

Edaravone Ameliorates Renal Warm Ischemia-Reperfusion Injury by Downregulating Endoplasmic Reticulum Stress in a Rat Resuscitation Model

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Background: This study was conducted to explore whether the effect of edaravone (5-methyl-2-phenyl-2,4-dihydro-3H-pyrazol3-one, EDR) can ameliorate renal warm ischemia-reperfusion injury (IRI) by modulating endoplasmic reticulum stress (ERS) and its downstream effector after cardiac arrest (CA) and cardiopulmonary resuscitation (CPR) in a rat model.

Methods: The rats (n=10) experienced anaesthesia and intubation followed by no CA inducement were defined as the Sham group. Transoesophageal alternating current stimulation was employed to establish 8 min of CA followed by conventional CPR for a resuscitation model. The rats with successful restoration of spontaneous circulation (ROSC) randomly received EDR (3 mg/kg, EDR group, n=10) or equal volume normal saline solution (the NS group, n=10). At 24 hr after ROSC, serum creatinine (SCR), blood urea nitrogen (BUN) levels, and cystatin-C (Cys-C) levels were determined and the protein level of glucose-regulated protein (GRP78), C/EBP homologous protein (CHOP), extracellular signal-regulated kinase (ERK), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), Bax/Bcl-2, and caspase-3 were detected by Western blot method.

Results: At 24 hrs after ROSC, SCR, BUN and Cys-C were obviously increased and the proteins expression, including GRP78, CHOP and p-ERK1/2, cleaved-caspase 3 Bax/Bcl-2 ratio, were significantly upregulated in the NS group compared with the Sham group ($p < 0.05$). The remarkable improvement of these adverse outcomes was observed in the EDR group ($p < 0.05$).

Conclusion: In conclusion, we found that EDR ameliorates renal warm IRI by down-regulating ERS and its downstream effectors in a rat AKI model evoked by CA/CPR. These data may provide evidence for future therapeutic benefits of EDR against AKI induced by CA/CPR.

Keywords: renal warm ischemia-reperfusion injury, edaravone, endoplasmic reticulum stress, cardiac arrest, cardiopulmonary resuscitation

Introduction

During ischemia, multiple signalling pathways, which are closely relative with inflammatory and metabolic, play pro-apoptosis roles in cells.¹ However, the subsequent blood perfusion restoration may cause more severe injury known as ischemia-reperfusion injury (IRI). Renal ischaemia-reperfusion injury (IRI) is known as one of the most common causes of acute kidney injury (AKI) and secondary to various clinical conditions, such as kidney grafting and resuscitation.^{2,3} Reactive oxygen species (ROS) play an important role in the development of IRI. It has been reported that

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ROS burst induces endoplasmic reticulum stress (ERS), mitogen-activated protein kinases (MAPK) and cell death.^{4,5}

ER is an intracellular organelle that plays a pivotal role in protein synthesis and folding, Ca^{2+} storage and signalling.⁶ As ER is stimulated by Ca^{2+} overload, ischaemia or hypoxia, its homeostasis changes followed by ERS.⁷ Growing evidences showed that when the stimuli is excessive or persistent, ERS of renal tubule epithelial cells is an initial response and plays a major pathogenic role in renal IRI.^{8–10} Thus, ERS inhibition may be a novel treatment for renal IRI.

Extracellular signal-regulated kinase 1/2 (ERK1/2) is a MAPK that is phosphorylated rapidly following renal injury.¹¹ As a downstream effector mechanism of ERS, activation of ERK1/2 has been suggested to be a regulator of renal IRI.^{12–15} For example, ERS may cause cell death at least by a classical BAX/BAK-dependent apoptotic response that can be inhibited by the ERK1/2 signalling pathway.¹⁶ However, it is unclear whether the ERS could modulate the ERK signalling pathways to ameliorate renal warm IRI evoked by the process of cardiac arrest/cardiopulmonary resuscitation (CA/CPR).

Edaravone (5-methyl-2-phenyl-2,4-dihydro-3H-pyrazol-3-one, EDR) is a novel free-radical scavenger that has been shown to prevent ERS induced by hypoxia and ischaemia.^{17,18} Although EDR is identified most recently as a protective factor for the development of renal IRI caused by renal arterial or hilar clamping,^{19–21} poor evidence can answer that whether EDR has the same protective effect in renal warm IRI evoked by CA/CPR. Experimental evidence suggests that GRP78 and CHOP activation is correlated with apoptosis as an ERS downstream event in renal IRI, and ERK activation is an important downstream mechanism of ERS. Therefore, we aimed to verify the hypothesis that EDR play a protective role on renal IRI by downregulated GRP78/CHOP/ERK pathway in a rat CA/CPR model.

Materials and Methods

Preparation of Experimental Rats

This animal study was approved by the Animal Ethics Committee of Guangxi Medical University (Animal Experimental Ethical Inspection no. 201811030). All animals received treatment in strict adherence to the National Research Council's 1996 Guidelines for the Care and Use of Laboratory Animals. Anaesthetics were titrated in all surgical procedures to avoid unnecessary pain. Male Sprague-Dawley rats weighing 200–230 g were purchased from the

Experimental Animal Center of Guangxi Medical University (China, Nanning). Animals were maintained at constant temperature ($23 \pm 2^\circ\text{C}$) with a 12 h light-dark cycle and free access to water and food.

Experimental Cardiac Arrest Rat Model Animal Preparation

All rats fasted for 12 h but had free access to water before the operation. Experimental rats were intraperitoneally injected with sodium pentobarbital (45 $\mu\text{g/g}$) for anaesthesia, and an additional dose of 10 $\mu\text{g/g}$ was supplemented at hourly intervals. Standard Lead II Electrocardiograph was used to monitor heart rhythm. A twenty-gauge catheter containing 5 IU/mL of sodium heparin saline was inserted into the right femoral vein for drug delivery, and another identical catheter was inserted into the right femoral artery for haemodynamic monitoring. Pressure transducers were connected to a four-channel physiological recorder (BL-420 E Biosystems, Chengdu Technology & Market Co. Ltd., China). After the 5 mins baseline electrocardiograph and physiologic measurements, temperature probes were placed into the rectum. During the experiment, the rectal temperature was adjusted to approximately 37°C using a heat lamp or ice pack.

Renal Warm Ischemia-Reperfusion Injury Induced by the Cardiac Arrest/Cardiopulmonary Resuscitation Model

The rat cardiac arrest (CA) model was established according to our previously reported method.²² Briefly, CA was induced by alternating current (12 V) from a stimulator through a pacing electrode placed in the oesophagus, as confirmed by a decrease in mean arterial pulse pressure (<10 mmHg) and by the appearance of asystole on the electrocardiograph (ECG). Cardiopulmonary resuscitation was initiated 8 min after the induction of CA with mechanical chest compressions (180 per minute) and effective ventilation (TV 8 mL/kg, respiration rate 40/min, and positive end-expiratory pressure 0 cm H₂O, oxygen concentration 100%) using a small animal ventilator with capacity control mode. After 1 min of CPR, one dose of epinephrine (0.4 $\mu\text{g/g}$) was given through the left femoral vein catheter. When ROSC was clarified by ECG activity with visible systole²³ and mean arterial pressure (MAP) ≥ 50 mmHg for ≥ 1 min, chest compressions were stopped. If ROSC is not achieved within 3 min of the onset of cardiopulmonary resuscitation, it is defined as a failure, and the animal is excluded from the study. After achieving ROSC, rats randomly received edaravone (3 mg/kg, $n=10$, EDR group) or equal volume normal saline

solution (n=10, NS group). The sham-operated rats only received the same experimental preparation without CA induction (n=10). The rats were individually fed in cages with dry litter and placed in a quiet room with air conditioning-adjusted temperature (room temperature 26°C).

Renal Function Analysis

The serum of the experimental rats was taken from the carotid artery at 24 h after ROSC. The values of serum creatinine (SCR), blood urea nitrogen (BUN), and cystatin-C (Cys-C) were monitored in the Department of Laboratory, the Second Affiliated Hospital of Guangxi Medical University.

Western Blot Analysis

Rats from each experimental group were anaesthetized and then sacrificed to take kidneys for Western blot detection at 24 hrs after ROSC. The expression levels of GRP 78, p-PERK, and CHOP in renal tissues were assessed by Western blot analysis. The prepared kidney tissues were weighed and homogenized in a glass homogenizer containing 1:10 (w/v) ice-cold whole cell lysis buffer (Beyotime Biotechnology, China, P0013B). The lysed protein was collected and centrifuged at $14,000 \times g$ for 15 mins at 4°C. The BCA Protein Assay Kit (Beyotime Biotechnology, China, P0010) was used to determine total tissue protein concentration. The tissue total protein (10–20 μ L) of protein lysates was separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Millipore, USA, 0.22- μ m pore diameter). The membranes were blocked with PBST containing 5% bovine serum albumin for 1 h and then incubated with primary antibody overnight at 4°C. β -Actin was used for normalization. The primary antibodies were as follows: primary antibodies ERK 1/2 (ab184699) and p-ERK1/2 (ab76299) were purchased from Abcam Plc, Cambridge, UK, and MFN2 (11925). β -Actin (CST, 4970S), GRP 78 (CST, 3183S), CHOP (CST, 2895S), Bax (CST, 14796S), Bcl-2 (Abcam, 182858), and GAPDH (Abcam, 181602). The membrane was washed three times with PBST and then incubated with secondary antibody (Cell Signalling Technology, USA, #5151, 1:15,000). Membranes were quantified by using a Western blot detection system with a Li-cor Odyssey Scanner imaging densitometer, and the results of the bands detected were quantified using ImageJ software (v1.33, NIH, Bethesda, MD, USA).

Statistical Analysis

All data are expressed as the mean $\pm$ standard deviations. Statistical analysis software is SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables between groups were compared using the Student's *t*-test. Groups were compared using one-way ANOVA followed by the Student–Newman–Keul test for post hoc comparisons. $p < 0.05$ was considered statistically significant.

Results

Edaravone Improves Renal Function After CA/CRP

To examine the kidney function, we compared Cys-C, SCR, BUN and Cys-C in all groups. The values of Cys-C, SCR and BUN were significantly increased in NS group compared with the Sham group ($p < 0.05$), suggesting an adverse outcome of renal function. Treatment of edaravone obviously decreased the level of Cys-C, SCR and BUN ($p < 0.05$), suggesting a protective effect on renal function (Figure 1).

EDR Reduced Endoplasmic Reticulum Stress Induced by CA/CPR

We perform the Western blot detection to determine the effect of EDR on the endoplasmic reticulum stress (ERS) induced by CA/CPR. The result showed that the expression of glucose-regulated protein (GRP78) and C/EBP homologous protein (CHOP) presented remarkable upregulation in the NS group ($p < 0.05$), while treatment of EDR significantly reduced the level of GRP78 and CHOP ($p < 0.05$). (Figure 2).

EDR Inhibits Phosphorylation of Extracellular Signal-Regulated Kinase 1/2

As shown in Figure 3, compared with the sham group, p-ERK1/2 was significantly elevated in the NS group ($p < 0.05$), while the expression of p-ERK1/2 was reduced in EDR group ($p < 0.05$).

EDR Decreases Caspase-3 and the Bax/Bcl-2 Ratio

Compared with the Sham group, cleaved caspase-3 and the Bax/Bcl-2 ratio were significantly upregulated in the NS group ($p < 0.05$); by the contrast, the expression of cleaved caspase-3 and the Bax/Bcl-2 ratio were significantly decreased in the EDR group. ($p < 0.05$). (Figure 4).

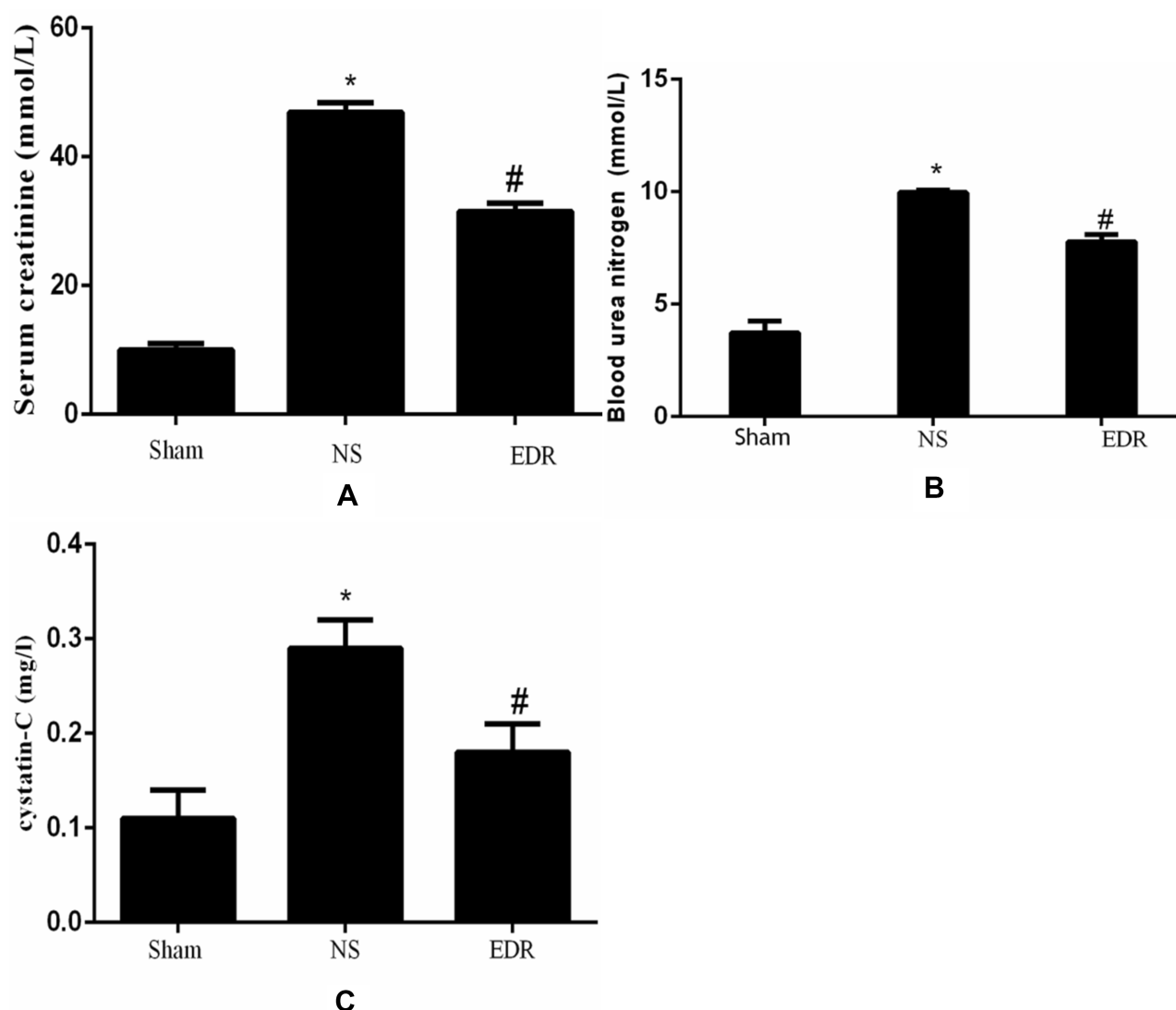


Figure 1 Comparison of SCR (A), BUN (B), and Cys-C (C) among the 3 groups. Data are expressed as the mean $\pm$ SD ($n = 10$ for each group). * $P < 0.05$ Vs the Sham group, # $P < 0.05$ Vs the NS group.

Abbreviations: SCR, serum creatinine; BUN, blood urea nitrogen; Cyst-C, Cystatin-C; NS, normal saline; EDR, edaravone.

Discussion

In this study, we found that renal warm ischaemia/reperfusion injury (IRI) induced by cardiac arrest/cardio-pulmonary resuscitation (CA/CPR) substantially upregulated the expression of Glucose Regulated Protein 78 (GRP78) and C/EBP-homologous protein (CHOP) at 24 hrs post-ROSC. EDR treatment ameliorated renal dysfunction and protected against renal damage, including a significant reduction in SCR, BUN, and Cys-C. Compared to the NS group, GRP78, CHOP, p-ERK1/2, caspase-3 expression and Bax/Bcl-2 ratio were significantly decreased in the EDR group, suggesting protective effect of EDR against endoplasmic reticulum stress (ERS) and apoptosis.

In our previous study, rats subjected to CA/CPR presented with excessive ROS production in the brain tissue.⁵ Overdose of ROS-induced ERS, which plays an important role in the development of several organ IRI in rats.^{24–26} while EDR can be used to reduce or block ROS-induced ERS to reduce organ IRI.²⁷ Hence, the purpose of our current study is to investigate the renal protective potential of EDR, a potent free-radical scavenger against renal warm IRI induced by CA/CPR.

The current data are consistent with previous results demonstrating that EDR pre-treatment protects against warm IRI in a variety of tissues and organs, including the heart and brain.^{28,29} In addition, a recent clinical study suggests that EDR may be a useful medication to protect kidney function

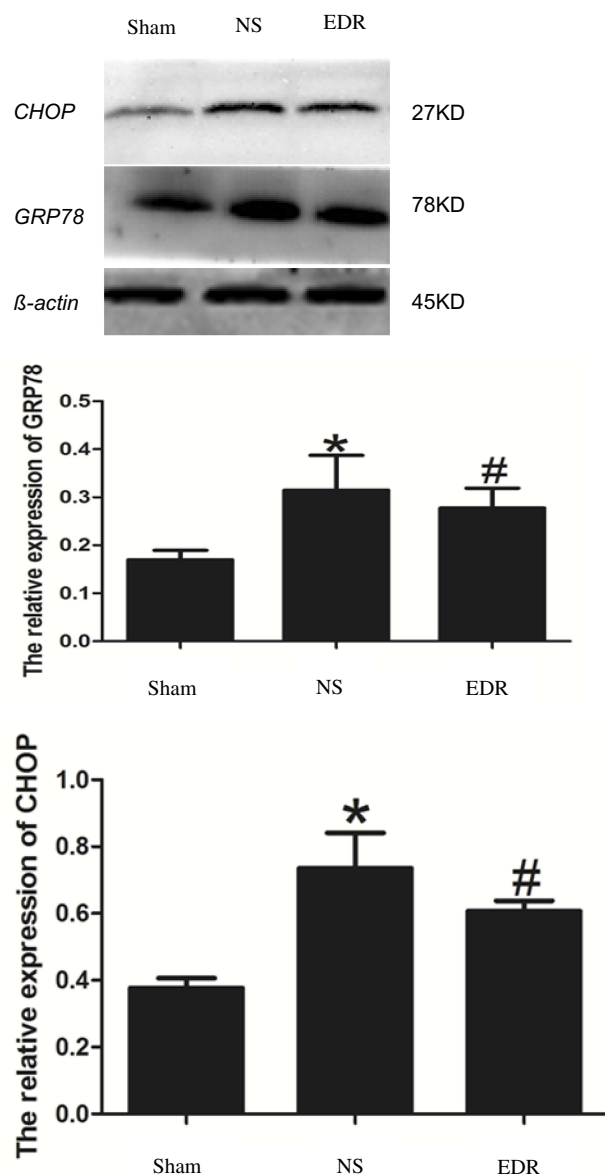


Figure 2 Western blot detection of CHOP and GRP78 among the 3 groups. Data are expressed as the mean $\pm$ SD. * $p < 0.05$ versus the Sham. # $p < 0.05$ versus the NS group.

Abbreviations: GRP78, glucose-regulated protein; C/EBP, homologous protein; NS, normal saline; EDR, edaravone.

in patients with acute ischaemic stroke.²¹ Overall, our findings are consistent with recently published data demonstrating that EDR exerts beneficial effects against organ ischaemic damage, including renal IRI.³⁰ As mentioned in the previous study, the animal model of the protective effect of EDR on renal IRI is mostly due to clamping of the renal artery or renal pedicle. However, there may be high incidence of vascular injury, infection, hilar injury, venous congestion, cold renal ischaemia during animal operation, and the observation period is only at the early phase of reperfusion.^{31–33} Importantly, these models did not fully mimic the most common clinical features of AKI

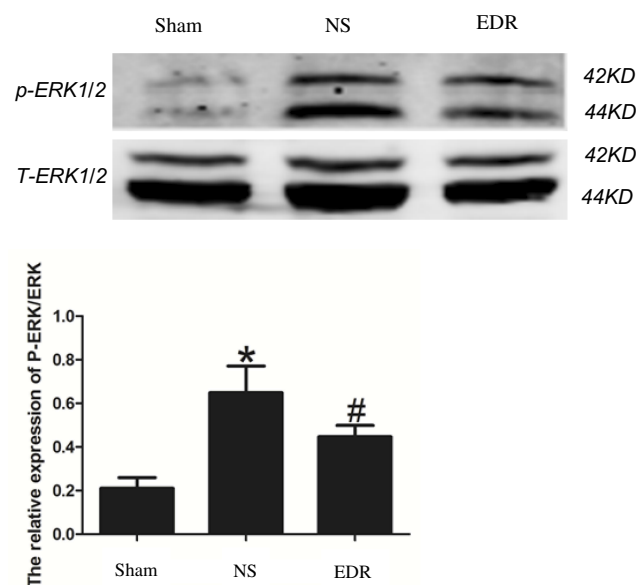


Figure 3 Western blot of total and phosphorylated ERK1/2 among the 3 groups. Data are expressed as the mean $\pm$ SD. * $p < 0.05$ versus Sham. # $p < 0.05$ versus NS group.

Abbreviations: ERK, extracellular signal-regulated kinase; NS, normal saline; EDR, edaravone.

after ROSC. Hence, we used the methods of transoesophageal alternating current stimulation to establish 8 min of CA in a rat model followed by conventional CPR to avoid the adverse effects and create a model of renal warm IRI that is close to clinical characteristics.

As an ERS downstream event, activation of GRP78/CHOP pathway is the key cellular response of ERS-induced apoptosis.^{9,34,35} Downregulation of GRP78/CHOP by intermedin significantly decreased apoptosis.¹⁰ In addition, a previous study revealed that EDR ameliorated early renal dysfunction and injury evoked by ischaemia/reperfusion in mice subjected to 45 min of bilateral renal IRI.³³ However, it is unclear whether EDR exerts protection against ERS in renal warm IRI evoked by CA/CPR. Our current research showed that EDR can ameliorate renal warm IRI by down-regulating GRP78 and CHOP expression, which was similar with previous studies regarding with EDR protection on dilated cardiomyopathy, spatial memory,^{28,29} cerebral ischaemia³⁶ and autoimmune myocarditis.³⁷

Studies have suggested that EDR can alleviate oxidative damage by inhibiting ERK1/2 activation.^{38,39} ERK1/2 is a member of the MAPK family, which plays an important role in cell survival and death.⁴⁰ Inhibition of ERK by EDR can alleviate oxidative damage. What is more, the ERK1/2 signalling pathway has also been shown to be

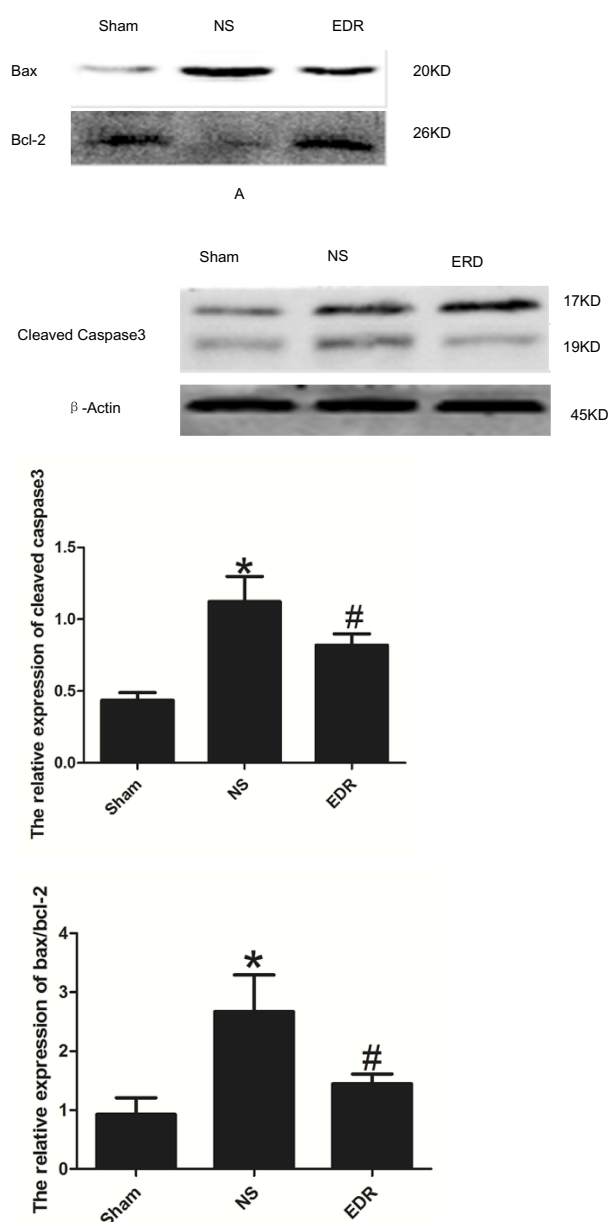


Figure 4 Western blot of caspase-3 (A) and the Bax/Bcl-2 (B) among the 3 groups. Data are expressed as the mean $\pm$ SD. * p < 0.05 versus Sham. # p < 0.05 versus NS group.

Abbreviations: NS, normal saline; EDR, edaravone.

involved in ERS-mediated apoptosis.^{41,42} Studies have demonstrated that IR dramatically increases phosphorylated ERK1/2 levels in the kidney.^{43,44} In addition, multiple studies proved that ERK activation was commonly protective in renal IRI.^{44,45} By the contrast, IRI dramatically increased phosphorylated ERK1/2 expression and promoted apoptosis have also been reported.⁴⁶ In our present work, we found that the expression of phosphorylated ERK1/2 was significantly elevated which accompanied with renal function decrease at 24 h after ROSC in NS

group. This result was consistent with our previous research, in which we found that inhibiting the expression of phosphorylated ERK1/2 can protect the brain from IRI in rat experienced CA/CPR.⁴⁷

Apoptosis is widely considered to be the main mechanism that induces cell death in renal IRI.^{48,49} Bax and caspase-3 are the most important downstream effectors for the ERK pathway^{50,51} that have also been demonstrated to involve in ERS-induced apoptosis.^{52–54} In renal cells, ischaemia activates Bax⁵⁵ and inhibits Bcl-2,⁵⁶ resulting in an increased Bax/Bcl-2 ratio.⁵⁷ On the other hand, caspases are well-known drivers of apoptotic cell death, cleaving cellular proteins that provide critical links in cell regulatory networks controlling dying cells.⁵⁸ Active caspase-3 leads to DNA fragmentation and formation of apoptotic bodies.⁵⁹ Caspase-3 and Bax have been demonstrated to mediate ERS-induced apoptosis.^{52–54} Our previous study demonstrated that inhibition of ischaemia-induced ERK1/2 kinase activity can reduce Bax and caspase-3 expression and improve organ function.⁴⁷ In the present study, we showed that EDR treatment decreased phosphorylation of ERK1/2 and apoptotic parameters including caspase-3 and the Bax/Bcl-2 ratio. These results suggested a reno-protective effect of EDR on a CA/CPR model. As mentioned previously, ERK activation is an important downstream mechanism of ERS.¹² We hold the opinion that EDR acts as an anti-apoptotic agent by downregulating GRP78/CHOP/ERK signal pathway.

Graphene was discovered in 2004 and its application in nanomaterials has been developing rapidly for drug delivery.⁶⁰ Graphene nanomaterials can enhance the efficacy in chemotherapy applications,⁶¹ increase drug loading capacity,⁶² and presents no toxicity to cells.⁶³ In addition, some improved graphene nanomaterials are able to cross the cell membrane and then accumulated more in the cell cytoplasm compared with the traditional ones. The capacity of transmembrane makes it possible to delivery drug to the targeted organ or cells more effectively.⁶⁴ Furthermore, a series of triggered drug delivery systems consist of graphene have been reported. The systems realize controlling drug delivery remotely and adjusting dosing regimens on demand.^{65,66} Therefore, it is worth to determine whether delivery of EDR targeting renal with graphene nanomaterials could obtain a better outcome in renal warm ischemia-reperfusion injury post CA/CPR.

In conclusion, we found that EDR ameliorates renal warm IRI by downregulating ERS and its downstream effectors in a rat AKI model evoked by CA/CPR. These data may provide evidence for future therapeutic benefits of EDR against AKI induced by CA/CPR.

Ethics Approval and Consent to Participate

The present study was approved by the Committee on the Ethics of Animal Experiments and Human Subject Research of the Guangxi Medical University.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

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