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Original Article

Cytoplasmic upregulation of Cyto c and AIF serve as biomarkers of mechanical asphyxia death

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Abstract: The certification of death due to mechanical asphyxia has been a complex problem in some cases. The use of protein expression to identify mechanical asphyxia death has recently attracted attention. Asphyxia creates an extremely hypoxic environment for cells, which should reactivate the mitochondria in the cells. Cyto c and AIF, located in the mitochondria, are transferred to the cytoplasm under hypoxia to trigger the apoptotic process. Based this phenomenon, we designed the animal asphyxia model and cell hypoxia model to examine whether Cyto c and AIF are expressed in the cytoplasm, and we used human samples to verify the results. We found that the two proteins were detectably expressed in the cytoplasm of mechanical asphyxia groups and were hardly detected in the cytoplasm of other groups. This is a promising finding that may shed light on the precise mechanisms associated with mechanical asphyxia.

Keywords: Mechanical asphyxia death, mitochondria, Cyto c, AIF, cytoplasm

Introduction

Mechanical asphyxia death, a common type of violent death is determined at the crime scene using superficial characterizations of the body like hanging groove and throttling marks, and internal signs like Tardieau spots and pink teeth. Occasionally, a conclusion cannot be reached when the superficial signs do not form (muzzle covered by soft materials or body in enclosed space without ventilation) and the internal characterizations are not specific enough to diagnose this type of death.

Biomarkers of mechanical asphyxia death have been prospectively explored. Bogomolov DV et al. [1] used standard histological and immunohistochemical methods to detect the expression of CD-117 antigen, which was upregulated in lung tissue from mechanical strangulation asphyxia cases. Cecchi R et al. [2] indicated that lung SP-A was related to an intense hypoxic stimulus, and HIF1-α was expressed in small-, medium-, and large-caliber lung vessels of the vast majority of mechanical asphyxia deaths and CO intoxications.

Heart and brain tissues are more sensitive to oxygen deprivation because of their negligible glycogen storage [3, 4]. In our previous study, Zeng et al. [3, 4] selected 119 differentially expressed mRNAs, examining the expression levels of them in 44 human cardiac tissue specimens from individuals who died of mechanical asphyxia, craniocerebral injury, hemorrhagic shock, or other causes. They found that the expression of dual-specificity phosphatase 1 (DUSP1) and potassium voltage-gated channel subfamily J member 2 (KCNJ2) increased in human cardiac tissues from the mechanical asphyxia group compared to control tissues.

The mitochondria, vital cellular organelles, provide energy for the metabolic process via oxidative phosphorylation. Oxygen deficiency inhibits oxidative phosphorylation and triggers mitochondria-mediated apoptosis [6, 7] (Cytochrome C (Cyto C) and apoptosis inducing factor (AIF),

the propellants of apoptosis, are released from mitochondria to cell plasma [8, 9]). We suspected that the expression of Cyto c and AIF in neuron and cardiac cells altered more dynamically in asphyxia cases than in other cases, and these proteins could be transferred from mitochondria to cytoplasm in a period, which may shed a light on the identification of mechanical asphyxia in some ambiguous cases.

Here, we used a cell and animal hypoxia model to detect protein expression in the cytoplasm, and we used human samples to examine these mitochondria-related protein expression in the same way.

Materials and methods

Materials

Cell hypoxia model: We chose H9C2 cells as the hypoxia model. H9C2 was cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and incubated under hypoxic conditions (1% O_2 and 5% CO_2) for 0-72 h. The FBS in the medium was not removed because suffocation or hanging causes asphyxia rather than ischemia.

Animal model: Male SD rats of approximately 200 g were chosen for the experiment. All rats were anaesthetized using pentobarbital sodium (concentration 3 mg/ml, 2 ml). Six groups (n = 5) were designated. The control groups were: Decapitating, CO intoxication (rats were placed in a 3 L container for 20 min, where CO was continuously passaged at 30 ml/min), brain injury (according to Feeney's free-falling device [10]), hemorrhagic shock (a cut in the arteria carotis). The experimental groups were: Hanging and suffocating (placed in a sealed container). We collected the heart and brain samples at 0 h, 24 h, 48 h, and 72 h for the mitochondrial membrane potential (MMP) test. The samples gathered at 0 h were used in other experiments. All experiments met the qualifications instituted by the animal ethics committee of Fudan University.

Human samples

All human samples were obtained from the Bureau of Public Security of Shanghai (every tissue was from anatomic corpses according to the Declaration of Helsinki). Samples were grouped by cause of death: control group: brain injury (n = 10), hemorrhagic shock (n = 10), intoxication (n = 4: alcohol (1), cyanide (1), CO intoxication (2)); experimental group: mechanical asphyxia (n = 8: hanging (2), strangulation (2), suffocation (2), and drowning (2)). The postmortem interval of samples was in 24 h.

Mitochondria extraction

We used the mitochondria extraction kit (tissues) (Lot.C3606, Beyotime, China) to extract mitochondria from tissues according to the manufacturer's instructions. In brief, 80 mg of brain tissue was cut completely and washed in PBS three times in a 1.5-ml tube. Then, 640 μl A liquor and two beads were added to the tube. The tube was put into a lapping machine for 20 s. The tube was centrifuged at 600 × g for 5 min. The supernatant was transferred to a new tube. The tube was centrifuged at 11,000 × g for 10 min. The supernatant contained cytoplasm, and it was transferred to a new tube. The precipitate contained mitochondria. Heart tissue was treated slightly differently. After washing with PBS, the cut heart tissue was digested with 0.25% trypsin for 20 min on ice. The other steps were the same as the brain protocol. Mitochondria in H9C2 were extracted using the mitochondria extraction kit (cell) (Lot. C3601, Beyotime, China) according to the manufacturer's instructions. Briefly, the digested 2 × 10^7 cells were ground in a lapping machine for 20 s. The tube was centrifuged at 600 × g for 5 min. The supernatant was transferred to a new tube. The tube was centrifuged at 11,000 × g for 10 min. The supernatant contained cytoplasm, and it was transferred to a new tube. The precipitate contained mitochondria.

The function of mitochondria

In this assay, mitochondrial function including the mitochondrial membrane potential (MMP), ATP levels, and reactive oxygen species (ROS) levels were measured using the reagent kit according to the manufacturer's instructions. To measure MMP, we used the mitochondrial membrane potential assay kit with JC-1 (Lot.C-2006, Beyotime, China). To measure the ATP level, we used the Rh123 kit (Dnjindo, Japan). To measure the ROS level, we used the reactive oxygen species assay kit (Lot.S0033, Beyotime, China). H9C2 proliferation was measured using

5-ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Kit with Alexa Fluor 488 (Lot.0071S, Beyotime, China). We used a fluorescence microscope to analyze the fluorescence level in H9C2. The membrane potential results from tissue mitochondria were detected using a microplate reader.

Protein assay

We used 50 μl of radio immunoprecipitation assay (RIPA) lysis buffer to treat the precipitate and the bicinchoninic acid assay (BCA) (Lot. P0010, Beyotime, China) to quantify the concentration of the protein in mitochondria and cytoplasm. The concentration of each sample was diluted to 2 μg/ul by adding 5 × loading buffer and RIPA lysis buffer. Samples were boiled at 100°C for 10 min.

Western blotting

Acrylamide gel electrophoresis (15%) was used to analyze all protein samples (electrophoresis: 80 V for 20 min/120 V for 2 h; transmembrane: 300 mA for 2 h). Polyvinylidene fluoride (PVDF) membranes (Merck, Germany) were incubated in 5% bovine serum albumin (BSA) containing phosphate buffer tween (PBST) for 1 h. The PVDF membranes were incubated with the primary antibody, cytochrome c oxidase subunit (COX4)/β-actin/cytochrome c (Cyto C)/apoptosis induce factor (AIF)/glyceraldehyde phosphate dehydrogenase (GAPDH) (concentration: 1:1000, from rabbit, except the β-actin from mouse) (Lot.bs-1533R/bs-0016M/bs-0013R/bs-0037R/bs-00755R, Bioss, China) overnight at 4°C. The PVDF membranes were washed in PBST 3 times for 45 min. The PVDF membranes were incubated in secondary antibody with horseradish peroxidase (HRP) (goat anti rabbit/mouse) (concentration: 1:1000) (Servicebio, China). The membranes were washed in PBST 3 times for 45 min. The membranes were covered with luminol-electrogenerated chemiluminescence (ECL) (Tanon™ High-sig ECL Western Blotting Substrate, Tanon, China) and imaged using a Tanon 4600S (Tanon, China).

Cell immunofluorescence

Detection of mitochondrial fusion and fission related proteins: The H9C2 were washed in PBST 3 times in 24 well plates. The cells were incubated in 4% paraformaldehyde for 15 min and washed 3 times with PBST. The H9C2 were permeated with 1% Triton for 20 min and incubated with 5% donkey serum diluted in PBST for 1 h. H9C2 was incubated with the primary antibodies against mitofusin 2 (Mfn2) and dynamin-related protein 1 (Drp1) (1:200) (Abcam, UK) overnight at 4°C and washed 3 times in PBST. The cells were covered by the secondary antibody tagged with red fluorescence (1:200) (Yeason, China) for 1 h. The cells were washed in PBST. We used the fluorescence microscope to observe H9C2 and record the images.

Detection of apoptosis related proteins: H9C2 was plated in 24 well plate. We designed two groups for this assay: hypoxia and hypoxia with N-Acetyl-L-cysteine (NAC) (concentration: 5 mM) to detect the effect of hypoxia on cell apoptosis progress and the NAC can or not defy the effect. The cells were incubated in 4% paraformaldehyde for 15 min and washed 3 times with PBST. The H9C2 were permeated with 1% Triton for 20 min and incubated with 5% donkey serum diluted in PBST for 1 h. H9C2 was incubated with the primary antibodies against B-cell lymfoma-2 (bcl-2) (Beyotime, China) and cleaved caspase-3 (CST, USA) overnight at 4°C and washed 3 times in PBST. The cells were covered by the secondary antibody tagged with red fluorescence (1:200) (Yeason, China) for 1 h. The cells were washed in PBST. We used the fluorescence microscope to observe H9C2 and record the images.

Statistical analysis

All images were analyzed using the software ImageJ (2006 version). The data were analyzed using the software Graphpad Prism 7.0 (*t*-test/one-way ANOVA). $P < 0.05$ was considered statistically significant.

Results

The identification of mitochondria

The mitochondria extracted from human tissue, animal tissue and cell were identified using the mitochondrial marker Cox4. The cytoplasm was identified using the cytoskeletal protein β-actin. The results showed that the precipitate contained mitochondria without cytoplasm components, and the cytoplasm was not contaminated (**Figures 1**, S1).

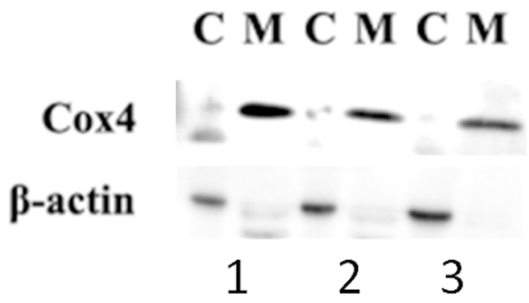


Figure 1. The identification of mitochondria. The cox4 expressed in "M" (mitochondria) lane and was not detected in "C" (cytoplasm) lane. The β-actin showed in "C" (cytoplasm) lane, not detected in "M" lane. This figure showed the mitochondria was extracted successfully. M: mitochondria; C: cytoplasm; 1: animal samples; 2: cell samples; 3: human samples.

MMP levels in the animal model

MMP changes in cardiac tissue: At 0 h, the MMP levels in the experimental groups were significantly lower than the decapitated group ($P < 0.05$). At 24 h, the MMP level in the hanging group was the highest, and it was higher in the suffocation group than in other groups (except for the brain injury group) ($P < 0.01$). At 48 h, the MMP level was the highest in the experimental groups ($P < 0.05$). At 72 h, the MMP level in the experimental groups was higher than that in the CO intoxication group. No manifest difference was observed in other groups ($P < 0.01$). The MMP trend in all groups is shown in **Figure 2A-G**. The MMP levels in all groups declined dramatically during the first 24 h. Subsequently, the MMP level in the hanging and suffocation groups increased sharply and decreased suddenly. Meanwhile, the MMP level in other groups remained slightly increased between 24 h and 72 h.

MMP changes in brain tissue

At 0 h, the MMP level in the hanging group was lower than in the decapitated group, and it was also higher than those in other groups ($P < 0.01$). The MMP level was the highest in the suffocation group ($P < 0.05$). At 24 h, the MMP level in the experimental group was the highest ($P < 0.05$). At 48 h, the MMP level in the hanging group was lower than the brain injury group, and it was higher than in that in the decapitated and CO intoxication groups ($P < 0.01$). The MMP level in the suffocation group was lower than that in the brain injury group, but it was higher than that in other groups ($P < 0.05$). At 72 h, the MMP level in the hanging group was lower than that in the hemorrhagic shock group, and it was higher than that in the CO intoxication group ($P < 0.01$). The MMP level was the highest in the suffocation group ($P < 0.01$). The MMP levels in the hemorrhagic shock, brain injury, hanging and suffocation groups increased sharply, and they decreased slightly in other groups during the first 24 h. The MMP level in all groups (except for the CO intoxication group) sharply declined between 24 to 48 h. The MMP level in all groups (except for the brain injury group) was up-regulated between 48 to 72 h. The MMP change was represented in **Figure 2H-N**.

MMP level in human samples

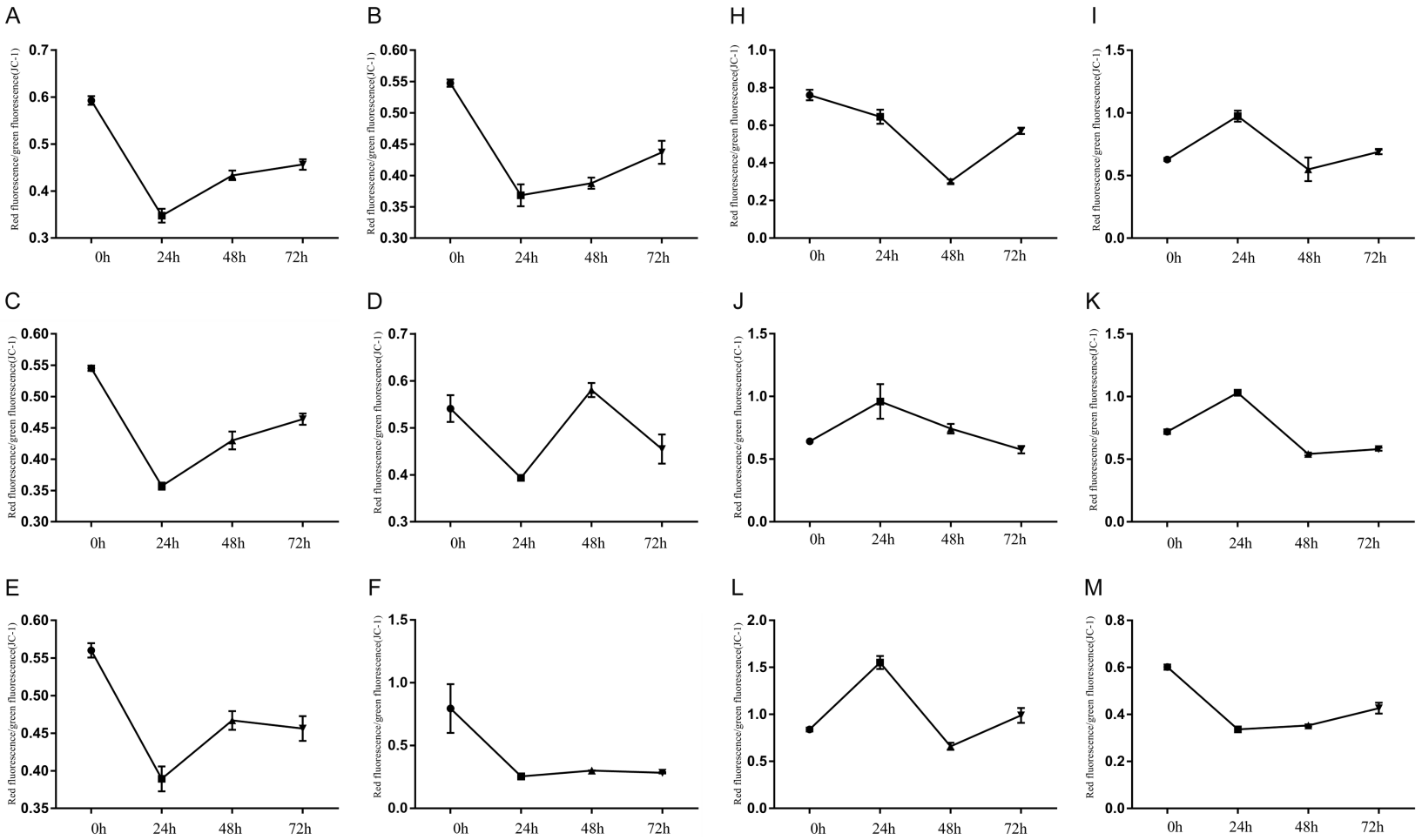
In heart tissues, the MMP level was higher in the mechanical asphyxia group than that in the hemorrhagic shock and intoxication groups ($P < 0.05$). In brain tissues, the MMP level in the mechanical asphyxia group was slightly higher than that in other groups; however, the difference was not significant ($P > 0.05$) (**Figure 2O**, **2P**).

MMP and ATP levels in H9C2

The MMP level decreased at 12 h and increased at 24 h. The MMP level at 0 h and 24 h was higher than the other time points. The ATP level was highest at 12 h during the hypoxia period ($P < 0.01$). The ATP level decreased dramatically afterward (**Figure 3**).

Expression of mitochondria-related proteins in the animal model

Asphyxia promoted Cyto c and AIF expression in the cytoplasm and mitochondria of the myocardium: AIF expression in the cytoplasm of the hanging group was higher than that in the hemorrhagic shock and brain injury groups ($P < 0.01$). AIF expression in the cytoplasm of the suffocation group was higher than that in the hemorrhagic shock group ($P < 0.05$). AIF expression in the mitochondria of the hanging group was higher than that in the brain injury group; however, it was lower than that in the CO intoxication group ($P < 0.05$). AIF expression was higher in the suffocation group than that in the hemorrhagic shock and brain injury groups ($P < 0.05$) (**Figures 4A-E**, S2A-C).


A
B
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Red fluorescence/green fluorescence(JC-1)
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24h
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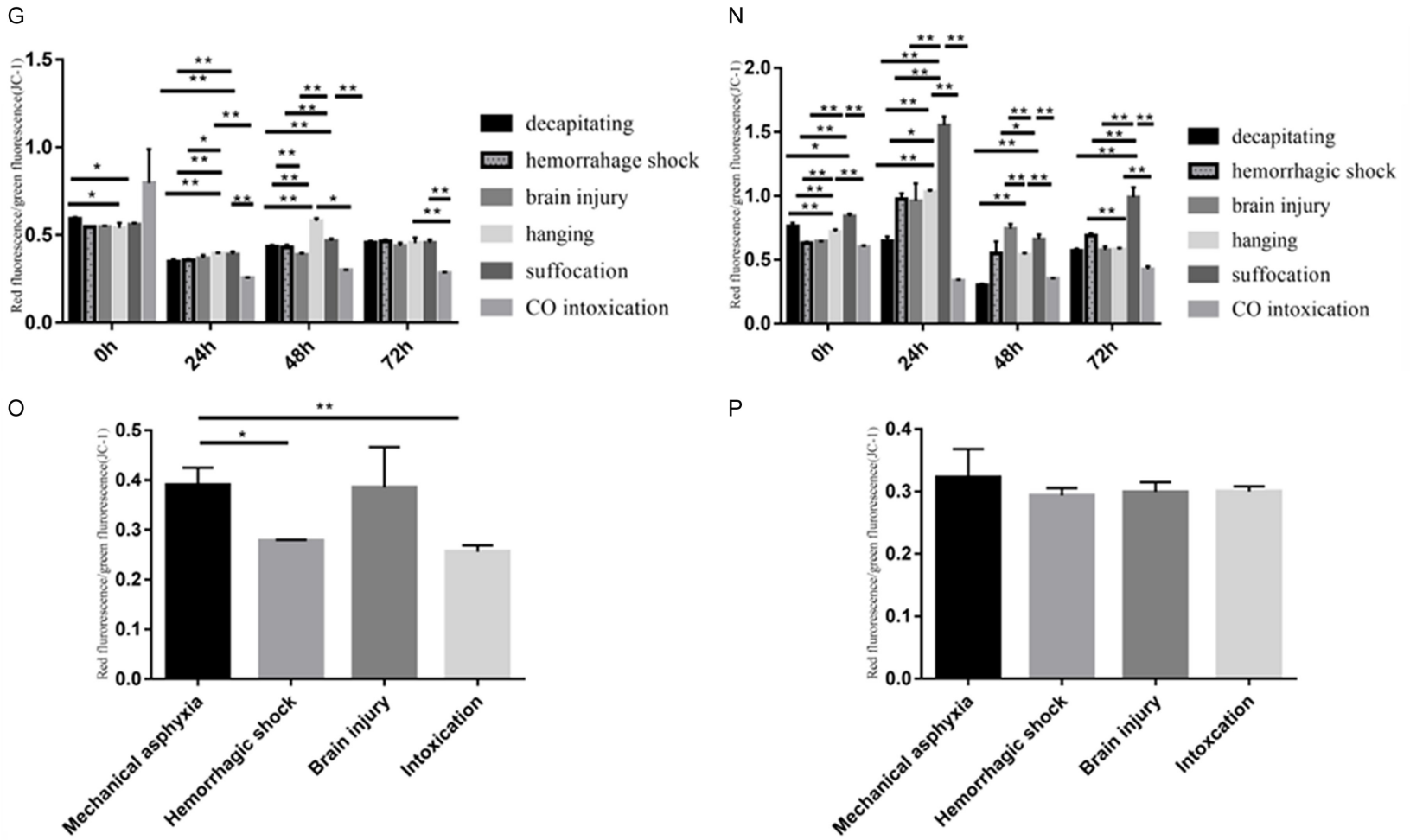


Figure 2. The MMP of myocardium in rat (A-G)/human samples (O) and of brain in rat (H-N)/human samples (P) in all groups. (A) The change of MMP in decapitating group; (B) The change of MMP in hemorrhagic shock group; (C) The change of MMP in brain injury group; (D) The change of MMP in hanging group; (E) The change of MMP in suffocation group; (F) The change of MMP in CO intoxication group. All figures showed the level of MMP in all group declined dramatically during the first 24 h and inclined gently subsequently; (G) The MMP of mycardium in all groups at 0 h, 24 h, 48 h and 72 h. At 0 h, the level of MMP in hanging and suffocation groups was lower than in decapitating group ($P < 0.05$). At 24 h and 48 h, the level of MMP in hanging and suffocation groups was higher than in other groups ($P < 0.05$). At 72 h, the level of MMP in hanging and suffocation groups was higher than in CO intoxication group ($P < 0.01$). (H) The change of MMP in decapitating group; (I) The change of MMP in hemorrhagic shock group; (J) The change of MMP in brain injury group; (K) The change of MMP in hanging group; (L) The change of MMP in suffocation group; (M) The change of MMP in CO intoxication group. (N) All the figures showed that the level of MMP in all groups (except in CO intoxication group) was inclined during first 24 h and declined subsequently. (G) The MMP of mycardium in all groups at 0 h, 24 h, 48 h and 72 h. At 0 h, the level of MMP

in hanging group was lower than in decapitating group but higher than in other groups ($P < 0.01$); the level of MMP in suffocation group was higher than in other groups ($P < 0.05$). (O) The level of MMP in heart tissue (human samples). The level of MMP in Mechanical asphyxia group was higher than in Hemorrhagic shock and Intoxication ($P < 0.05$). (P) The level of MMP in brain tissue (human samples). The level of MMP in all groups showed no significant difference. Data are expressed as $x \pm s$, $n = 5$, $^*P < 0.05$, $^{**}P < 0.01$.

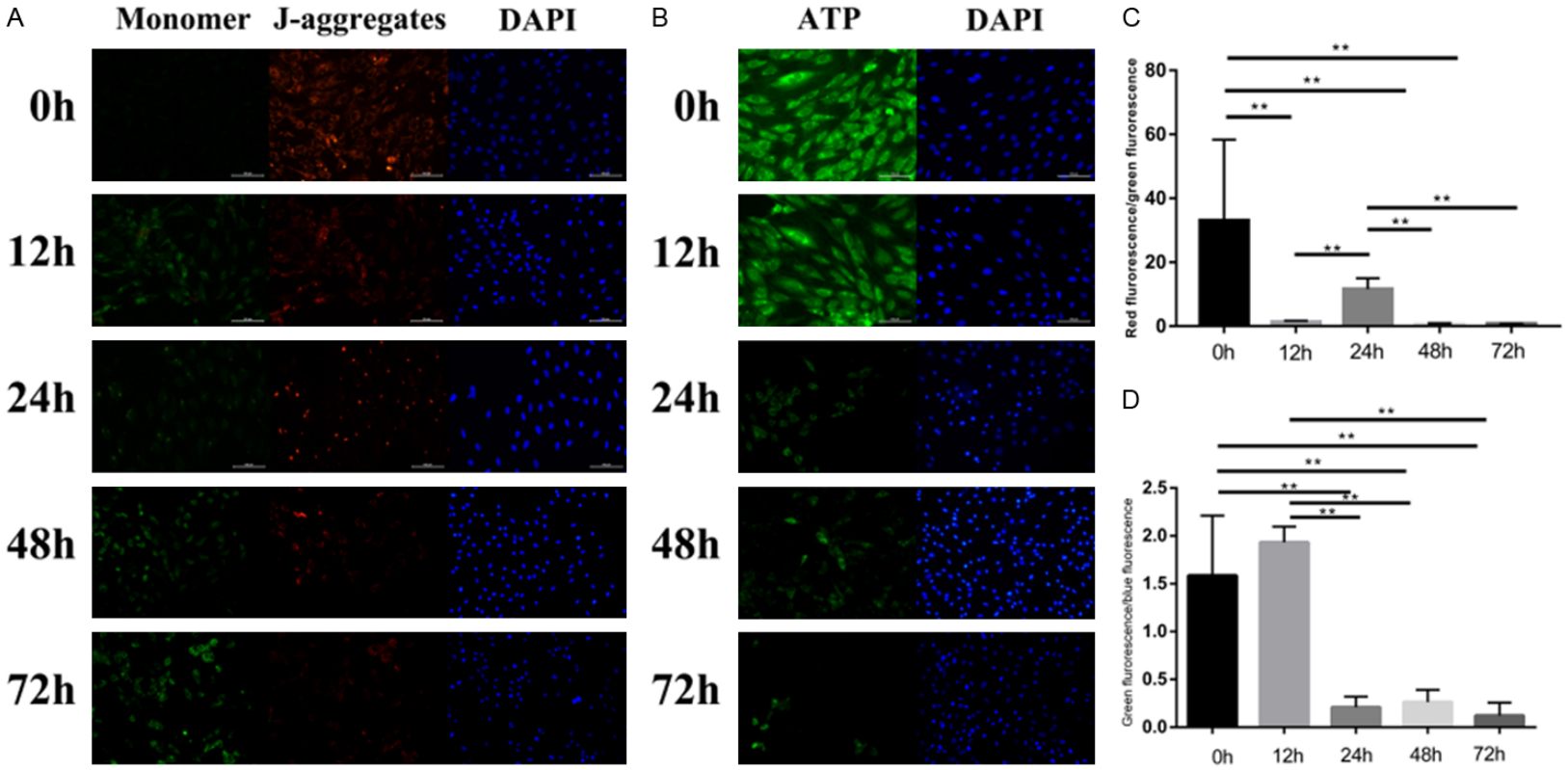


Figure 3. The level of MMP and ATP of mitochondria in H9C2. A, C. The level of MMP in H9C2 at 0 h, 12 h, 24 h, 48 h and 72 h. The level of MMP in H9C2 at 0 h was highest ($P < 0.01$); the level of MMP in H9C2 at 24 h was higher than at 12 h, 48 h and 72 h ($P < 0.01$). B, D. The amount of ATP in H9C2 at 0 h, 12 h, 24 h, 48 h and 72 h. The level of MMP in H9C2 at 0 h and 12 h was higher than at 24 h, 48 h and 72 h ($P < 0.01$). Data are expressed as $x \pm s$, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$.

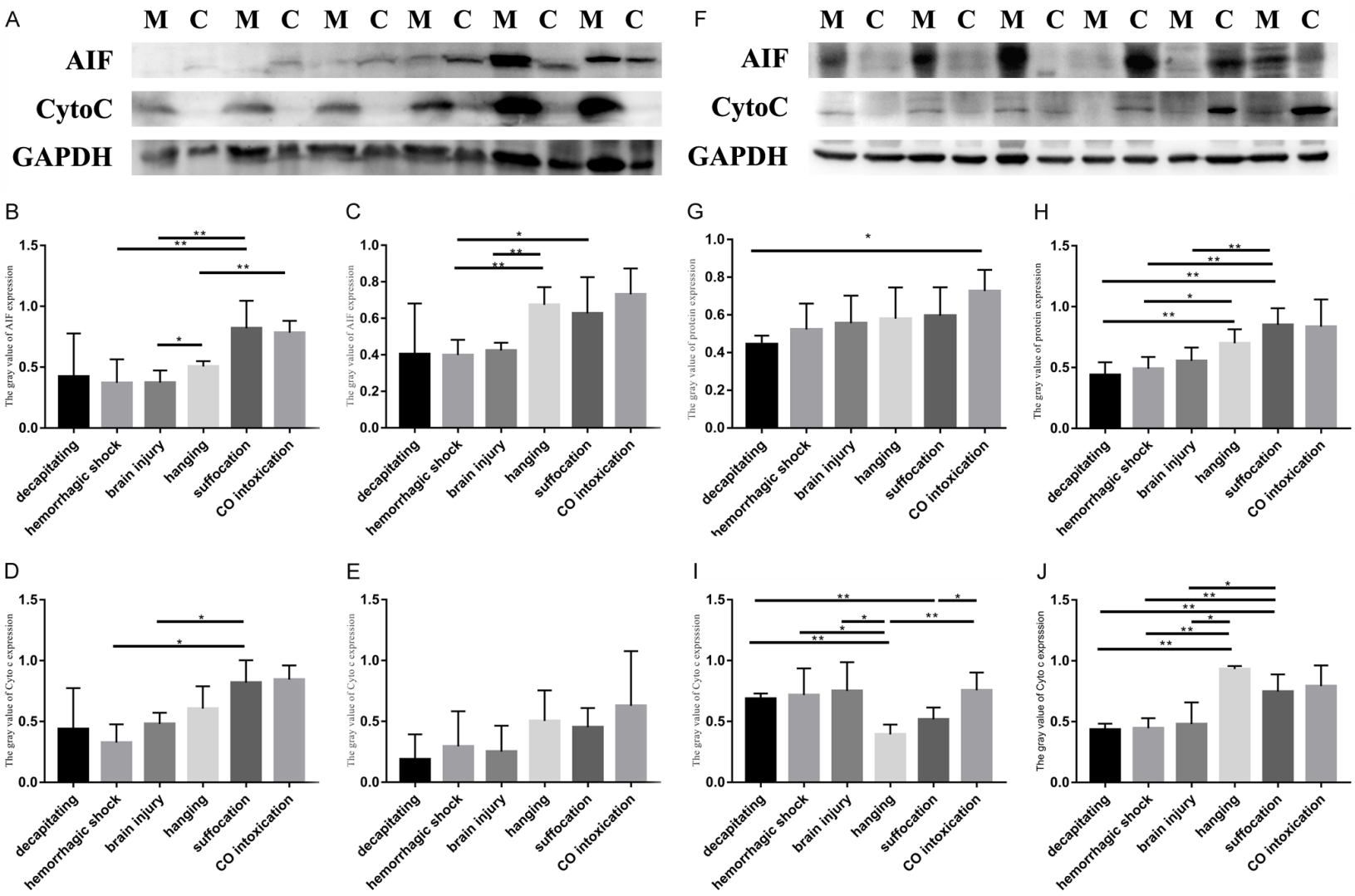


Figure 4. The expression of Cyto c and AIF in myocardium (A-E)/brain (F-J) (rat). (A) The expression of Cyto c and AIF in mitochondria and cytoplasm (myocardium); (B) The expression of AIF in mitochondria. The AIF expression of mitochondria in hanging group was higher than in brain injury group but lower than in CO intoxication group; (C) The expression of AIF in cytoplasm. The AIF expression of cytoplasm in hanging group was higher than in hemorrhagic shock and brain injury groups ($P < 0.01$); The AIF expression of cytoplasm in suffocation group was higher than in hemorrhagic group ($P < 0.05$). (D) The expression of Cyto c in mitochondria. The Cyto c expression of mitochondria in suffocation group was higher than in hemorrhagic and brain injury groups ($P < 0.05$). (E) The expression of Cyto c in cytoplasm. The Cyto c expression of all groups showed no difference. (F) The expression of AIF and Cyto c in mitochondria and cytoplasm (brain). (G) The expression of AIF in mitochondria. The AIF expression of mitochondria in CO intoxication group was higher than in decapitating group ($P < 0.05$). (H) The expression of AIF in cytoplasm.

The AIF expression of cytoplasm in hanging group was higher than in decapitating and hemorrhagic shock groups; the AIF expression of cytoplasm in suffocation group was higher than in decapitating, hemorrhagic shock and brain injury groups ($P < 0.05$). (I) The expression of Cyto c in mitochondria. The Cyto c expression of mitochondria in hanging group was lowest ($P < 0.01$); the Cyto c expression of cytoplasm in suffocation group was lower than in decapitating and CO intoxication group. (J) The expression of Cyto c in cytoplasm. The Cyto c expression of cytoplasm in hanging and suffocation groups was higher than decapitating, hemorrhagic shock and brain injury groups ($P < 0.05$). 1: decapitating; 2: hemorrhagic shock; 3: brain injury; 4: hanging; 5: suffocation; 6: CO intoxication. Data are expressed as $x \pm s$, $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Asphyxia promoted Cyto c and AIF expression in cytoplasm in the brain: AIF expression in the cytoplasm of the hanging group was higher than that in the decapitated and hemorrhagic shock groups ($P < 0.05$). AIF expression in the cytoplasm of the suffocation group was higher than that in the decapitated, hemorrhagic shock, and brain injury groups ($P < 0.01$). AIF expression showed no significant difference between the experimental and control groups. Cyto c expression in the cytoplasm of the experimental groups was higher than that in other groups (except for CO intoxication group) ($P < 0.05$). Cyto c expression in the mitochondria of the experimental groups was lower than in other groups ($P < 0.01$) (**Figures 4F-J**, S2D-F).

Expression of mitochondria-related proteins in human samples

We compared the expression of proteins in the cytoplasm with expression in mitochondria to normalize the gray value on different PVDF membranes.

In heart tissue, the ratio of Cyto c in the cytoplasm to Cyto c in the mitochondria was highest in the mechanical asphyxia group ($P < 0.01$). No trace of cytoplasmic expression was detected in the hemorrhagic shock, intoxication and drowning groups. The ratio of AIF in the cytoplasm to Cyto c in the mitochondria of the intoxication group was higher than that in the mechanical asphyxia and brain injury groups ($P < 0.05$) (**Figures 5A-F**, S3A-L).

In brain tissue, the Cyto c was expressed in the cytoplasm in the mechanical asphyxia, intoxication, and drowning groups. However, no obvious difference was found among the three groups. No trace of cytoplasmic expression was observed in the hemorrhagic shock or brain injury groups. AIF was significantly expressed in the cytoplasm of the mechanical asphyxia, intoxication, and drowning groups; however, no significant difference was observed in the three groups. No trace of cytoplasmic AIF expression was detected in the other groups (**Figures 5G-L**, S4A-L).

Hypoxia changed the expression of Cyto c and AIF in mitochondria and cytoplasm

Cyto c expression in mitochondria was increased during hypoxia treatment. Cyto c expression in mitochondria was highest at 48 h ($P < 0.05$). Cyto c expression was observed in cytoplasm at 24 h. AIF expression in mitochondria was up-regulated during hypoxia treatment. AIF expression was the highest at 48 h. No obvious AIF expression was found in the cytoplasm of H9C2 between 0 and 72 h of hypoxia treatment (**Figures 6**, S5A-C).

Hypoxia changed the expression of bcl-2 and cleaved caspase-3

The expression of bcl-2 in hypoxia group increased gently from 0-24 h and sharply decreased at 48 h. In hypoxia group, the bcl-2 expression at 24 h was slightly higher than at 0 h and 48 h ($P < 0.05$); no significant difference was observed between the bcl-2 expression at 12 h and 24 h. The expression of bcl-2 in NAC (5 mM) group showed similar trend, and the level of expression was higher than in hypoxia group at 12 h, 24 h and 48 h ($P < 0.05$); the expression of bcl-2 at 24 h was significant higher than at other time points ($P < 0.05$) (**Figure 7A-C**). In hypoxia group, the expression cleaved caspase-3 at 24 h was obvious higher than the cleaved caspase-3 expression at 0 h and 12 h ($P < 0.05$); the cleaved caspase-3 expression was slightly decreased at 48 h. However, no significant difference was observed between cleaved caspase-3 expression at 24 h and at 48 h. The expression of cleaved caspase-3 in NAC group showed similar trend, and the level of expression was lower than in hypoxia group at each time point ($P < 0.05$) (**Figure 7D-F**).

The function of mitochondria in H9C2

The ROS level was increased by hypoxia. The ROS level was the highest at 48 h ($P < 0.01$). The EDU level peak increased at 24 h ($P <$

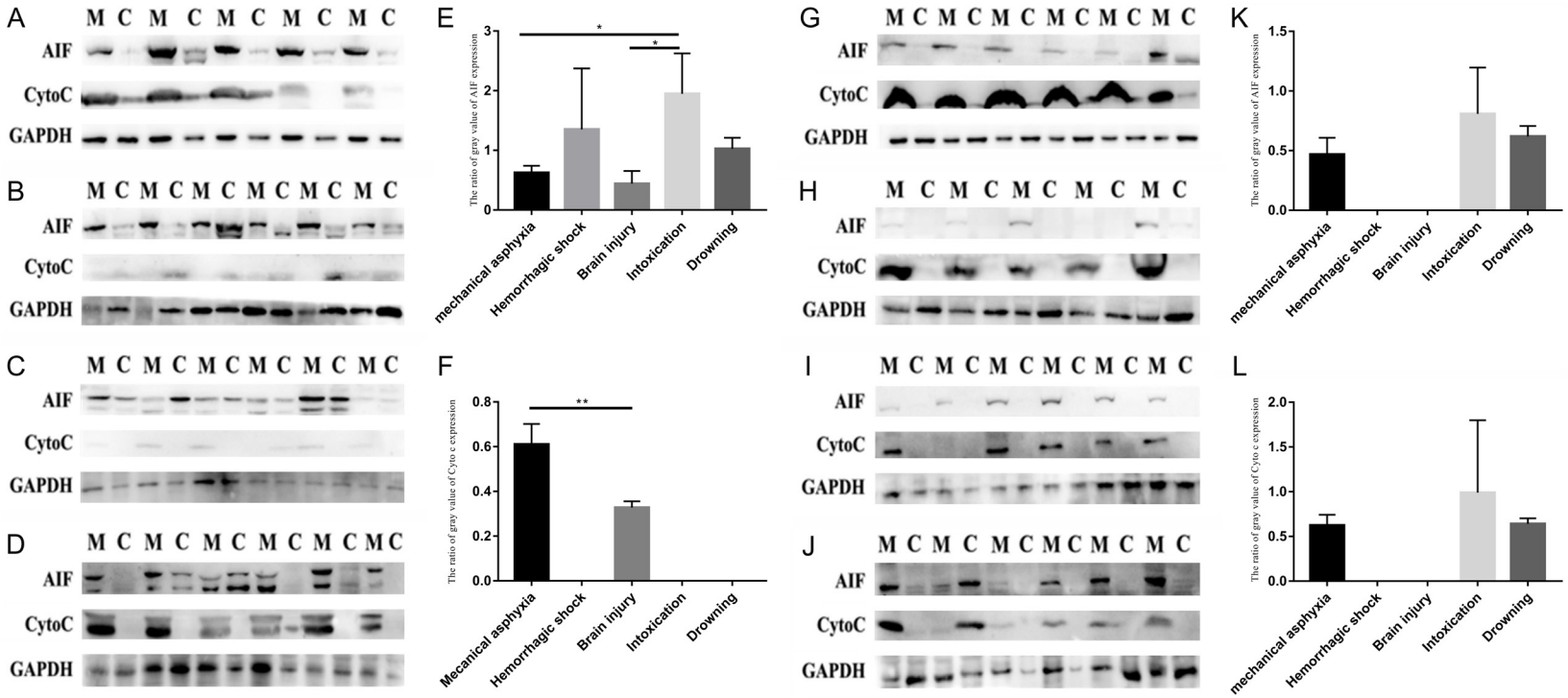


Figure 5. The expression of Cyto c and AIF in human tissues in all groups (heart: A-F; brain: G-L). (A) The expression of Cyto c and AIF in mitochondria and cytoplasm in mechanical asphyxia group; (B) The expression of Cyto c and AIF in mitochondria and cytoplasm in brian injury group; (C) The of Cyto c and AIF in mitochondria and cytoplasm in hemorrhagic shock group; (D) The expression of Cyto c and AIF in mitochondria and cytoplasm in alchol intoxication group, cyanide intoxication group, drowning group (n = 2), CO intoxication group (n = 2); (E) The ratio of AIF in cytoplasm/ AIF in mitochondria. The ratio in intoxication group was higher than in mechanical asphyxia and in brain injury group. (F) The ratio of Cyto c in cytoplasm/Cyto c in mitochondria; (G) The expression of Cyto c and AIF in mitochondria and cytoplasm in mechanical asphyxia group; (H) The expression of Cyto c and AIF in mitochondria and cytoplasm in brian injury group; (I) The expression of Cyto c and AIF in mitochondria and cytoplasm in hemorrhagic shock group; (J) The expression of Cyto c and AIF in mitochondria and cytoplasm in alcohol intoxication group, cyanide intoxication group, drowning group (n = 2), CO intoxication group (n = 2); (K) The ratio of AIF in cytopasm/AIF in mitochondion. The ratio in mechanical asphyxia group was higher than in hemorrhagic shock and brain injury groups ($P < 0.05$). (L) The ratio of Cyto c in cytoplasm/Cyto c in mitochondria. The ratio in mechanical asphyxia group was higher than in hemorrhagic shock and brain injury groups ($P < 0.05$). Data are expressed as $x \pm s$, n = 3, *$P < 0.05$, **$P < 0.01$.

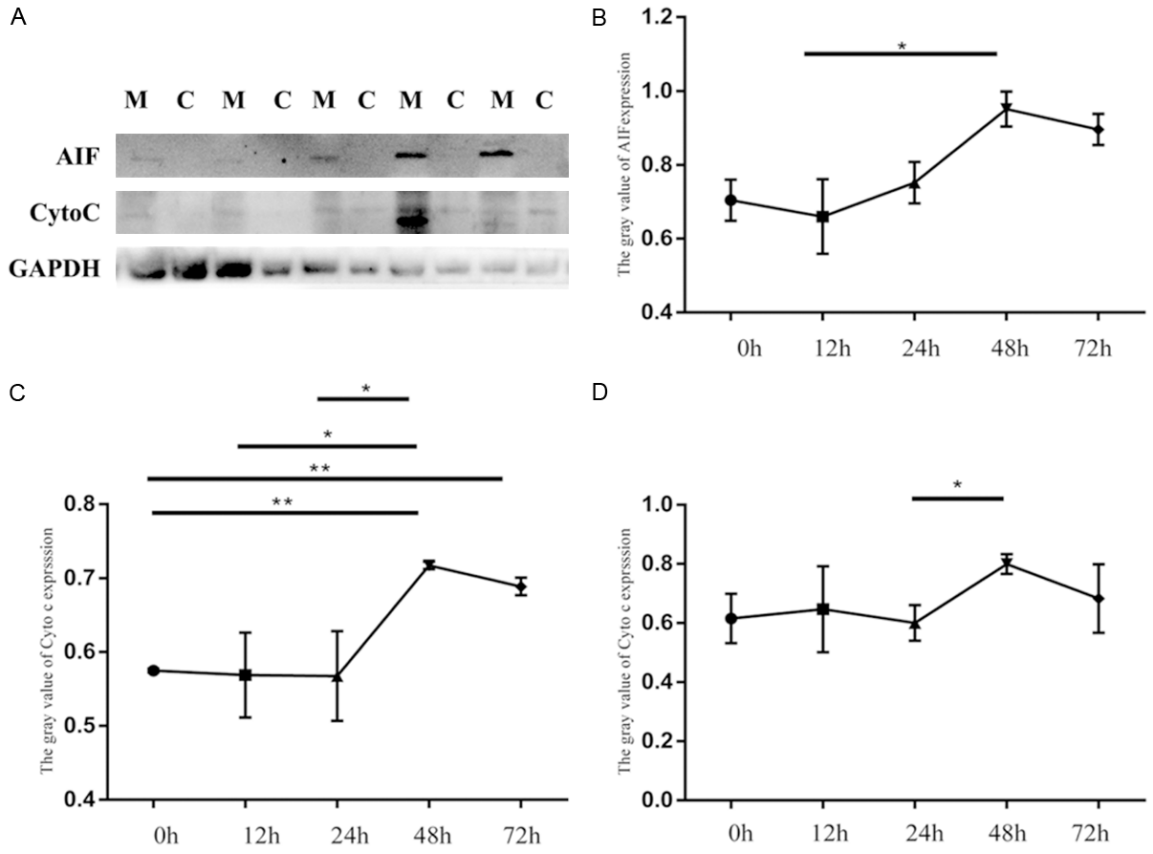


Figure 6. The expression of Cyto c and AIF in H9C2 (cell line). A. The expression of Cyto c and AIF in mitochondria and cytoplasm at 0 h, 12 h, 24 h, 48 h and 72 h. The Cyto c in cytoplasm was observed at 24 h. The AIF in Cytoplasm was not observed at all time points. B, C. The expression of AIF in mitochondria. The AIF expression in mitochondria was increased with during 72 h. C. The expression of Cyto c in mitochondria. The Cyto c expression in mitochondria was increased at 48 h. D. The expression of Cyto c in cytoplasm. The Cyto c expression in cytoplasm was increased at 48 h. Data are expressed as x ± s, n = 3, *P < 0.05, **P < 0.01.

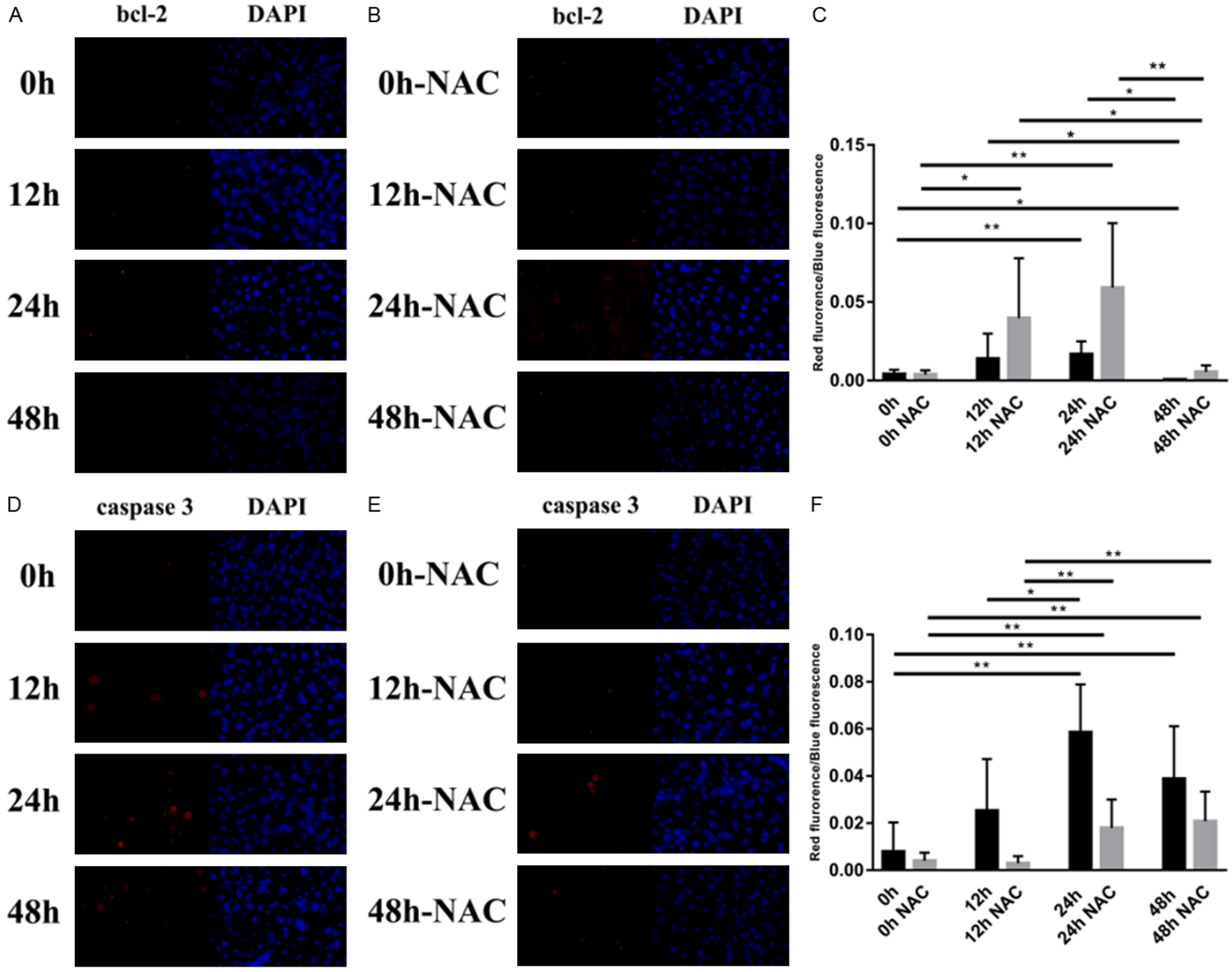
0.01). Hypoxia inhibited Mfn2 expression and induced DRP1 expression. Mfn2 expression was the highest at 0 h (P < 0.01), and the Drp1 expression peaked at 12 h (P < 0.01) (**Figure 8**).

Discussion

The diagnosis of mechanical asphyxia depends on crime scene investigation, superficial and internal signs on the body. However, superficial traces and internal signs are not crystal enough to identify the cause of death in some ambiguous cases. For instance, visible traces might not be left on the body if the corpse was suffocated by soft materials or if the body was in an oxygen-deprived space.

Some studies conveyed that protein expression may help diagnose mechanical asphyxia death, hypoxia can induce or inhibit the expression of some proteins in tissues and cells. Hypoxia-inducible factor 1 (HIF-1) is the most common protein in this field [11, 12]. Recently, mitochondria-related proteins attracted tremendous attention for many researchers in this subject [13-15]. Mitochondria are an vital cellular organelles, and the electron respiratory chain obtains oxygen and produces energy stored as ATP. Hypoxia leads to mitochondrial dysfunction, including changes in MMP, ROS, ATP, and the expression of proteins related to apoptosis. The neuron and myocadiac cells are the most sensitive to oxygen deprivation [16, 17]. Here, we chose brain and heart as target tissues.

All tissues and cells go through hypoxia conditions after regardless of the cause of death. Live cells gradually consume the oxygen diluted in the blood. Few studies focused on this subject. The cells in a body that died from extreme hypoxia will survive for a while with lower con-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bcl-2
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48h-NAC
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E
caspase 3
DAPI
0h-NAC
12h-NAC
24h-NAC
48h-NAC
F
Red flurorence/Blue fluorescence
0.10
0.08
0.06
0.04
0.02
0.00
0h
0h NAC
12h
12h NAC
24h
24h NAC
48h
48h NAC

Figure 7. The expression of bcl-2 and cleaved caspase-3 in H9C2. A. The expression of bcl-2 in hypoxia group. B. The bcl-2 expression in NAC group. C. In hypoxia group, the bcl-2 expression at 24 h was higher than at 0 h and 48 h ($P < 0.05$) and showed no significant difference with at 12 h; the expression of bcl-2 at 48 h was the lowest. In NAC group, the bcl-2 expression at 24 h was highest ($P < 0.01$); the bcl-2 expression at 12 h was higher than at 0 h and 48 h ($P < 0.05$). The expression of bcl-2 in NAC group was higher than in hypoxia group at 12 h, 24 h and 48 h. D. The cleaved caspase-3 expression in hypoxia group. E. The cleaved caspase-3 expression in NAC group. F. In hypoxia group, the cleaved caspase-3 at 24 h was higher than 0 h and 12 h ($P < 0.05$); the cleaved caspase-3 expression at 48 h was higher than at 0 h ($P < 0.01$) and showed no significant difference with expression at 12 h. In NAC group, the cleaved caspase-3 expression at 48 h was higher than at 0 h and 12 h, and showed no significant difference with expression at 24 h. Data are expressed as x ± s, n = 3, *$P < 0.05$, **$P < 0.01$.

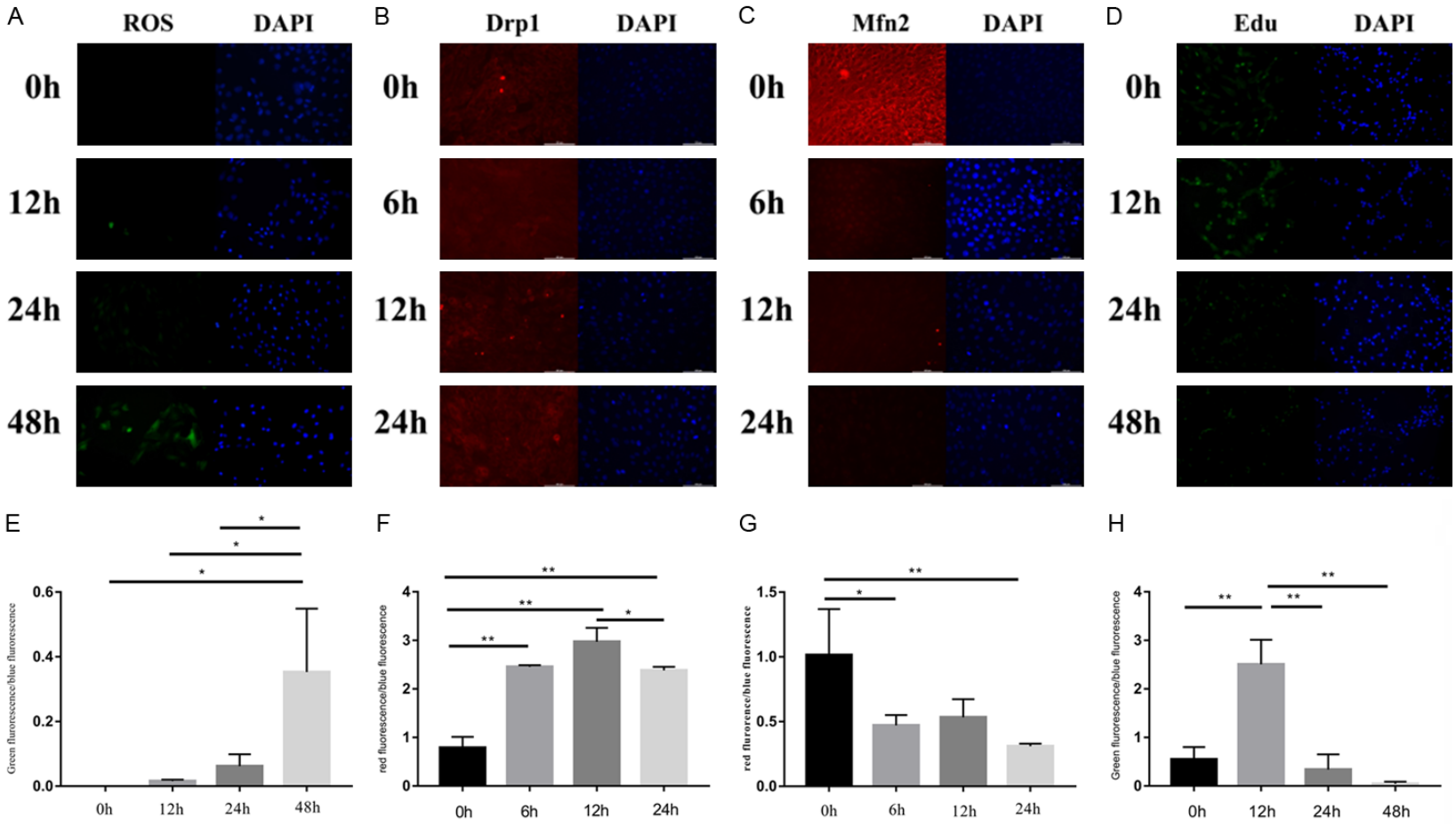

Figure 8. The function of mitochondria in H9C2. A, E. The level of ROS in H9C2 at 0 h, 24 h, 48 h and 72 h. B, F. The expression of DRP1 in H9C2 at 0 h, 6 h, 12 h, and 24 h. The Drp1 expression at 12 h was the highest ($P < 0.05$). C, G. The expression of MFN2 in H9C2 0 h, 6 h, 12 h, and 24 h. The Mfn2 expression at 0 h was highest ($P < 0.05$). D, H. The level of EDU in H9C2 at 0 h, 24 h, 48 h and 72 h. The level of EDU at 24 h was highest ($P < 0.01$). Data are expressed as x ± s, n = 3, *$P < 0.05$, **$P < 0.01$.

centrations of oxygen. We suspected that the lower oxygen concentration could influence the function of mitochondria in alive cells in a different way.

MMP level change when oxygen is scarce. Maintaining MMP level is crucial for protecting mitochondrial function. In our results, the MMP level decreased in most groups before increasing in rat myocardium within 72 h after death. In brain tissue, the MMP level increased before decreasing within 72 h after death.

In rat tissues, the MMP level in experimental groups was higher than in other groups. The results from cell and human sample experiments confirmed this phenomenon. The MMP level in H9C2 was highest at 24 h during the hypoxia treatment. The MMP level in the mechanical asphyxia group of human cardiac tissues was higher than in the other groups. The results were opposite to the conclusion published, that is hypoxia reduced MMP level in tissues and cells [18, 19].

On the contrary, Kelli I Korski et [15] found that the level of MMP in the cardiac progenitor cells clearly increased in hypoxia; however, the mechanism was unknown. It has been reported that MMP levels in brain tissue were maintained in a long-term hypoxia model [20], because NO production induced by hypoxia can protect MMP levels in brain tissue. The amount of ATP, a marker evaluating the function of mitochondria, was examined. The ATP levels were highest at 12 h and sharply decreasing at 24 h. However, the MMP level decreased at 12 h and increased slightly at 24 h. These results indicate that MMP and ATP may not change at same time. The cells may maintain the MMP level by consuming ATP. These hypotheses may explain why the MMP level can be maintained at a high level.

The cytoplasmic expression of Cyto c and AIF were increased in the experimental groups. Cyto c and AIF are the major triggers of apoptosis in cells [21, 22]. Their translocation (Cyto c and AIF from mitochondria to cytoplasm) is a key step of apoptosis initiation [23]. In animal experiments, cytoplasmic Cyto c and AIF expression of brain tissue significantly increased in experimental groups, but it was hardly detectible in control group cytoplasm.

We discovered similar conditions in human samples. Myocardial and brain Cyto c and AIF expression was up-regulated in the cytoplasm of mechanical asphyxia cases and drowning cases. The expression of the two proteins was significantly down-regulated or not detected in the cytoplasm of other groups. In brain tissue, no trace of Cyto c or AIF was observed in the cytoplasm of drowning cases. In the cyanide intoxication case, Cyto c and AIF were detected in the cytoplasm.

We also detected Cyto c and AIF expression in H9C2. We found that Cyto c was detected in the cytoplasm at 24 h, 48 h and 72 h, and the AIF expression was not detected in the cytoplasm. The results indicated that AIF translocated after Cyto c translocation. Moreover, the ROS level gradually increased from 0 to 72 h. Low concentration ROS could stimulate cell proliferation [23], which can be reflected by EdU levels. The EdU level peaked at 24 h. Bcl-2, located in outer membrane of mitochondrion, is anti-apoptotic protein which stabilize permeability of membrane of mitochondria to keep Cyto c and AIF unreleased [24]. Our results showed that bcl-2 was increased from 0 to 24 h preventing Cyto c from translocation. The results implied that cell could defy the apoptosis (induced by hypoxia) by increasing bcl-2 expression. However, the self-rescue can not last for 48 h. The expression of bcl-2 was decreased sharply at 48 h meanwhile the Cyto c was detected in cytoplasm at 24 h to trigger the apoptosis [25]. The pro-apoptotic protein, cleaved caspase-3 was activated by Cyto c in cytoplasm, whose expression reach the peak at 24 h. NAC, as an antioxidant, delayed the apoptosis by increasing bcl-2 expression and decreasing the expression of cleaved caspase-3. Hypoxia may also lead to changes in mitochondrial dynamics. Mfn2 is a key mitochondrial fusion protein, and Drp1 is the key mitochondrial fission protein [23, 26]. The expression of Mfn2 slightly decreased during hypoxia, and Drp1 expression peaked at 12 h. These results show that hypoxia induced mitochondria fission and disturbed the balance between fusion and fission [27].

Conclusion

Here, we had some interesting findings. First, functioning mitochondria were detectable for a

while after death (at least 24 h). The results implied that the mitochondria keep the level of MMP by consuming ATP. Second, Cyto c and AIF could be biomarkers for the mechanical asphyxia. They were expressed manifestly in cytoplasm in mechanical asphyxia group, which can be used for identification of mechanicanl asphyxia death when the superficial signs do not form. Third, the cells located in the brain of corpse survive for a while. The apoptotic progress was not triggered immediately after death. Our study still had limitations. The human samples must be extensively examined, and the change in mitochondrial function after death should be comprehensively verified to elucidate the mechanism behind the phenomenon.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

DUSP1, dual-specificity phosphatase 1; KCNJ2, potassium voltage-gated channel subfamily J member 2; Cyto C, cytochrome c; AIF, apoptosis induce factor; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; RIPA, radio immunoprecipitation assay; BCA, bicinchoninic acid assay; PVDF, Polyvinylidene fluoride; BSA, bovine serum albumin; PBST, phosphate buffer tween; COX4, cytochrome c oxidase subunit; GAPDH, glyceraldehyde phosphate dehydrogenase; HRP, horseradish peroxidase; ECL, luminol-electrogenerated chemiluminescence; Mfn2, mitofusin 2; Drp1, dynamin-related protein 1; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; ROS,reactiveoxygenspecies;EdU,5-ethynyl-2'-deoxyuridine.

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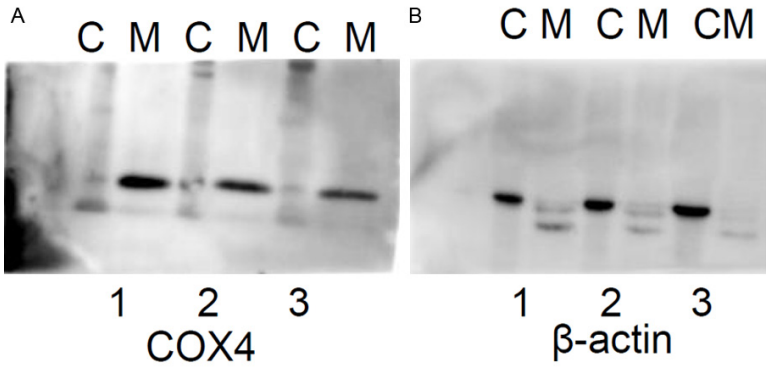

Figure S1. The identification of mitochondria. A: The cox4 expressed in "M"(mitochondria) lane and was not detected in "C"(cytoplasm) lane. B: The β-actin showed in "C"(cytoplasm) lane, not detected in "M" lane. This figure showed the mitochondria was extracted successfully. M: mitochondria; C: cytoplasm; 1: animal samples; 2: cell samples; 3: human samples.

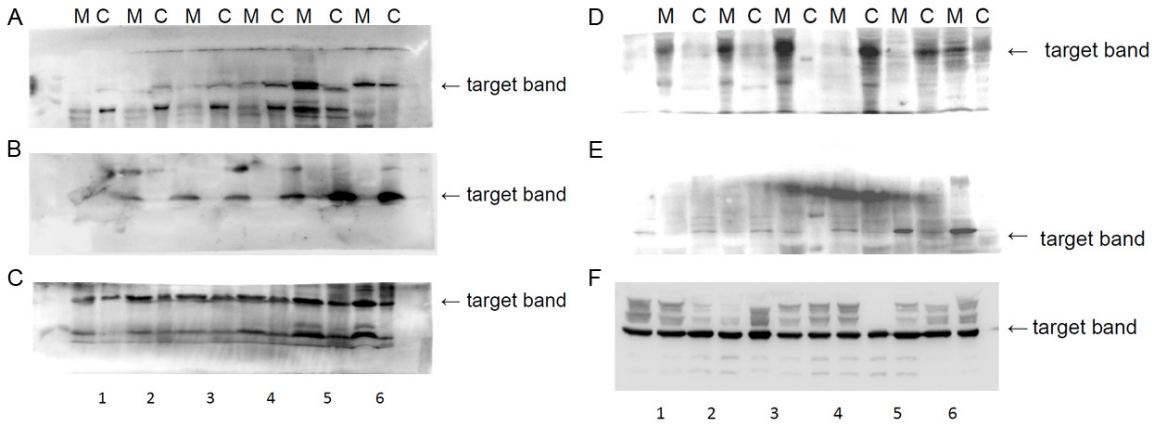

Figure S2. The expression of Cyto c and AIF in myocardium (A-C)/brain (D-F) (rat). (A) The expression of AIF in mitochondria and cytoplasm(myocardium); (B) The expression of Cyto c in mitochondria and cytoplasm(myocardium); (C) The expression of GAPDH in mitochondria and cytoplasm(myocardium); (D) The expression of AIF in mitochondria and cytoplasm(brain); (E) The expression of Cyto c in mitochondria and cytoplasm(brain); (F) The expression of GAPDH in mitochondria and cytoplasm(brain). 1: decapitaing; 2: hemorrhagic shock; 3: brain injury; 4: hanging; 5: suffocation; 6: CO intoxication.

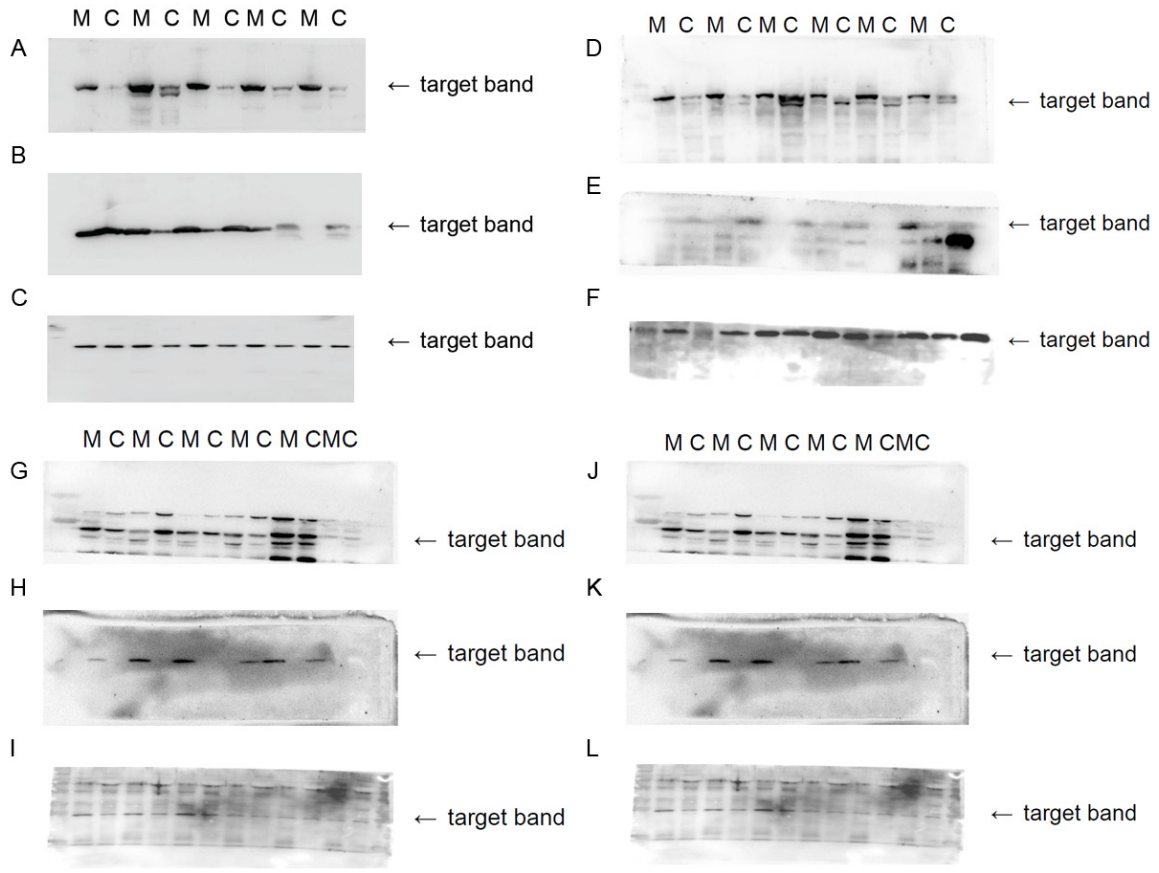

Figure S3. The AIF, Cyto c and GAPDH expression in mechanical asphyxia group (human heart). A: The expression of AIF; B: The expression of Cyto c; C: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in brain injury group (human heart). D: The expression of AIF; E: The expression of Cyto c; F: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in hemorrhagic shock group (human heart). G: The expression of AIF; H: The expression of Cyto c; I: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in alchole intoxication, cyanide intoxication, drowning and CO intoxication group (human heart). J: The expression of AIF; K: The expression of Cyto c; L: The expression of GAPDH.

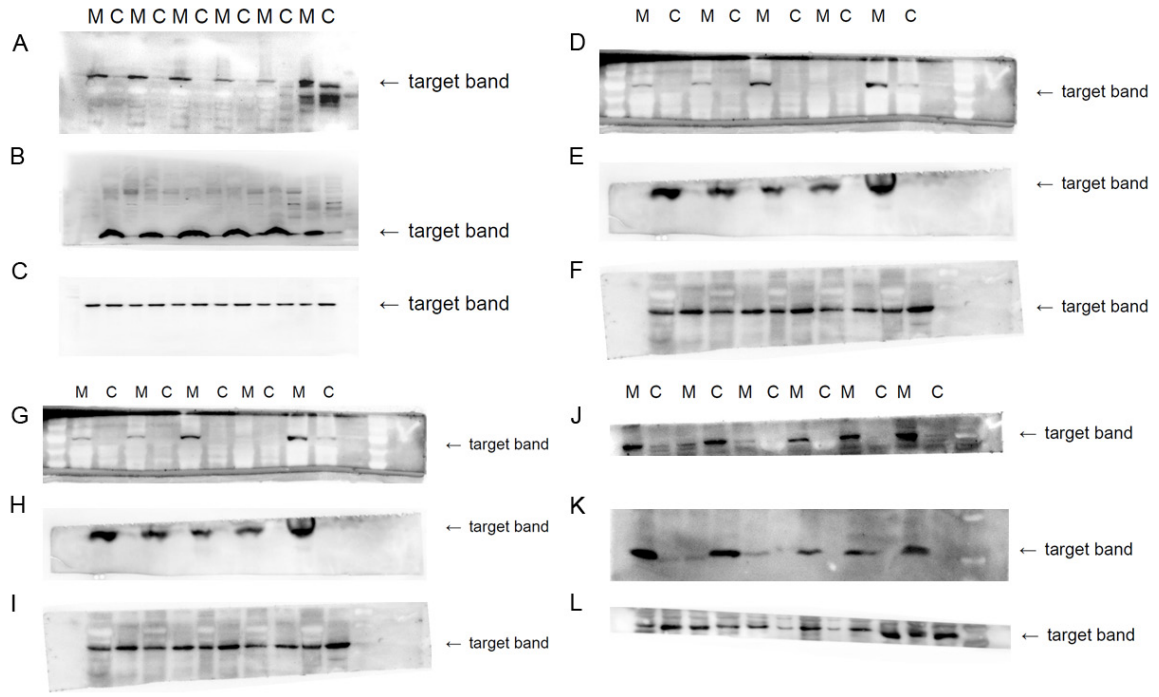

Figure S4. The AIF, Cyto c and GAPDH expression in mechanical asphyxia group (human brain). A: The expression of AIF; B: The expression of Cyto c; C: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in brain injury group (human brain). D: The expression of AIF; E: The expression of Cyto c; F: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in hemorrhagic shock group (human brain). G: The expression of AIF; H: The expression of Cyto c; I: The expression of GAPDH. The AIF, Cyto c and GAPDH expression in alchole intoxication, cyanide intoxication, drowning and CO intoxication group (human brain). J: The expression of AIF; K: The expression of Cyto c; L: The expression of GAPDH.

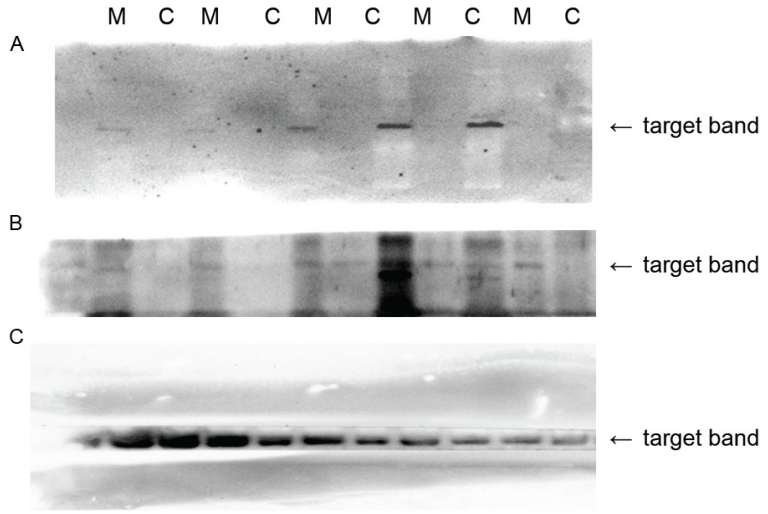

Figure S5. The AIF, Cyto c and GAPDH expression in mechanical asphyxia group (human brain). A: The expression of AIF; B: The expression of Cyto c; C: The expression of GAPDH.