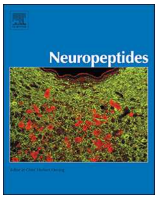
Neuropeptides 76 (2019) 101936

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep



Protective effects of ghrelin on brain mitochondria after cardiac arrest and resuscitation

Hongying Xu[a], Yong Li[b], Rongqiang Liu[a], Lin Wu[a], Chunling Zhang[a], Nan Ding[a], Aiying Ma[a], Jincheng Zhang[c], Xuemeng Xie[a,*]

[a] Department of Critical Care Medicine, Affiliated Hospital of Jining Medical University, Jining 272029, PR China
[b] Department of Emergency Medicine, Affiliated Hospital of Jining Medical University, Jining 272029, PR China
[c] Department of Critical Care Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, PR China

ARTICLE INFO

Keywords:
Ghrelin
Brain injury
Cardiac arrest
Cardiopulmonary resuscitation
Mitochondrial dysfunction
Mitochondrial unfolded response

ABSTRACT

Mitochondrial dysfunction plays a critical role in brain injury after cardiac arrest (CA) and cardiopulmonary resuscitation (CPR). Our recent study demonstrated that ghrelin protected against post-resuscitation brain injury with an elevated expression of mitochondrial uncoupling protein 2 (UCP2). However, the effects of ghrelin on mitochondrial dysfunction after CA are not clear. In the present study, the protective role of ghrelin was evaluated on mitochondrial dysfunction and the subsequent damage induced by CA in rats. In addition, mitochondrial unfolded protein response (UPRmt), an intrinsic cytoprotective pathway, was observed at the same time. Either vehicle (saline) or ghrelin (80 µg/kg) was injected blindly immediately after 6 min of CA and successful resuscitation. Neurological deficit was evaluated 6 h after CA and then cortex was collected for assessments. As a result, we found that ghrelin significantly improved the neurological deficit score in rats after CA. The functional analysis of isolated mitochondria revealed that ghrelin improved the mitochondrial ATP synthesis capacity and significantly reduced the reactive oxygen species (ROS) leakage after 6 h of CA. Concomitantly, we observed an increased ATP level and an attenuated oxidative stress in ghrelin treated animals. Moreover, ghrelin markedly improved the mitochondrial morphology compared with the vehicle animals. Further research revealed that ghrelin treatment significantly activated the UPRmt as demonstrated by the increased expression of heat shock protein 60 (HSP60), heat shock protein 10 (HSP10), caseinolytic protease 1 (CLPP1), and high-temperature requirement protein A2 (HTRA2). Our results suggest that ghrelin protected against cerebral mitochondria dysfunction after CA and the mechanism may involve a UPRmt pathway.

1. Introduction

Cardiac arrest (CA) remains a leading cause of death that affects more than a million individuals worldwide every year, despite the recent advancement in cardiopulmonary resuscitation (CPR) and intensive care (Lim et al., 2004). Brain injury is the cause of death in 68% of the patients after out-of-hospital CA and 23% after in-hospital CA (Laver et al., 2004). Functional outcomes of the survivors are variable, but poor-quality survival is common, with only 3%–7% able to return to their previous level of functioning (Edgren et al., 1989). To date, therapeutic hypothermia is regarded as the only effective treatment for post-resuscitation brain injury, but even it has not yet been shown to lead to a high level of neurological function recovery (Cronberg et al., 2009). Therefore, the development of new therapeutic options is an indispensable need for the patients surviving CA.

Recent data have indicated that mitochondria play a critical role in cerebral ischemia and reperfusion injury induced by CA and CPR (Ayoub et al., 2008; Jiang et al., 2014). Impaired mitochondrial function leads to reduced ATP production, disrupted calcium homeostasis and, in particular, the overproduction of reactive oxygen species (ROS). ROS can damage mitochondria by initiating the peroxidation of intra-mitochondrial lipids and proteins, inhibiting the activity of mitochondrial respiratory enzymes, and breaking mitochondrial DNA, which induces further damage on the mitochondrial function and leads to cell apoptosis or necrosis. Therefore, targeting mitochondria could be a promising strategy for CA and CPR.

We have recently shown that the treatment with ghrelin, a 28-amino acid peptide, improved the neurological performance and prevented the neuronal degeneration in the rats after CA (Xie et al., 2015). Moreover, ghrelin significantly reduced the oxidative stress possibly by an

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[*] Corresponding author at: Department of Critical Care Medicine, Affiliated Hospital of Jining Medical University, No. 89, Guhuai Road, Jining 272029, PR China.
E-mail address: 3404534595@qq.com (X. Xie).

https://doi.org/10.1016/j.npep.2019.05.007
Received 30 October 2018; Received in revised form 23 April 2019; Accepted 14 May 2019

elevated expression of mitochondrial uncoupling protein 2 (UCP2). However, it is unclear whether ghrelin changes the mitochondrial functions after CA. Therefore, the present study was performed to assess the effects of ghrelin on mitochondrial dysfunction and the subsequent damage in a rat model of CA and CPR.

Mitochondria are dependent on a diverse compilation of proteins to carry out their vital functions. Brain ischemia induces the toxic accumulation of unfolded proteins in vulnerable neurons. When unfolded, misfolded, or unassembled proteins accumulate beyond the folding capacity, a specific mitochondrial unfolded protein response (UPRmt) could be initiated to boost folding and degradation capacity in response to unfolded and aggregated protein accumulations in mitochondria (Pellegrino et al., 2013). Previous studies have suggested that the UPRmt is involved in numerous diseases that share the common pathogenetic mechanism of mitochondrial dysfunction (Haynes et al., 2013; Pellegrino and Haynes, 2015). However, the role of the UPRmt in the brain injury after CA and CPR has not been elucidated. Therefore, we evaluated the effect of ghrelin on UPRmt after CA in the subsequent experiments. We hypothesized that ghrelin protected against cerebral mitochondria dysfunction induced by CA and CPR, and the protective mechanism might involve an up-regulation in UPRmt.

2. Materials and methods

2.1. Experimental animals

Experimental procedures were approved by the Animal Care and Use Committee of Jining Medical University. Male Sprague-Dawley rats weighing 250–350 g were purchased from the Animal Experimental Center of Jining Medical University. The rats were group housed in a temperature-controlled room on a 12 h light/dark cycle and fed a standard rat chow diet.

2.2. Model of cardiac arrest

Prior to the induction of cardiac arrest (CA), the rats were kept on fasting overnight but were allowed to have water ad libitum. The rats were anesthetized with pentobarbital sodium (45 mg/kg, *ip*) and supplemented with additional doses of 10 mg/kg during the entire experimental procedures. The trachea was orally intubated and mechanically ventilated (tidal volume 0.65 ml/100 g, respiratory rate 55 min^{-1}, $FiO_2 = 0.21$; HX-300S, Chengdu Taimeng Technology Corp, China). Polyethylene catheters (PE50) were advanced through the left femoral artery and vein into the abdominal aorta and the inferior vena cava. The heart rate and electrocardiogram were continuously recorded. The rectal temperature was monitored by a temperature probe and maintained between 36.5 °C and 37.5 °C with heating lamps. The CA was induced by transcutaneous electrical epicardium stimulation, which was described previously (Pan et al., 2013). Briefly, two disposable acupuncture needles were placed between the fourth rib of the left sternal border and the third rib of the right sternal border. The stimulator electrode was connected, and a 60 Hz current of 2 mA was delivered. Circulatory arrest was defined by the onset of mean arterial pressure decline to 20 mm Hg. Ventilation was stopped. Current flow continued for 3 min to prevent spontaneous defibrillation. After 6 min of CA, advanced cardiac life support including mechanical ventilation (tidal volume 0.65 ml/100 g, respiratory rate 100 min^{-1}, $FiO_2 = 1.0$), chest compression (approx. 200 beats/min), and the application of adrenaline (20 μg/kg) was started. If the return of spontaneous circulation (ROSC) could not be achieved immediately, cardiopulmonary resuscitation (CPR) was continued, and adrenaline administrations were repeated after 60 s each. ROSC was defined as maintenance of the systolic aortic blood pressure of at least 60 mm Hg for an interval exceeding 10 consecutive minutes. If ROSC could not be achieved within 5 min, resuscitation efforts were stopped. After ROSC, mechanical ventilation was continued for the next 2 h. The endotracheal tube and catheters were then removed. After recovery from the anesthesia, animals were returned to their cages with close observation.

The rats were consecutively allocated to the sham, sham + ghrelin, CA + vehicle or CA + ghrelin group by a random sequence generator. Enrollment and randomization were performed until 13 animals per group reached 6 h survival. Sham experiments were conducted with the same experimental procedure except for CA.

In addition, to explore the changes of mitochondrial unfolded protein response (UPRmt) following different duration of CA, 2 subgroups ($n = 5$ for each) were set up following the same protocol as CA + vehicle group except different CA intervals (1 min and 3 min).

Rat ghrelin, purchased from AnaSpec, Inc. (USA), was dissolved in distilled water (1 mg/ml), and stored at − 20 °C until the time of preparation for administration. Immediately before administration, ghrelin was diluted again with 0.9% saline to the final concentration of 0.05 mg/ml. Ghrelin (80 μg/kg) was injected intravenously immediately after ROSC. Animals in the vehicle group received the same amount of saline (1.6 ml/kg).

2.3. Assessment of neurological outcome

Neurological outcome was assessed 6 h after CA using a neurological deficit scoring (NDS) system (Neumar et al., 1995). The score is composed of 6 items representing the level of consciousness, respiration, cranial nerves, motor, sensory and coordination. The scoring system ranged from 0 to 100 (normal: 100; brain death: 0).

2.4. ATP assay

ATP synthesis is a basic physiological function of mitochondria. After CA and CPR, mitochondria dysfunction causes ATP synthesis deficiency. We first detected the cortex ATP level to evaluate the effect of ghrelin on mitochondria function. A 20 mg cortex sample was homogenized in boiling distilled water, and the lysate was centrifuged at 4000*g* for 10 min. The ATP level in the supernatant was measured by a biochemical kit (Nanjing Jiancheng Biochemical Ltd., China) according to the manufacturer's instructions. The protein concentration was determined using the BCA Protein assay. ATP levels were presented as nmol/mg protein.

2.5. Mitochondrial ATP synthesis

To observe ghrelin's effect on mitochondria ATP synthesis function directly and dynamically, functional analysis on isolated mitochondria was conducted. After 6 h of observation, sham-operated and resuscitated animals were killed with a pentobarbital sodium overdose and perfused transcardially with 0.9% saline. Brains were removed, and frontal cortex tissues were collected immediately. Cortex mitochondria were isolated using a commercially available tissue mitochondria isolation kit (Beyotime, China) according to the manufacturer's protocol. A 3 μl of mitochondrial suspension (15 mg protein/ml) was diluted again to 0.5 mg protein/ml in an experimental buffer containing 130 mM KCl, 5 mM KH_2PO_4, 20 mM MOPS, 0.001 mM $Na_4P_2O_7$, and 0.1% BSA (pH adjusted to 7.15 with KOH). The rate of mitochondrial ATP synthesis was determined by chemiluminescence utilizing the reaction of firefly luciferase and luciferin with ATP, as described previously (Riess et al., 2014). The final working solution contained an experimental buffer, 0.2 μM diadenosine pentaphosphate, 30 μM ADP, 10 μg/ml mitochondria, 0.1 mg/ml luciferin, and 1.25 μg/ml luciferase. Addition of pyruvate/malate or succinate (5 mM each) initiated the reaction. The chemiluminescence representing the ATP production was measured by Biotek Synergy 2 (USA) with excitation at 328 nm and emission at 532 nm after 120 s. Background fluorescence was measured in the absence of mitochondria, and the results were calculated as relative fluorescence units (RFU) in the mitochondrial sample minus background RFU and were expressed as RFU/min/μg protein.

2.6. MDA and SOD assay

Mitochondria are the primary intracellular source of ROS, while oxidative stress is the leading cause of systemic I/R injury after CA and CPR. We first tested indicators of oxidative stress, MDA and SOD, to evaluate ghrelin's effect on mitochondria ROS production. A 10 mg frozen cortex sample was homogenized in a lysis buffer (0.01 M Tris-HCl, 0.1 mM EDTA, 0.01 M sucrose, 0.8% NaCl, pH = 7.4), and the proteins were quantified using BCA reagent. The levels of MDA were measured by a spectrophotometric kit (Nanjing Jiancheng Biochemical Ltd., China) at 532 nm based on a thiobarbituric acid (TBA) test. The total SOD activity in the hippocampus was measured by a spectrophotometric kit (Nanjing Jiancheng Biochemical Ltd., China) at 550 nm based on the reduction of nitroblue tetrazolium (NBT) by O_2^-. The activity of SOD was calculated in terms of units defined as the amount of SOD that inhibits the reduction of NBT by 50%.

2.7. Measurement of ROS generation leading to hydrogen peroxide production

To observe ghrelin's effect on mitochondria ROS production directly and dynamically, functional analysis on isolated mitochondria was conducted then. Reactive oxygen species (ROS) generation leading to hydrogen peroxide (H_2O_2) production was measured in the isolated mitochondria following the reaction of H_2O_2 with Amplex red (Sigma) in the presence of horseradish peroxidase (HRP) at 30 °C (Han et al., 2008). Briefly, 15 µl of mitochondrial suspension (15 mg protein/ml) was centrifuged again, and the mitochondrial pellet was suspended in 200 µl of KRP buffer (145 mM NaCl, 5.7 mM Sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM Glucose, pH 7.35) and was kept on ice prior to use. 100 ml of a solution containing HRP and Amplex red (0.1 unit/ml and 50 mM, respectively, in KRP buffer) was added, and the reaction was initiated by adding 20 µl of the mitochondrial mixture at 37 °C. For negative control, 20 µl of KRP was added alone. Fluorescence intensity was measured with excitation at 525 nm and emission at 590 nm after 5 min. Background fluorescence was measured in the absence of mitochondria, and the results were calculated as RFU in the mitochondrial sample minus background RFU and were expressed as RFU/min/mg protein.

2.8. Transmission electron microscopy (TEM)

Mitochondria are structurally complex organelles that provide multiple essential metabolic functions. Mitochondrial dysfunction is associated with structure disruption after CA and CPR. Transmission electron microscopy was used to evaluate mitochondria morphology changes. Fresh cortex tissues ($1\ mm^3$) were fixed in cold 2% glutaraldehyde (0.1 mol/L phosphate buffer, pH 7.4), postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol solutions, and embedded in epoxy resin. Afterward, they were cut into ultrathin sections (80 nm) and stained with uranyl acetate and lead citrate. The resulting micrographs were viewed under a TEM (Hitachi Ht7700, Japan). The pathological data were assessed by an independent investigator blinded to the experimental groups.

2.9. Western blot

Heat shock protein 60 (HSP60), Heat shock protein 10 (HSP10), Caseinolytic Protease 1 (CLPP1), and High-temperature requirement protein A2 (HTRA2) are main indictors of UPRmt in mammals. Western blot were used to detect changes of the above proteins. A 50 mg frozen cortex sample was homogenized in a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, China), and the proteins were quantified using the BCA assay reagent. A total of 30 µg of protein in each sample was separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (200 mA, 80 min; Millipore-upstate, USA). The membranes were blocked with 5% nonfat milk for 2 h and then incubated with primary antibody (HSP10, HTRA2, and CLPP1: Proteintech, USA, diluted 1:1000; HSP60: Proteintech, USA, diluted 1:2000; Growth Hormone Secretagogue Receptor -1α[GHSR-1α]: Boster, China, diluted 1:2000; GAPDH: Boster, China, diluted 1:45,000) overnight at 4 °C. After washing with Tris-Buffered Saline and Tween, the membranes were incubated with secondary antibody (Boster, China, diluted 1:50,000) for 2 h at 37 °C. The specific bands were detected with an enhanced chemiluminescence detection kit (Thermo Scientific, USA). The immunoreactive bands were visualized on film and scanned, and then the bands were analyzed by Image-Pro Plus (version 6.0). The quantitative data were expressed as the target protein optical density (OD)/β-actin OD ratio.

2.10. Immunohistochemistry

Changes in the expression of HSP60, HSP10, CLPP1, and HTRA2 proteins were confirmed by the immunohistochemistry. Immunohistochemical staining was performed strictly according to the manufacturer's instructions. Briefly, the rats were killed with a pentobarbital sodium overdose and perfused transcardially with cold normal saline. Brains were removed and fixed in 4% paraformaldehyde overnight. Brains were embedded in paraffin, and coronal 4-µm brain sections were cut at the level of the hippocampus. Paraffin sections were then deparaffinized and rehydrated using xylene and an alcohol series. Antigen retrieval was performed in a microwave for 10 min. After the incubation with 0.3% H_2O_2 in methanol and 10% normal goat serum, the brain sections were incubated overnight at 4 °C with diluted primary antibodies (HSP10, HTRA2, and CLPP1: Proteintech, USA, diluted 1:70; HSP60: Proteintech, USA, diluted 1:100). After washing with PBS, the sections were incubated with the appropriate secondary antibodies for 60 min. The immunoreactivity was visualized with a DAB development kit (Beyotime, China) and washed with PBS. Brain tissue sections from the sham group that had been processed for IHC without primary antibodies were used as negative controls. A blinded observer analyzed the stained sections with light microscopy in 5 random non-overlapping high-power fields (×400) in the frontal cortex and the representative pictures were captured then. Three slices per animal were analyzed.

2.11. Statistical analysis

Only data obtained from the animals who had a 6 h survival rate were analyzed. Statistical analysis was performed with SPSS Statistics 17.0 program. Data were reported as mean ± SD. Normally distributed data were analyzed with One-way ANOVA with Student–Newman–Keuls post-hoc test. The difference in survival rate was analyzed by Fisher's exact test. *P*-values $< .05$ were considered as significant differences.

3. Results

3.1. Cardiac arrest, cardiopulmonary resuscitation, and survival

We lost 2 animals due to technical failure before cardiac arrest (CA) was induced. Successful resuscitation could not be reached in 6 rats after 6 min of CA. Five animals (3 for CA + vehicle and 2 for CA + ghrelin ; survival rate: 13/15 versus 13/16, $P < .05$) died after the return of spontaneous circulation (ROSC) before reaching the 6 h survival.

Before the induction of CA, there was no difference between the groups in body weight or hemodynamic variables. The transcutaneous electrical stimulation caused circulatory arrest within 20 s in all the animals. No significant difference was observed with regard to the inducing period, cardiopulmonary resuscitation (CPR) duration or

Table 1
Characteristics of each group before and after CA or sham operation.

	Sham	Sham + vehicle	CA + vehicle	CA + ghrelin
	(n = 13)	(n = 13)	(n = 13)	(n = 13)
Body weight (g)	339 ± 16	332 ± 17	329 ± 17	337 ± 19*
MAP at baseline (mm Hg)	112 ± 11	124 ± 16	116 ± 13	112 ± 10*
Heart rate at baseline (bpm)	386 ± 21	384 ± 25	378 ± 18	373 ± 14*
Inducing period (s)	–	–	14 ± 5	15 ± 4*
CPR duration (s)	–	–	166 ± 44	175 ± 46*
Adrenaline i.v. (μg)	–	–	1.9 ± 0.8	2.0 ± 0.8*
MAP at 2 h after CA or sham operation (mm Hg)	116 ± 14	113 ± 13	88 ± 12	86 ± 6*
Heart rate at 2 h after CA or sham operation (bpm)	386 ± 23	380 ± 24	355 ± 12	360 ± 11*

Comparison of the body weight, mean arterial blood pressure, and heart rate revealed no difference between the groups (n = 13). No difference was detected with regard to the inducing period, CPR duration, or adrenaline dosage between the CA + ghrelin group and the CA + vehicle group (n = 13) (Compared with the CA + vehicle group, $^*P > .05$).

adrenaline dosage between the CA + vehicle group and the CA + ghrelin group.

The CA resulted in a marked decrease in mean arterial blood pressure and heart rate. However, no difference was observed between the CA + vehicle and the CA + ghrelin group (Table 1). After ROSC, adequate spontaneous respiration recovered gradually in 2 h in all the rats. After catheter removal, most animals remained anesthetized but were able to breathe spontaneously.

In the subgroup of 1 min CA (n = 5) and the subgroup of 3 min CA (n = 5), all the rats survived until 6 h after ROSC.

3.2. Ghrelin improved neurological outcome 6 h after CA

The NDS is lower both in the CA + vehicle and CA + ghrelin groups than that observed in the sham groups. However, there was an improved NDS in the CA + ghrelin group compared to that of the CA + vehicle group (CA + vehicle: 58.8 ± 5.4, CA + ghrelin: 74.4 ± 7.3; $P < .05$) (Fig. 1).

3.3. Ghrelin elevated the ATP levels in cortex and improved the ATP synthesis capacity in isolated mitochondria 6 h after CA

The ATP levels were drastically decreased 6 h after ROSC both in the CA + ghrelin and CA + vehicle groups compared with the sham groups. However, the treatment with ghrelin resulted in an increase in ATP compared with the CA + vehicle group (CA + vehicle: 7.8 ± 0.9 nmol/mgprot, CA + ghrelin: 11.5 ± 1.8 nmol/mgprot; $P < .05$). No difference was detected between the sham group and the sham + ghrelin group (sham: 16.9 ± 0.6 nmol/mgprot, sham + ghrelin: 16.0 ± 0.7 nmol/mgprot; $P > .05$) (Fig. 2A). CA and CPR resulted in an impairment of mitochondrial ATP synthesis capacity. For complex I substrates (pyruvate/malate), the rate of ATP synthesis was improved in mitochondria isolated from the CA + ghrelin group (CA + vehicle: 87.0 ± 27.9 RUF/min/μgprot, CA + ghrelin: 123.1 ± 16.0 RUF/min/μgprot; $P < .05$). No difference was detected between the sham group and the sham + ghrelin group (sham: 197.1 ± 18.6 nmol/mgprot, sham + ghrelin: 199.7 ± 14.1 nmol/mgprot; $P > .05$)(Fig. 2B). For complex II substrate (succinate), the rate of ATP synthesis was improved in mitochondria isolated from the CA + ghrelin group (CA + vehicle: 81.2 ± 22.1 RUF/min/μgprot, CA + ghrelin: 128.7 ± 10.1 RUF/min/μgprot; $P < .05$). No difference was detected between the sham group and the sham + ghrelin group (sham: 199.8 ± 17.5 nmol/mgprot, sham + ghrelin:193.2 ± 12.5 nmol/mgprot; $P > .05$). (Fig. 2C).

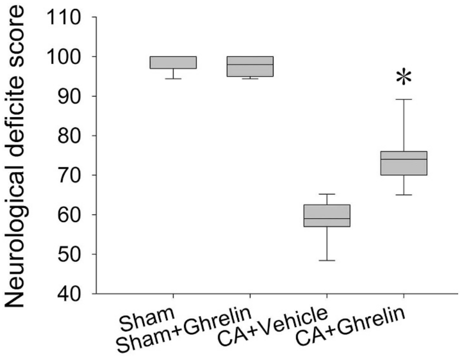

Fig. 1. Neurological deficit score in sham, sham + ghrelin, CA + vehicle and CA + ghrelin groups (n = 13) at 6 h after sham operation or CA.
There was an improved NDS in the CA + ghrelin group compared with the CA + vehicle group. ($^*P < .05$, by SNK-test) Results are expressed as median, 1st/3rd quartile (boxes), minimum/interquartile/maximum (whiskers).

3.4. Ghrelin decreased oxidative stress in cortex and generation of reactive oxygen in isolated mitochondria 6 h after CA

In resuscitated animals, a higher level of MDA and the lower SOD activity were observed compared with sham-operated animals. Meanwhile, the ghrelin treatment after CA was associated with a lower level of MDA (CA + vehicle: 25.0 ± 5.0 nmol/mgprot,CA + ghrelin: 14.0 ± 2.2 nmol/mgprot; $P < .05$) (Fig. 3A) and a higher level of the SOD activity (CA + vehicle: 99.7 ± 12.8 U/mgprot, CA + ghrelin: 142.8 ± 10.3 U/mgprot; $P < .05$) (Fig. 3B). No difference was detected between the sham group and the sham + ghrelin group in MDA (sham: 4.7 ± 0.9 nmol/mgprot, sham + ghrelin: 4.9 ± 0.9 nmol/mgprot; $P > .05$) and SOD (sham: 181.1 ± 16 nmol/mgprot, sham + ghrelin: 179.9 ± 13.1 nmol/mgprot; $P > .05$). Using the Amplex Red probe for fluorometric H_2O_2, we found that cortex mitochondria produced increasing levels of H_2O_2 at 6 h after reperfusion. However, ghrelin decreased the generation of reactive oxygen in isolated mitochondria compared with the vehicle group (CA + vehicle: 1038.6 ± 194.7 RUF/min/mgprot, CA + ghrelin: 725.0 ± 218.0 RUF/min /mgprot; $P < .05$) No difference was detected between the sham group and the sham + ghrelin group (sham: 301.4 ± 21.6 nmol/mgprot, sham + ghrelin: 292.0 ± 15.2 nmol/mgprot; $P > .05$) (Fig. 3C).

3.5. Ghrelin mitigated the damage of cortex mitochondria 6 h after CA

In the sham groups, we observed little, if any, swelling and no membrane disruption. No difference was detected between the sham group and the sham + ghrelin group. The CA and CPR induced dramatic

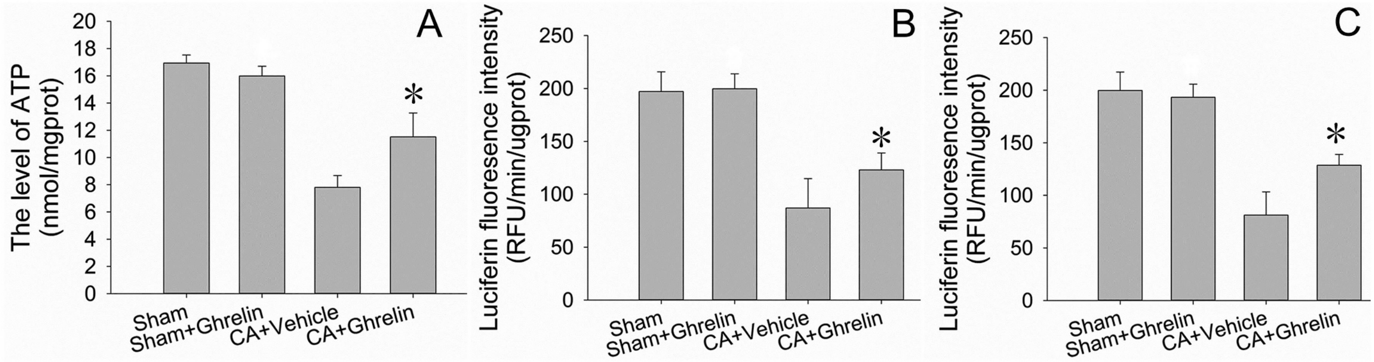

Fig. 2. Effect of ghrelin on cortex ATP level and mitochondrial ATP synthesis rate 6 h after CA or sham operation. ($n = 5$).
Treatment with ghrelin after CA resulted in an increase in ATP in the cortex compared with the CA + vehicle group (*$P < .05$, by SNK-test). (A) For both complex I (B) and complex II (C) substrates, the rate of ATP synthesis was improved in the mitochondria isolated from the CA + ghrelin group 6 h after CA. (Compared with the CA + vehicle group, *$P < .05$, by SNK-test).

ultrastructural alterations including loss of internal membrane contact, mitochondrial matrix swelling, and disruption of mitochondria cristae. However, when treated with ghrelin, mitochondrial alterations were improved obviously (Fig. 4).

3.6. *Ghrelin enhanced the expression of HSP60, HSP10, CLPP1, and HTRA2 in cortex 6 h after CA*

In the subgroups of 1 min CA and 3 min CA, the expressions of heat shock protein 60 (HSP60), heat shock protein 10 (HSP10), caseinolytic Protease 1 (CLPP1), and high-temperature requirement protein A2 (HTRA2) in the cortex, 6 h after ROSC, were significantly higher than the sham group. However, in the CA + vehicle group of 6 min CA, the expressions of HSP60, HSP10, CLPP1, and HTRA2 in the cortex, 6 h after ROSC, were significantly lower than that in the subgroups. Moreover, the expression of HSP60 (CA + vehicle: 0.39 ± 0.04, CA + ghrelin: 0.56 ± 0.05; $P < .05$), HSP10 (CA + vehicle: 0.65 ± 0.06, CA + ghrelin: 0.78 ± 0.06; $P < .05$), CLPP1 (CA + vehicle: 0.18 ± 0.03, CA + ghrelin: 0.23 ± 0.04; $P < .05$), and HTRA2 (CA + vehicle: 0.37 ± 0.04, CA + ghrelin: 0.45 ± 0.05; $P < .05$) were higher after treating with ghrelin compared with the CA + vehicle group (Fig. 5A). Changes in the expression of HSP60, HSP10, CLPP1, and HTRA2 proteins were confirmed by the immunohistochemistry (IHC). All the proteins were located in the cytoplasm of neurocytes. Compared to those from the sham group, the samples from the CA + vehicle group exhibited notably higher immunoreactivity for HSP60, HSP10, CLPP1, and HTRA2 proteins. Ghrelin administration increased the above protein expression 6 h after ROSC compared to the CA + vehicle group (Fig. 5B).

3.7. *Ghrelin receptor GHSR-1α expression was reduced significantly after CA and reversed by ghrelin treatment*

Neuroprotective effects of ghrelin are proved to be mediated through activation of GHSR-1α. GHSR-1α was detected in the rat cortex by western blot. Compared with sham group, CA groups showed a significantly reduced expression of GHSR-1α that was reversed by ghrelin treatment. (CA + vehicle: 0.20 ± 0.03, CA + ghrelin: 0.48 ± 0.06; $P < .05$) (Fig. 6).

4. Discussion

Our previous study has demonstrated that ghrelin treatment (80 μg/kg) immediately after ROSC improved the neurological performances with reserved neuronal viability and inhibited neuronal apoptosis in rats 72 h after CA (Xie et al., 2015). The present 6-h study was designed to investigate the effects of ghrelin on mitochondrial dysfunction and UPRmt. Our results suggest that ghrelin protected against cerebral mitochondria dysfunction after CA and the mechanism may involve a UPRmt pathway.

After the return of spontaneous circulation (ROSC), especially in the early stage of post-ROSC, the mitochondrial oxidative phosphorylation is slowed down, resulting in the reduced generation of ATP, and thereby electrons are diverted to the Q-cycle through the electron transport chain, generating excessive ROS. The products exceed the scavenging capacity of the endogenous antioxidant system in mitochondria, and consequently, oxidative stress occurs. At the same time of generating ROS, mitochondrial electron transport chain per se could

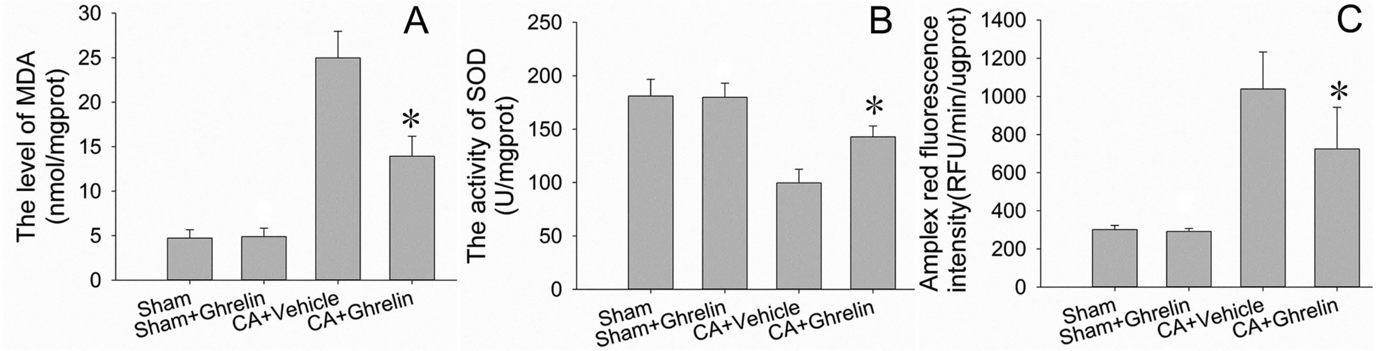

Fig. 3. Effect of ghrelin on cortex oxidative stress and mitochondrial reactive oxygen generation 6 h after CA or sham operation. ($n = 5$).
Animals treated with ghrelin showed a lower level of MDA (A) and a higher level of SOD activity after CA and CPR (Compared with the CA + vehicle group, *$P < .05$, by SNK-test)(B). Ghrelin treatment decreased the generation of reactive oxygen after CA compared with the CA + vehicle group (*$P < .05$, by SNK-test) (C).

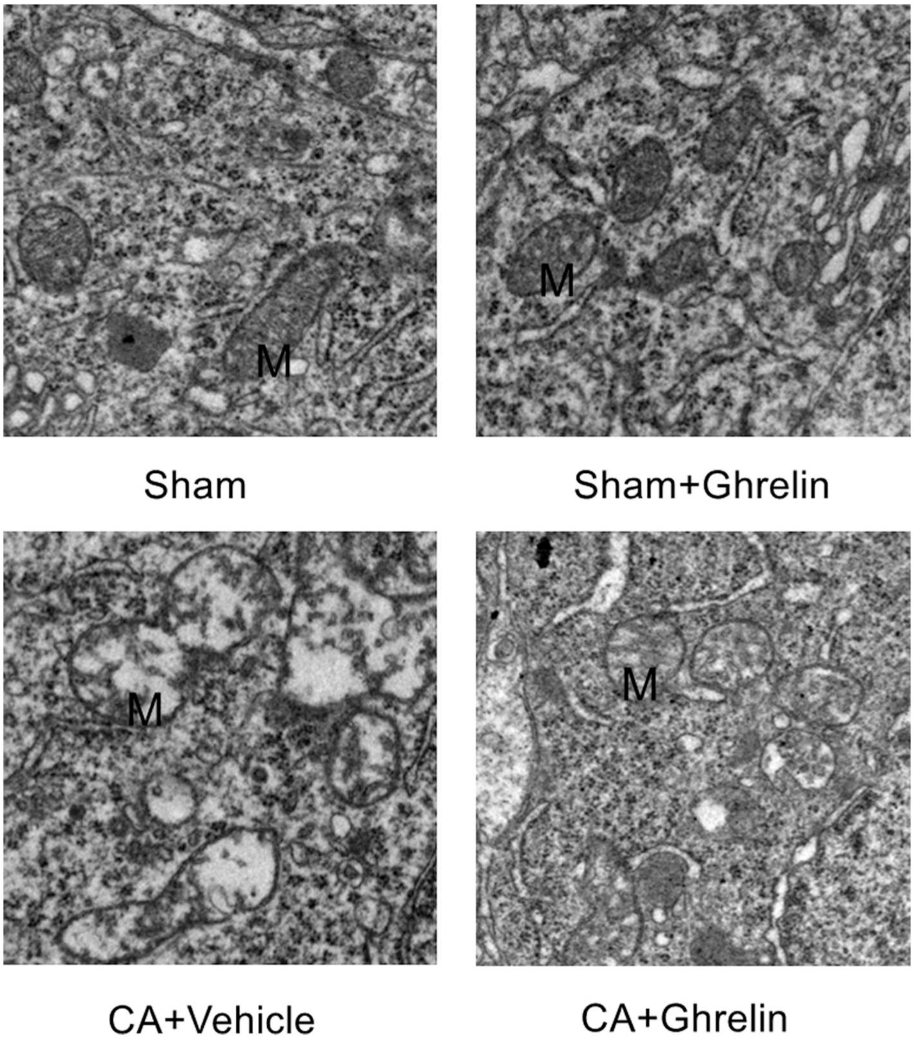

Fig. 4. Effect of ghrelin on the ultrastructural changes of cortex mitochondria 6 h after CA or sham operation. (n = 3).
The ultrastructural changes of cortex mitochondria from the sham, sham + ghrelin, CA + vehicle, and CA + ghrelin groups. Ultrastructural alterations including loss of internal membrane contact, mitochondrial matrix swelling, and disruption of mitochondria cristae were found after CA and CPR. Ghrelin markedly improved the mitochondrial morphology after CA compared with the CA + vehicle animals. No difference was detected between the sham group and the sham + ghrelin group. All images are captured at 5000× magnification. M: mitochondria.

be impaired by ROS (Ayoub et al., 2008). So, mitochondria might initially act as the target organelles of CA-induced ischemia, and subsequently as the effectors contributing to post-resuscitation brain injury.

Consistent with the other reports (Han et al., 2008; Jiang et al., 2014), our results revealed a great impairment of mitochondrial ATP synthesis capacity and a steep increase of ROS generation from mitochondria early after CA. These data indicated that mitochondrial dysfunction developed and lasted in the brain as long as 6 h after CA, in which the mitochondrial electron transport complexes are shifted mostly toward imbalanced ROS versus energy production.

Ghrelin, a peptide released by the stomach that plays a major role in regulating energy metabolism, has been reported to alleviate brain damage by anti-oxidative and anti-apoptosis effects in a series of models of neuronal injury (Jiao et al., 2017; Liu et al., 2018; Yu et al., 2016). However, it is unknown whether ghrelin changes the mitochondrial functions during neuronal ischemia. In the present study, particular attention has been paid to analyzing the ghrelin action on mitochondrial functions and morphology. Here we first report that the ghrelin treatment significantly attenuated mitochondrial dysfunction and improved the mitochondrial morphology in a rat model of cerebral ischemia and reperfusion induced by CA and CPR, which could be an important mechanism of ghrelin's neuroprotective effects.

UPRmt is a cytoprotective process activated in response to outside stress to stabilize the structure of mitochondrial proteins (Haynes et al., 2013). When unfolded proteins accumulate in the mitochondrial matrix, c-Jun N-terminal kinase (JNK) is activated, which phosphorylates as c-Jun. The activation of c-Jun triggers CHOP10 expression. In turn, the transcription factor regulates the expression of stress-resolving genes such as mitochondrial proteases and chaperones including HSP60, HSP10, and CLPP1. In the inter-membrane space, the UPRmt is mediated by the activation of ERα, triggered by the AKT-dependent phosphorylation. Mitochondrial stress resolves by an increase in the expression of HTRA protease (Pellegrino et al., 2013).

UPRmt is activated in the brain after ischemia/reperfusion injury, but possibly at a low level. Jessie S. Truettner reported that after a transient (15 min) ischemic insult, the subcellular responses to the accumulation of unfolded proteins vary between the cellular compartments and are most prevalent in the cytoplasm and, to a lesser degree, in the mitochondrial matrix (Truettner et al., 2009). Researchers proposed that the UPRmt is activated at low levels of mitochondrial stress, but begin to fade as the level or duration of stress exceeds a certain point, which brings mitophagy or apoptosis. In both the circumstances, UPRmt could not be induced sufficiently and the protective effects are limited (Pellegrino et al., 2013).

In accordance with the theory above, in the present study, UPRmt is significantly activated after 1 min CA and 3 min CA. However, when CA intervals are prolonged to 6 min, the expression of UPRmt indicator genes was significantly inhibited when compared with the subgroups. These data may indicate the inadequacy of UPRmt activation as the level or the duration of mitochondrial stress increases.

Moreover, we found that the ghrelin treatment significantly promoted the expression of UPRmt indicator genes after CA. Actually, recent studies by others also suggest that the up-regulation of UPRmt is possibly beneficial in ischemia/reperfusion injuries. Kurt and

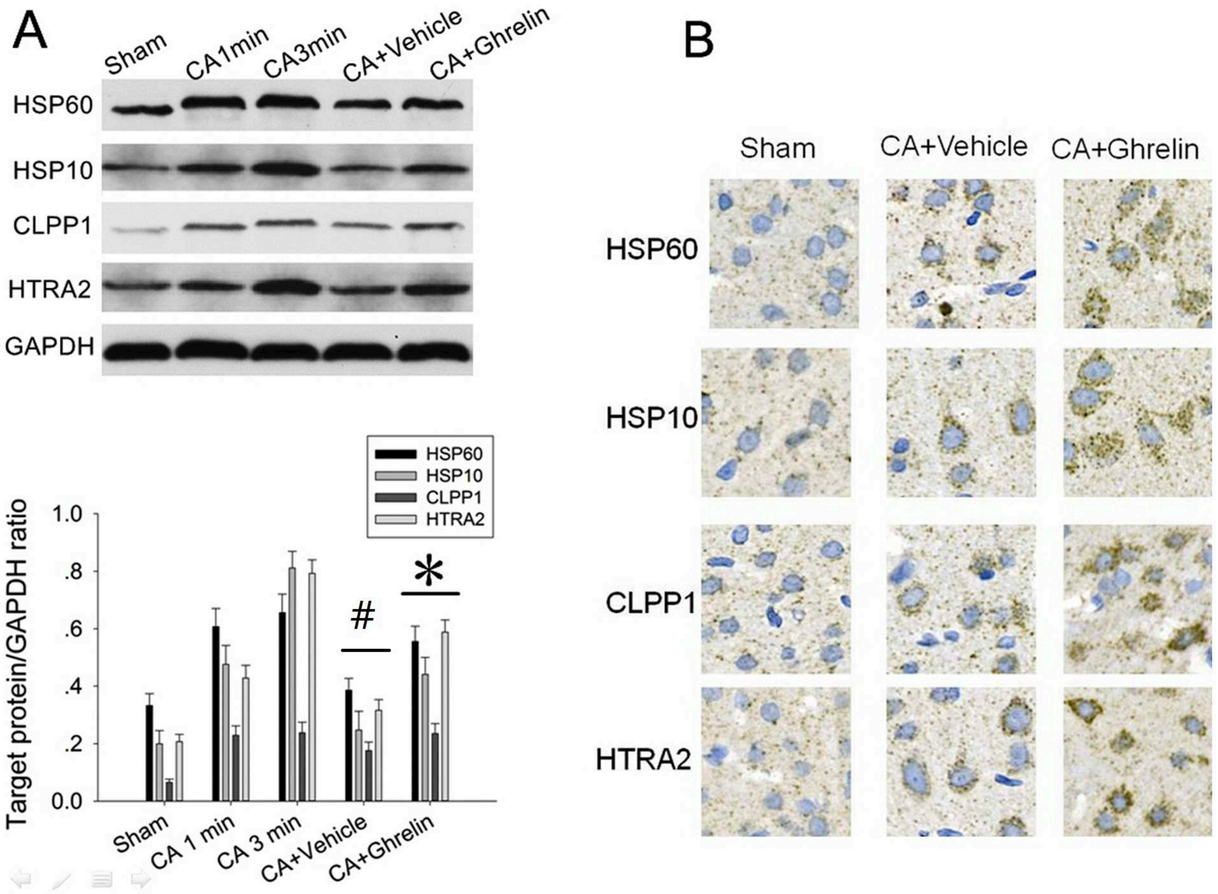

Fig. 5. Western blot and immunohistochemistry for HSP60, HSP10, CLPP1, and HTRA2 expression in the cortex 6 h after CA or sham operation ($n = 5$).
In the CA + vehicle group of 6 min CA, the expressions of HSP60, HSP10, CLPP1, and HTRA2 were significantly lower than that in the subgroups of 1 min CA and 3 min CA. ($^{\#}P < .05$, by SNK-test). Ghrelin promoted HSP60, HSP10, CLPP1, and HTRA2 expressions in the cortex compared with the CA + vehicle group (A). ($^{*}P < .05$, by SNK-test)(A). In situ immunostaining demonstrated that HSP60, HSP10, CLPP1, and HTRA2 levels were significantly enhanced by ghrelin (B).

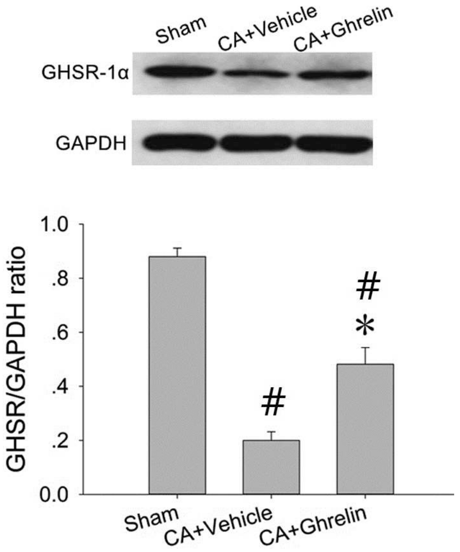

Fig. 6. Changes of expression of GHSR-1α in cortex and effects of ghrelin. ($n = 3$).
CA groups showed a significantly reduced expression of GHSR-1α (Compared with Sham: $^{\#}P < .05$, by SNK-test) that was reversed by ghrelin treatment. (Compared with the CA + vehicle group, $^{*}P < .05$, by SNK-test).

colleagues reported that the overexpression of HSP60 or HSP10 using an adenoviral vector in cardiac myocytes protects mitochondrial functions and prevents apoptotic cell deaths induced by the simulated ischemia-reoxygenation (Lin et al., 2001). Interestingly, in a rat model of middle cerebral artery occlusion, aminoguanidine, an inhibitor of the inducible nitric oxide synthase, further up-regulated the expressions of HSP60 and HSP10 and reduced ischemic brain damage (Kim and Lee, 2007). Based on these pieces of evidence, we think it would be safe to postulate that the mitochondrial protection by ghrelin may involve a UPRmt pathway.

Neuroprotective effects of ghrelin are proved to be mediated through activation of GHSR-1a (Frago, 2011). Western blotting experiments for cortex GHSR-1a expression were conducted in this study. We found GHSR-1a expression were decreased after CA/CPR, which was prevented by ghrelin administration. This study suggests that ghrelin may upreglulate the GHSR in the cortex. The notion was supported by the evidence that GHSR mRNA in hypothalamus was upregulated by ghrelin administrated intracerebrovetricularly in rat (Nogueiras et al., 2004).

The detailed mechanisms of how ghrelin promotes the level of UPRmt are still unknown and need to be elucidated in the future. Firstly, the ghrelin receptor, Growth Hormone secretagogue receptor (GHSR-1α), belongs to a family of receptors operating via the pathways including JNK and PKB (Frago, 2011), which are directly involved in the UPRmt. Secondly, UCP2 is thought to provide a controlled leak of protons across the inner membrane of the mitochondrion, which could lead to a moderate decrease in the mitochondrial membrane potential (MMP). And the decrease of MMP is thought to be a key factor in the early activation of UPRmt (Pellegrino et al., 2013).

5. Conclusions

Our results suggest that ghrelin protected against cerebral mitochondria dysfunction after cardiac arrest (CA) and the mechanism may involve a mitochondrial unfolded protein response (UPRmt) pathway.

Conflict of interest statement

None.

Acknowledgments

This work was supported by grants from the Doctoral Scientific Research Foundation of Affiliated Hospital of Jining Medical University (No. 2016-BS-013)and the National Natural Science Foundation of China (No. 81272063 and 81601658).

Declarations of interest

None.

Competing interests to declare

None.

References

Ayoub, I.M., Radhakrishnan, J., Gazmuri, R.J., 2008. Targeting mitochondria for resuscitation from cardiac arrest. Crit. Care Med. https://doi.org/10.1097/ccm.0b013e31818a89f4.

Cronberg, T., Lilja, G., Rundgren, M., Friberg, H., Widner, H., 2009. Long-term neurological outcome after cardiac arrest and therapeutic hypothermia. Resuscitation. https://doi.org/10.1016/j.resuscitation.2009.06.021.

Edgren, E., Kelsey, S., Sutton, K., Safar, P., Group, T.B.R.C.T.I.S, 1989. The presenting ECG pattern in survivors of cardiac arrest and its relation to the subsequent long-term survival. Acta Anaesthesiol. Scand. https://doi.org/10.1111/j.1399-6576.1989.tb02905.x.

Frago, L., 2011. Neuroprotective actions of ghrelin and growth hormone secretagogues. Front. Mol. Neurosci. https://doi.org/10.3389/fnmol.2011.00023.

Han, F., Da, T., Riobo, N.A., Becker, L.B., 2008. Early mitochondrial dysfunction in electron transfer activity and reactive oxygen species generation after cardiac arrest. Crit. Care Med. https://doi.org/10.1097/ccm.0b013e31818a8a51.

Haynes, C.M., Fiorese, C.J., Lin, Y.F., 2013. Evaluating and responding to mitochondrial dysfunction: the mitochondrial unfolded-protein response and beyond. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2013.02.002.

Jiang, J., Fang, X., Fu, Y., Xu, W., Jiang, L., Huang, Z., 2014. Impaired cerebral mitochondrial oxidative phosphorylation function in a rat model of ventricular fibrillation and cardiopulmonary resuscitation. Biomed. Res. Int. https://doi.org/10.1155/2014/192769.

Jiao, Q., Du, X., Li, Y., Gong, B., Shi, L., Tang, T., Jiang, H., 2017. The neurological effects of ghrelin in brain diseases: beyond metabolic functions. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2016.12.010.

Kim, S.W., Lee, J.K., 2007. NO-induced downregulation of HSP10 and HSP60 expression in the postischemic brain. J. Neurosci. Res. https://doi.org/10.1002/jnr.21236.

Laver, S., Farrow, C., Turner, D., Nolan, J., 2004. Mode of death after admission to an intensive care unit following cardiac arrest. Intensive Care Med. https://doi.org/10.1007/s00134-004-2425-z.

Lim, C., Alexander, M.P., LaFleche, G., Schnyer, D.M., Verfaellie, M., 2004. The neurological and cognitive sequelae of cardiac arrest. Neurology. https://doi.org/10.1212/01.WNL.0000144189.83077.8E.

Lin, K.M., Lin, B., Lian, I.Y., Mestril, R., Scheffler, I.E., Dillmann, W.H., 2001. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. Circulation. https://doi.org/10.1161/01.CIR.103.13.1787.

Liu, S., Chen, S., Ren, J., Li, B., Qin, B., 2018. Ghrelin protects retinal ganglion cells against rotenone via inhibiting apoptosis, restoring mitochondrial function, and activating AKT-mTOR signaling. Neuropeptides. https://doi.org/10.1016/j.npep.2017.11.007.

Neumar, R.W., Bircher, N.G., Sim, K.M., Xiao, F., Zadach, K.S., Radovsky, A., Katz, L., Ebmeyer, E., Safar, P., 1995. Epinephrine and sodium bicarbonate during CPR following asphyxial cardiac arrest in rats. Resuscitation. https://doi.org/10.1016/0300-9572(94)00827-3.

Nogueiras, R., Tovar, S., Mitchell, S.E., Rayner, D.V., Archer, Z.A., Dieguez, C., Williams, L.M., 2004. Regulation of growth hormone secretagogue receptor gene expression in the arcuate nuclei of the rat by leptin and ghrelin. Diabetes. https://doi.org/10.2337/diabetes.53.10.2552.

Pan, H., Chen, D., Liu, B., Xie, X., Zhang, J., Yang, G., 2013. Effects of sodium hydrosulfide on intestinal mucosal injury in a rat model of cardiac arrest and cardiopulmonary resuscitation. Life Sci. https://doi.org/10.1016/j.lfs.2013.05.012.

Pellegrino, M.W., Haynes, C.M., 2015. Mitophagy and the mitochondrial unfolded protein response in neurodegeneration and bacterial infection. BMC Biol. https://doi.org/10.1186/s12915-015-0129-1.

Pellegrino, M.W., Nargund, A.M., Haynes, C.M., 2013. Signaling the mitochondrial unfolded protein response. Biochim. Biophys. Acta Mol. Cell Res. https://doi.org/10.1016/j.bbamcr.2012.02.019.

Riess, M.L., Matsuura, T.R., Bartos, J.A., Bienengraeber, M., Aldakkak, M., McKnite, S.H., Rees, J.N., Aufderheide, T.P., Sarraf, M., Neumar, R.W., Yannopoulos, D., 2014. Anaesthetic postconditioning at the initiation of CPR improves myocardial and mitochondrial function in a pig model of prolonged untreated ventricular fibrillation. Resuscitation. https://doi.org/10.1016/j.resuscitation.2014.09.019.

Truettner, J.S., Hu, K., Liu, C.L., Dietrich, W.D., Hu, B., 2009. Subcellular stress response and induction of molecular chaperones and folding proteins after transient global ischemia in rats. Brain Res. https://doi.org/10.1016/j.brainres.2008.10.032.

Xie, X., Zhang, J., Chen, D., Pan, H., Wu, Z., Ge, D., Yang, G., 2015. Effects of ghrelin on postresuscitation brain injury in a rat model of cardiac arrest. Shock. https://doi.org/10.1097/SHK.0000000000000337.

Yu, J., Xu, H., Shen, X., Jiang, H., 2016. Ghrelin protects MES23.5 cells against rotenone via inhibiting mitochondrial dysfunction and apoptosis. Neuropeptides. https://doi.org/10.1016/j.npep.2015.09.011.