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The effect of mitochondrial calcium uniporter on mitochondrial fission in hippocampus cells ischemia/reperfusion injury

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ABSTRACT

The mitochondrial calcium uniporter (MCU) transports free Ca^{2+} into the mitochondrial matrix, maintaining Ca^{2+} homeostasis, thus regulates the mitochondrial morphology. Previous studies have indicated that there was closely crosstalk between MCU and mitochondrial fission during the process of ischemia/reperfusion injury. This study constructed a hypoxia reoxygenation model using primary hippocampus neurons to mimic the cerebral ischemia/reperfusion injury and aims to explore the exactly effect of MCU on the mitochondrial fission during the process of ischemia/reperfusion injury and so as the mechanisms. Our results found that the inhibitor of the MCU, Ru360, decreased mitochondrial Ca^{2+} concentration, suppressed the expression of mitochondrial fission protein Drp1, MIEF1 and Fis1, and thus improved mitochondrial morphology significantly. Whereas spermine, the agonist of the MCU, had no significant impact compared to the I/R group. This study demonstrated that the MCU regulates the process of mitochondrial fission by controlling the Ca^{2+} transport, directly upregulating mitochondrial fission proteins Drp1, Fis1 and indirectly reversing the MIEF1-induced mitochondrial fusion. It also provides new targets for brain protection during ischemia/reperfusion injury.

1. Introduction

Presently, the incidence and mortality rate of cerebrovascular diseases increase year by year, which urgently needs new treatment strategies to improve. Pathological physiological process of cerebral ischemia/reperfusion injury (IRI) plays a vital role in the development of these diseases [1,2]. Therefore, how to reduce or avoid brain IRI is capital to reduce the risk of cerebrovascular diseases. Brain is an important organ, which is highly susceptible to be-ischemic damaged. Blood flow reconstruction and the ischemia area blood supply increase are necessary conditions of cerebral ischemia repair. Cerebral ischemia-reperfusion injury is a pathological physiological phenomena which refers to cerebral ischemia and restored blood flow to the ischemic areas, resulting that these areas not only fail to restore function, but further are injured [3].

Previous studies have shown that Mitochondria, as the power plants of the cell, are highly dynamic organelles [4]. Mitochondrial morphology is controlled by two opposing processes: fusion and fission, the balance of which can form a new individual and network structure, seriously affects the function of Mitochondrial and also regulates apoptosis of cells [5]. Recent researches indicated that mitochondrial division was regulated by many factors, among which, Drp1 and Fis1 were considered to be the important ones [6–8]. Drp1 mediates the split of mitochondrial depending on the Fis1's recruitment from cytoplasm to mitochondrial outer membrane and controls the mitochondria fusion/fission balance and also involves in the first step of apoptosis [5]. Mitochondrial division would be restrained if Fis1 or Drp1 was lacking [9,10]. Recent years, MIEF1-a novel integral mitochondrial outer membrane protein-has been found, and it promotes mitochondrial elongation and fusion. Elevated level of MIEF1 induces extensive mitochondrial fusion, whereas depletion of MIEF1 causes mitochondrial fragmentation [9]. Besides, as a second messenger, Ca^{2+} participates in pathophysiological processes such as energy metabolism and apoptosis [11]. It also controls the balance of the mitochondria fusion/fission through a variety of ways. Mitochondrial Ca^{2+} signaling has been shown to regulate mitochondrial fission by phosphorylation or dephosphorylation of Drp1 [12,13].

Abbreviations: I/R, ischemia/reperfusion; MCU, mitochondrial calcium uniporter; Drp1, dynamin-related protein1; MIEF1, mitochondrial elongation factor 1; Fis1, fission 1.

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Mitochondrial calcium uniporter is a protein located on the mitochondrial membrane [14], the mainly role of which is transferring Ca^{2+} from the cytoplasm to the mitochondrial matrix. Studies have found that inhibition of MCU can not only reduce nerve cell necrosis and apoptosis after cerebral ischemia, but also reduce brain edema and the expression of AQP4, the role of which is associated with the regulation of MPTP of mitochondria [15,16]. A current study of our laboratory indicated that Ru360(the specific inhibitors of MCU) could inhibit the MCU , thus reduce mitochondrial Ca^{2+} concentration and reduce the expression of Drp1, which protect the mitochondria using a ischemia/reperfusion injury model of living rats [17]. However, the exactly role of MCU in working for mitochondrial splitting during the process of ischemia/reperfusion injury is not clear. This study aims to explore deeply the role of MCU for the regulation of mitochondrial fission using a hippocampus neuron cell hypoxia reoxygenation model and provide new theoretical basis and therapeutic targets for clinical brain ischemia-reperfusion injury.

2. Materials and methods

2.1. Hippocampal neurons culture

The experiments were approved by the institution of Ethics Committee of Qingdao University Medical College (No. QUMC 2011-09). The hippocampal neurons were prepared from the neonatal Wistar Rats according to the methods described previously [18,19]. The rats purchased from the Experimental Animal Center of Qingdao Drug Inspection Institute (SCXK [LU] 20090007) were disinfection, and the brain was exposed where the hippocampus isolated from. Then the hippocampal tissue was digested for 20 min with trypsin (HyClone, UT, USA) at 37 °C. The cell pellet was resuspended in Dulbecco modified Eagle medium/F12 (Gibco, NY, USA) solution containing 20% (vol/vol) fetal bovine serum (HyClone) and plated at a density of 700,000 cells on 25-mm Petri dishes that were coated by poly-L-lysine. Cultures were maintained at 37 °C in a humidified incubator with 95% (v/v) O_2 and 5% (v/v) CO_2. Twenty-four hours after being plated, the medium was replaced of Neurobasal-A medium (Gibco) containing 2% (v/v) B27 supplement (Gibco) and 2 mM glutamine. Half of the medium was replaced every 3 days. On the 8th day, by NSE staining, cells were identified and observed under fluorescent microscopy (Leica, DMI 4000 B, Japan).

2.2. Neuronal cell hypoxia reoxygenation model (oxygen-glucose deprivation (OGD) model) and experimental grouping method

Presently, a lot of experiments and studies have shown that OGD experiment is a stroke model in vitro and the effect of hypoxia reoxygenation model was used to simulate the neurons [20–23]. OGD models: the culture medium was replaced by the glucose-free EBSS (Gibco), at the same time the cultivation bottles was shifted to an incubator with 5% (v/v) CO2 and 95% (v/v) N2 at a temperature of 37 °C.

The neurons were randomly divided into 6 groups:

(1) Control group: cells were continually cultured in normoxic gas mixture for 8 days without any treatment;
(2) I/R group: at the 8th day, cells were OGD for 6 h and returned to normoxic conditions for 20 h.
(3) I/R + Ru360 group: at the 8th day, the cells were pretreated with Ru360 (10 μM) for 40 min before OGD for 6 h, then returned to normoxic conditions for 20 h;
(4) I/R + Sper group: at the 8th day, the cells were treated with spermine (10 μM) for 40 min before OGD for 6 h, then returned to normoxic conditions for 20 h [24];
(5) Ru360 group: at the 8th day, the cells were treated with Ru360 (10 μM) for 6 h, then cultured with no drug for 20 h;
(6) Sper group: at the 8th day, the cells were treated with spermine (10 μM) for 6 h, then cultured with no drug for 20 h.

2.3. Determination of mitochondrial calcium

The extraction of mitochondria was as follows: The cells were digested with trypsin and collected, then incubated in mitochondrial separation reagent for 30 min. Sample was harvested by centrifugation (600 g, 5 min and 11,000, 20 min).

The isolated mitochondria were washed by Hanks solution, then the Fluo -3, AM (AAT Bioquest, Inc.US) [17] was added in and samples were incubated in 37 °C for 30 min. And then we washed the samples using Hanks solution three times to remove excess probe and incubated them in 37 °C for 30 min again. The fluorescence intensity was determined at the excitation 506 nm and emission 526 nm using a fluorescence microplate reader.

2.4. Transmission electron microscope observation

Hippocampal neurons in different groups were collected and fixed in glutaraldehyde with a 1% (w/v) solution of osmium tetroxide and then the fixed cells was embedded in Epon812-Araldite. Finally, ultrathin slices (50 nm thick) were cut and we observed the microstructure under a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

2.5. Protein extraction and western blots

Cells sediment was homogenized in ice cold RIPA buffer (containing 1/1000 PMSF). Soluble proteins were collected and centrifuged at 12,000 g. For each sample, 60 mg of soluble protein was separated by SDS-PAGE (with different concentrations appropriate for their differing molecular weight of the targeted proteins) at 80 V for 0.5 h and 120 V for 1 h using the Mini-PROTEAN 3 electrophoresis cell system (Bio-Rad). Proteins were then transferred to a PVDF membrane (Bio-Rad) by the semi-dry blotting method (Bio-Rad) and the Dunn carbonate transfer buffer that consisted of NaCHO3 (10 mM), Na2CO3 (3 mM), and 20% methanol. Membranes were blocked for 2 h by 5% (w/v) nonfat dry milk and then incubated overnight at 4 °C with the primary antibodies, mouse anti-Drp1 (1:1,000; Abcam, Ab56788), rabbit anti-MIEF1 (1:500; Abcam, Ab89944), rabbit anti-Fis1 (1:500; Abcam, ab71498), and mouse anti-GAPDH (1:500; Abcam, Ab8245). Membranes were subsequently incubated with peroxidase-conjugated secondary antibodies. Antibody binding was detected after incubation with HRP-linked secondary antibodies, with the membrane-bound antibodies visualized by luminal chemiluminescence ChemiDoc XRS (BIO-RAD).

2.6. Statistical analysis

SPSS18.0 statistical software was used to perform statistical analysis and the experimental results was expressed as means ± standard error. One-way ANOVA followed by the Tukey's post hoc test for multiple comparisons. $p < 0.05$ was considered statistically significant.

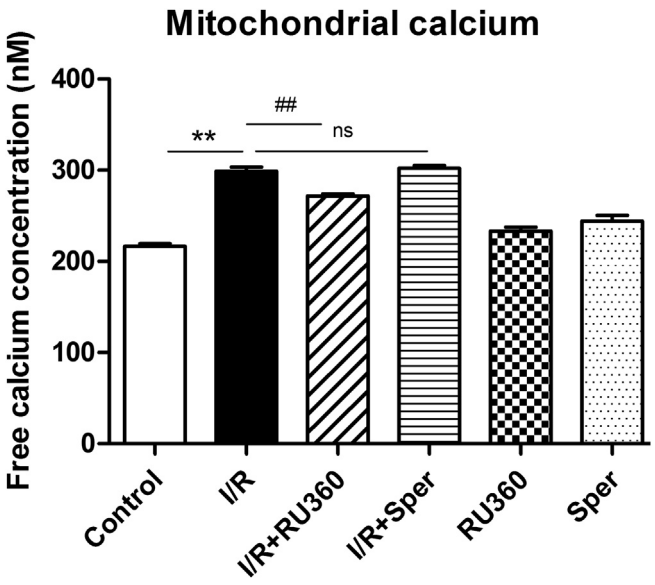

Fig. 1. The measurement of mitochondrial free calcium concentration. Mitochondrial free calcium concentration was measured using fluorescence labeling methods with probes. The fluorescence intensity was determined at the excitation 506 nm and emission 526 nm using a fluorescence microplate reader. Values are represented as the mean ± SE (n = 6 per group). $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs.I/R.

3. Results

3.1. The mitochondrial free calcium concentration

Previous study has shown that mitochondrial calcium uniporter (MCU) plays a crucial role and controls the activities of mitochondrial calcium, normal physiology and integrity for mitochondria [25]. The mitochondrial free calcium concentration in the I/R and I/R + Sper groups was significantly higher than the control group. However, no change was observed in the Ru360 and Sper groups. Inversely, the concentration of mitochondrial free calcium in the I/R + Ru360 group was significantly lower than in the I/R group (Fig. 1). The results indicated that inhibition of MCU activity may effectively reduce the Ca^{2+} overload.

3.2. Mitochondrial ultrastructure observation

As mitochondrial morphology is controlled by fusion and fission, which is correlated with status of MCU, we accessed mitochondrial ultrastructure using a JEM-1200EX transmission electron microscope. In the control group and the RU360 group, TEM micrographs showed intact mitochondria, with double membranes visible, cristae structurally intact, and round in shape(Fig. 2A and C). In contrast, the mitochondria of cells in the I/R group、the Sper group and the I/R + Sper group were swelling with hypodense matrices and ambiguous cristae and even vacuolization, which lost typical mitochondrial structure (Fig. 2B, E and F). Prospectively, the mitochondrial ultrastructures in the I/R + RU360 group were less dramatically destroyed and we could still observed clear cristae (Fig. 2D). These results were coincidence with calcium concentration change among different groups.

3.3. The expression of protein related to mitochondrial morphology

To explore the mechanisms of mitochondrial morphology regulation by MCU in the process of ischemia-reperfusion injury, we examined factors closely associated to mitochondria fission and fusion. Dynamin-related protein1 (Drp1) and Fis1 is central players controlling mitochondrial fission, whereas MIEF1 (mitochondrial elongation factor 1) is recently identified associated to mitochondria fusion. Compared to the control group, I/R group showed significantly increased expression of Drp1, Fis1 and MIEF1 (Fig. 3A). However, when I/R group was pretreated with Ru360, an inhibitor of MCU, expression of these three proteins decreased significantly compared to I/R group, almost correspond to control group. Using Ru360 alone did not change abundance of Drp1, Fis1 and MIEF1, which could exclude the effect of drug itself (Fig. 3). In I/R group pretreated with MCU agonist spermine, no significant differences of Drp1 and MIEF1 expression was observed (Fig. 3B and C), but Fis1 abundance decreased apparently(Fig. 3D). These results suggested that inhibition of MCU could inverse the change of Drp1, Fis1 and MIEF1 induced by ischemia-reperfusion injury. In addition, stimulation of MCU could not promote the high expression of these three proteins and then intensify the injury. We could presume that in the process of ischemia-reperfusion injury induced mitochondria fission, MCU is a necessary but not sufficient factor.

4. Discussion

In the present study, apoptosis was an important mechanism of the neuron ischemia-reperfusion injury. In addition, mitochondria is morphologically dynamic organelles [26,27], whose fission and fusion played an important role in mediating cell apoptosis signaling pathways [19]. The Ca^{2+} participated in apoptosis processes and increasing the concentration of Ca^{2+} in the cytoplasm and mitochondrial matrix was the critical factor to mitochondrial fission [28]. Imbalance of the mitochondrial fusion and fission dramatically changed mitochondrial morphology [29]. Our results indicated that the concentration of mitochondrial free calcium was markedly higher in the I/R + Sper and I/R groups than the control group and the mitochondrial ultrastructures had been destroyed dramatically in the I/R + Sper and I/R groups. However, in the I/R + Ru360 groups, the Ca^{2+} concentration was significantly lower and the mitochondrial cristae was more clearly than the I/R group. Therefore, we inferred that inhibition of the MCU reduced Ca^{2+} overload and mitochondrial fission.

Drp1 is a dynamin-like GTPase shuttling between the cytoplasm and the mitochondrial surface and mediating mitochondrial fission [30]. Its effect on mitochondria fission is controlled by calcium-dependent dephosphorylation [30]. Fis1 is also a fission-promoting protein, which is located at mitochondrial outer membrane and believed to serve as a receptor for recruitment of Drp1 to mitochondria [31,32]. In our research, the expression of Drp1 and Fis1 in I/R group is significantly increased accompanying with high free calcium concentration and severe mitochondrial fission. Conversely, when hippocampus cells were treated with MCU inhibitor Ru360 before ischemia-reperfusion injury, they expressed decreased level of Drp1 and Fis1 and improved mitochondrial morphology. Although these results indicated that MCU is necessary in the regulation of mitochondrial morphology during ischemia-reperfusion injury, we wondered that whether MCU is the only one factor controlling the process. So we used MCU agonist spermine before ischemia-reperfusion injury. There was no difference on Drp1 expression and mitochondrial morphology between I/R and I/R + Sper. Although decreased Fis1 expression was observed, the spermine treated control group (Sper) also showed decreased Fis1 expression (Fig. 3D), which could not exclude side effect of drug itself. Therefore, we could make conjecture that there may be

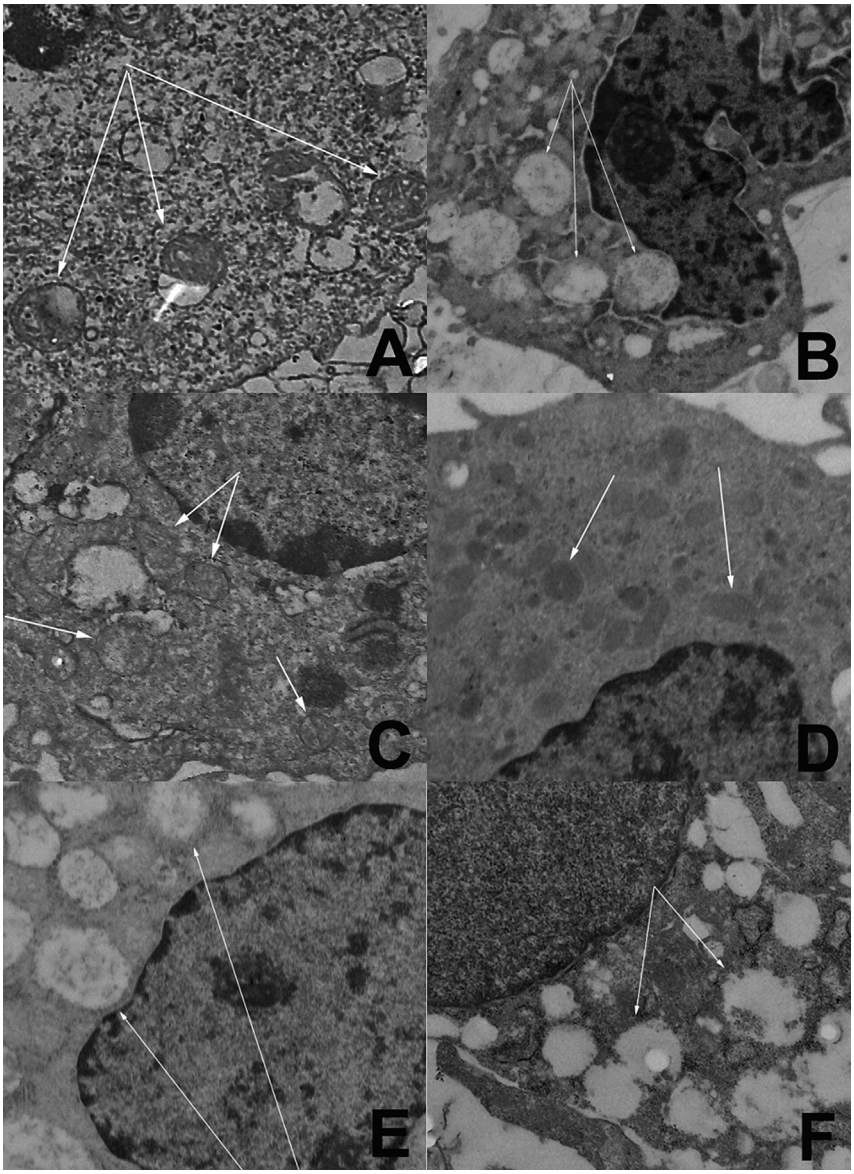

Fig. 2. Comparison of mitochondria ultrastructure. Images were captured using a JEM-1200EX transmission electron microscope (Jeol, Tokyo, Japan) with an amplification of 10 k. A: Control group; B: I/R group; C: RU360 group; D: I/R + RU360 group; E: Sper group; F: I/R + Sper group.

additional factor(s) controlling mitochondrial morphology in the process of ischemia-reperfusion injury synergetic with MCU, but MCU is the one indispensable.

Studies indicated that increased or reduced levels of Fis1 do not affect the amount of mitochondrial related Drp1 [33], which is accordant with our research. In I/R + Sper group, the expression of Fis1 was reduced but not as Drp1. Therefore, there is a possibility that additional factors interact with Drp1 and potentially contribute to the interaction recruitment of Drp1 to the mitochondrial surface [34]. MIEF1 is one of the recently identified factors to act in mitochondrial elongation and perinuclear clustering. Moreover, MIEF1 interacts with Drp1 and induces translocation of Drp1 from cytoplasm to mitochondria. Different from Fis1, MIEF1 acts as a suppressor to depress Drp1 and inhibits Drp1-mediated fission [35]. As is shown in Fig. 3, variation trend of Drp1 was coincidence with that of MIEF1. Separate from its interaction with Drp1, MIEF1 also interacts with Fis1, and elevated Fis1 levels reverse the MIEF1-induced mitochondrial fusion [9]. According to these evidences, we can explain the fact that elevated MIEF1 levels accompany with open of MCU and mitochondria fission, and vice versa. Since Fis1 shows a robust interaction with MIEF1, in contrast to a much weaker interaction observed between Fis1 and Drp1, we can presume that high abundance of Fis1 maybe “kidnap” MIEF1, reverse its function of mediating mitochondrial fusion and cripple its inhibition effect on Drp1 [9]. In other words, MCU regulated mitochondrial fission induced by ischemia-reperfusion injury through two pathways: 1) directly upregulating mitochondrial fission proteins Drp1, Fis1 and 2) indirectly reverse the MIEF1-induced mitochondrial fusion.

In conclusion, our results indicated that MCU may play an essential role in mitochondrial fission, but not sufficient for mitochondrial fission in hippocampus cells ischemia/reperfusion injury. Inhibiting MCU activity may decrease mitochondrial fission by preventing mitochondrial Ca^{2+} overload and thus protecting the cerebral cortex from ischemia/reperfusion injury.

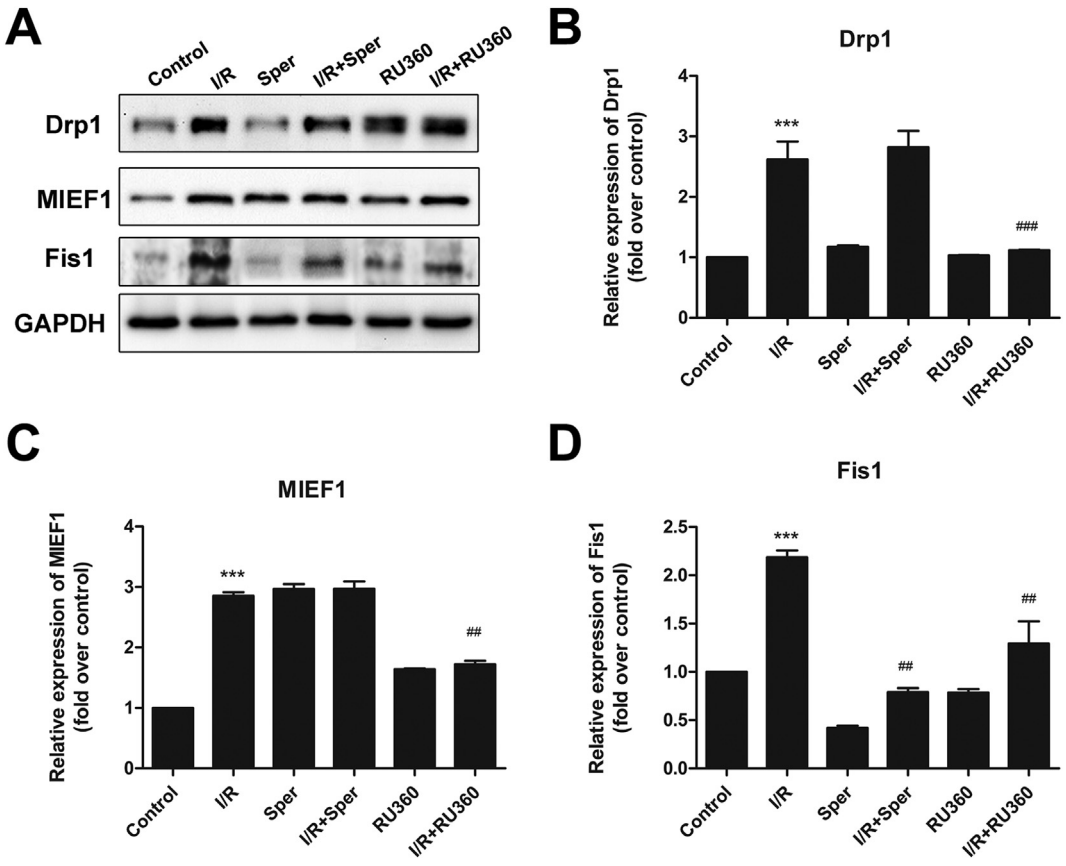

Fig. 3. Comparison of the expression of Drp1, MIEF1 and Fis1 using Western blot. (A) Western blot analysis of Drp1, MIEF1 and Fis1 of control, I/R, Sper, I/R + Sper group, Ru360 and I/R + Ru360 group. Bands were analyzed using Image J software and GAPDH was used as a loading control. Quantification of the Western blot analysis of (B) Drp1, (C) MIEF1 and (D) Fis1. Protein levels are relative to the control group. Results are typical of three independent experiments. Data represent means ± S.E. (n = 3). ***p < 0.001vs control; ##p < 0.01, ###p < 0.001vs I/R.

Conflict of interest

None.

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Transparency document

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