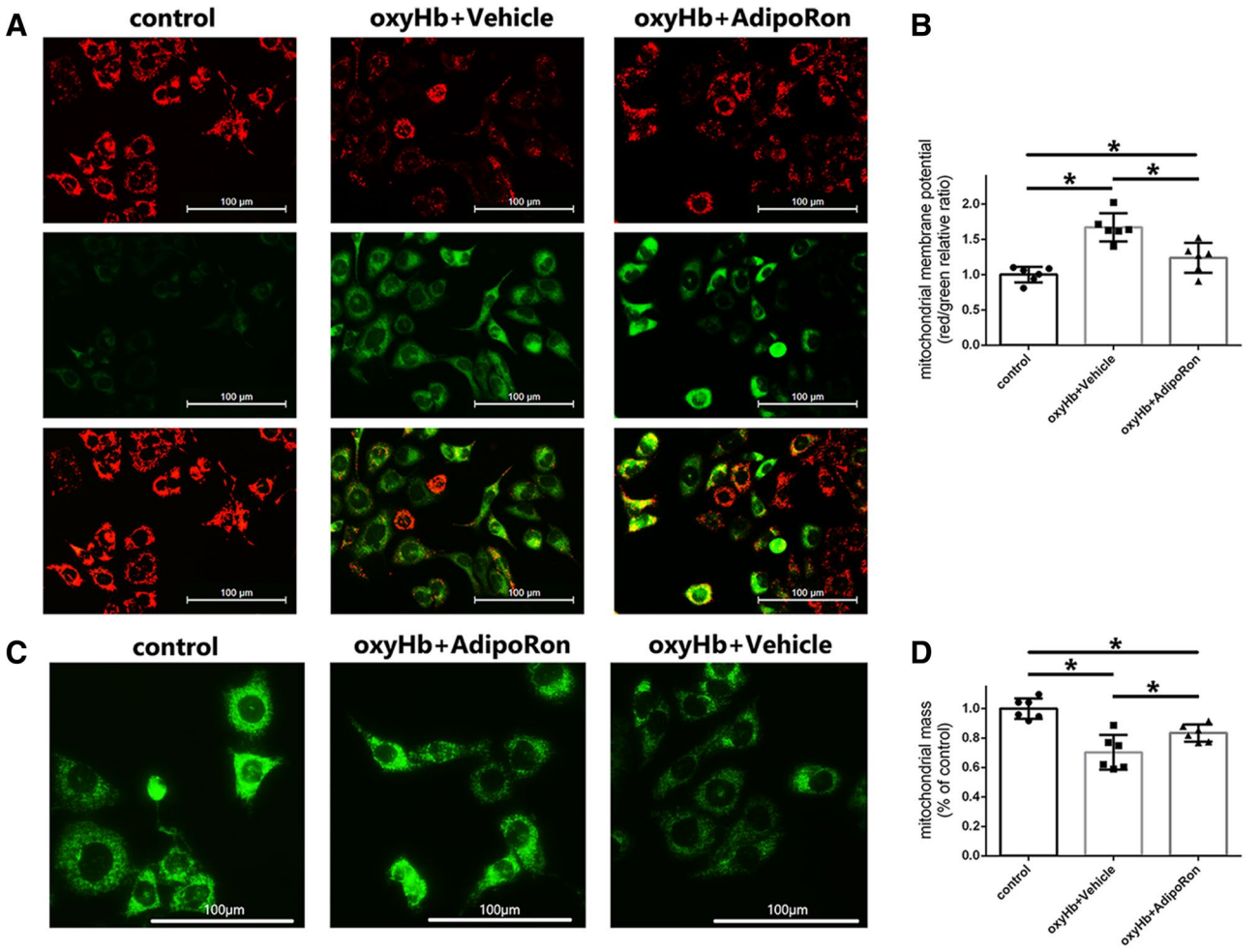

Fig. 4 AdipoRon improves mitochondrial permeability potential and mitochondrial mass in vitro. **a** The representative JC-1 staining images of SH-SY5Y cells (scale bars: 100 μm). **b** Quantification of Δψm (JC-1 staining) by flow cytometry, *P < 0.05. **c** The representative mitochondria images stained by Mitotracker™ Green (scale bars: 100 μm) **d** Quantification of mitochondrial mass (Mitotracker™ Green) by flow cytometry, *P < 0.05

[35]. The present study indicated that the administration of AdipoRon could attenuate ATP reduction and ROS accumulation in perihematoma tissues after experimental ICH. In addition, the in vitro experiments also proved that AdipoRon treatment could improve Δψm and mitochondrial mass in neurons. The above-mentioned experimental results indicated that AdipoRon could alleviate neuronal mitochondrial dysfunction after ICH.

The positive effect of AdipoRon on neuronal mitochondria subsequently result in neuron survival and neurological function recovery.

The results of immunofluorescence and western blot showed that AdipoR1, p-AMPK and PGC-1α were markedly expressed in neurons. The protein expression of AdipoR1, p-AMPK and PGC-1α were significantly increased after AdipoRon treatment.

As previously described, AdipoR1 activation enhance the phosphorylation of AMP-activated protein kinase (AMPK) and consequently exert anti-apoptosis, anti-inflammatory effects after ischemic stroke [10, 12]. AMPK is an important molecule involved in cellular energy homeostasis and metabolic pathways [13]. ICH-induced brain injury caused cellular ATP reduction which induced the phosphorylation of AMPK [14]. As the crucial downstream signal molecule of AMPK, transcriptional coactivators PGC-1α mediated numerous mitochondrial functions including mitochondrial biogenesis, Δψm and ROS production [36]. PGC-1α could improve mitochondrial mass and ATP production via activating NRF1/TFAM axis in Alzheimer's disease and ICH [15, 16]. The present study exhibited the similar results that NRF1 and TFAM protein expression were enhanced after the activation of AdipoR1–AMPK–PGC1α axis. Hence,

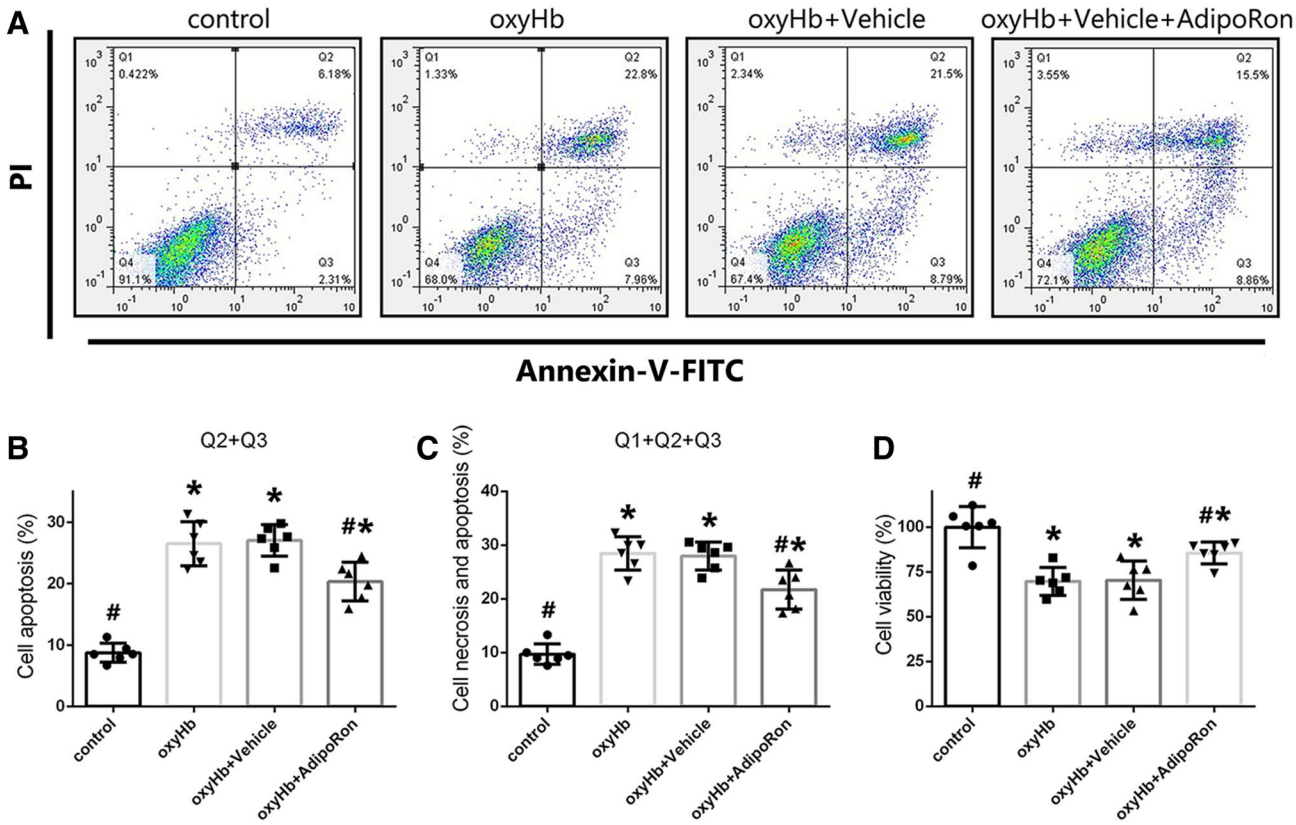

Fig. 5 AdipoRon attenuates neuronal apoptosis and necrosis, improves cell viability in vitro. **a** The representative images of flow cytometry (PI/Annexin-V double labeling). **b** Apoptotic rate. **c** The necrotic plus apoptotic rate. **d** Cell viability. $^{\#}P < 0.05$ versus oxyHb + vehicle group; $^{*}P < 0.05$ control group

we speculate that AdipoRon alleviated mitochondrial dysfunction after ICH via activating AdipoR1–AMPK–PGC1α pathway.

There were still numbers of limitations in our research. The present study only focused on the mitochondrial dysfunction. Actually, numerous complicated mechanisms and multiple factors participated in the pathophysiological process of ICH. Hence, in the next phase of the study, we should illuminate the effects of AdipoRon in inflammation, oxidative stress, white matter injury, etc. after ICH. The knockout mice might be used to further increase the credibility of the molecular pathways. Moreover, in the present study, several technology and methods e.g., ATP assay, ROS assay, JC-1 staining (Δψm) and mitochondrial fluorescent probe were used to detect the mitochondrial function. However, there were still some persuasive approaches that we were unable to include, e.g., mitochondrial complex activity assay, mitochondrial DNA test. The above-mentioned limitations should be solved in further researches.

Conclusion

In conclusion, the present study revealed that AdipoRon alleviated the neuronal apoptosis/necrosis and promoted neurological function recovery after ICH. The neuroprotective effects of AdipoRon may depend on the improvement of mitochondrial dysfunction via AdipoR1–AMPK–PGC1α pathway. These findings implicated a novel therapeutic strategy to attenuate ICH-induced brain injury.

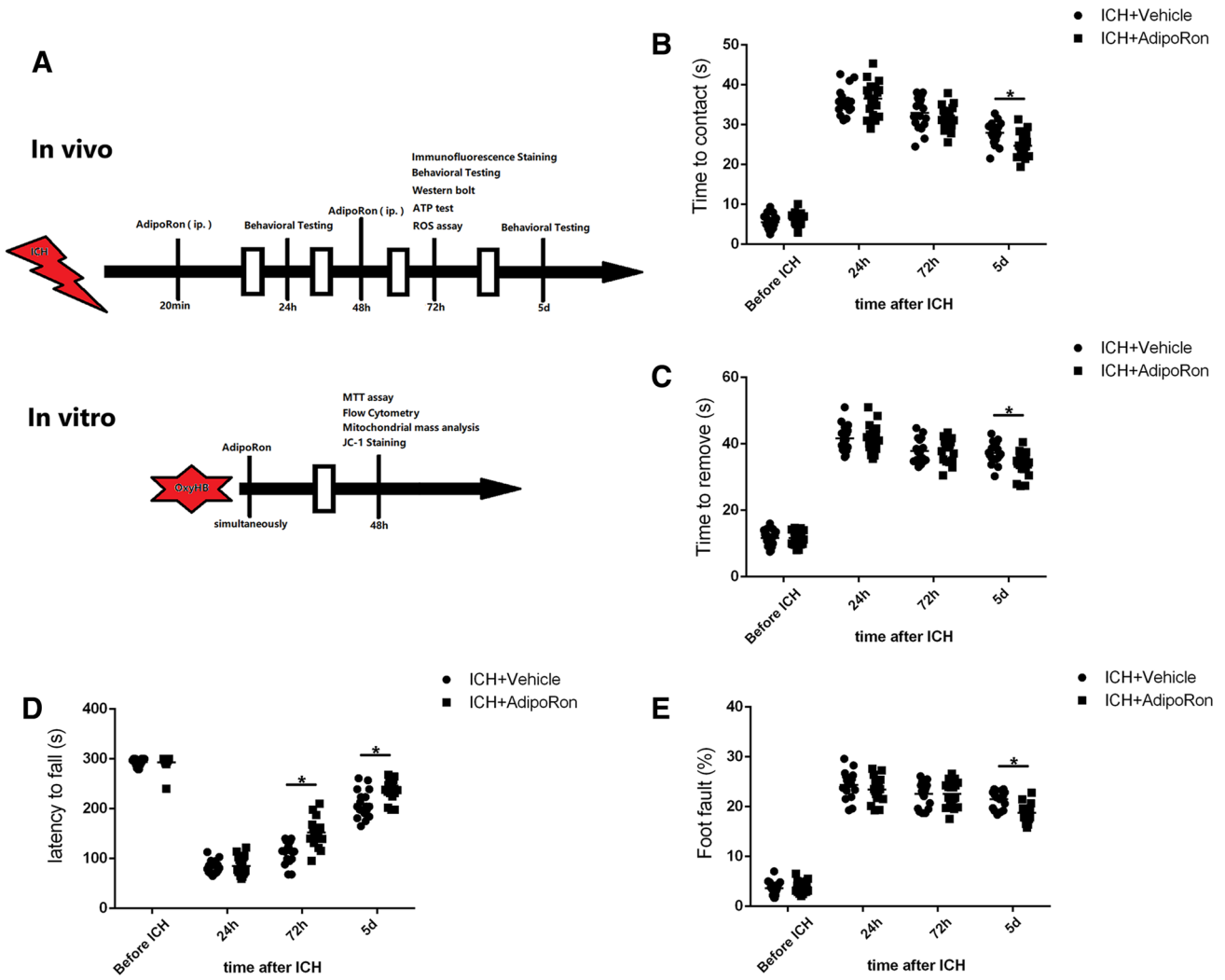

Fig. 6 AdipoRon improves neurological deficits in mice. **a** Experimental design. **b** Adhesive removal test (contact), *P < 0.05. **c** Adhesive removal test (contact), *P < 0.05. **d** Accelerated rotarod test, *P < 0.05 **e** foot fault test, *P < 0.05. n = 6 in each group (each test was repeated three times)

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethics Approval All animal experimental protocols were in compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

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