

Original Article

Zinc sulphate preconditioning alleviates rat myocardial hypoxia reoxygenation injury *in vitro*: activation of the Nrf2/ARE signaling pathway

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Abstract: Background: Zinc, as an important microelement, has been reported to alleviate myocardial ischemia reperfusion injury (MIRI). However, the mechanism was not clear yet. Aim: The purpose of this study was to determine if the Nuclear factor erythroid2-related factor/antioxidant response element (Nrf2/ARE) signaling pathway was involved in the cardioprotection of zinc sulfate (ZnSO₄) preconditioning. Methods: By utilizing the Langendorff perfusion system, we separately and randomly allocated the isolated Sprague-Dawley rat cardiomyocytes into the following four groups: Con group (13.75 h aerobic cultivation), H/R group (after normal cultured for 12 h, 45 min hypoxia plus 60 min reoxygenation was performed), ZnP group (cultured with 10 μM ZnSO₄ for 30 min before hypoxia) and L+ZnP group (cultured with 10 μM luteolin, a blocker of Nrf2/ARE pathway, for 11.5 h before ZnSO₄ preconditioning). After that, we tested the myocardial injury and the expression (genes and proteins) of Nrf2/ARE signaling pathway in each group. Results: We found that ZnSO₄ preconditioning eased the hypoxia/reoxygenation injury of cardiomyocytes, but luteolin canceled such protective effect. And the levels of Nrf2, NADH-quinone oxidoreductase-1 (NQO1), heme oxygenase 1 (HO-1) and superoxide dismutase 1 (SOD1) were increased in ZnP group (all $P < 0.05$ vs. Con), but significantly decreased in L+ZnP group (all $P < 0.05$ vs. ZnP). Conclusion: ZnSO₄ preconditioning is likely to alleviate rat myocardial hypoxia reoxygenation injury *in vitro* by activating the Nrf2/ARE signaling pathway.

Keywords: ZnSO₄ preconditioning, Nrf2, cardioprotection, hypoxia reoxygenation injury, Langendorff

Introduction

Myocardial ischemic diseases are threatening the public health, and restoring blood flow in ischemia area as soon as possible is the key to handle it. However, restoring perfusion of the ischemia tissue leads to myocardial ischemia reperfusion injury (MIRI), which has greatly impacted on various recanalization treatments [1].

The mechanism of myocardial protection is extremely complex, and many signal transduction pathways are involved in and coordinated with each other [2]. As a nuclear transcription factor, Nrf2 is a very important endogenous antioxidant stress factor. And as one of the many signal transduction pathways, the Nrf2/ARE sig-

nal pathway is widely distributed in the cardiovascular system and crucial in anti-oxidation stress response [3]. Our pervious study proved that *in vitro* model of rat, both ischemia postconditioning and pharmacological postconditioning can activate the Nrf2/ARE signaling pathway and alleviate MIRI [4].

As an important microelement, Zinc is the essential material of enzymes, proteins and transcription factors [5]. Report shows that pretreated with zinc, the cardiomyocytes can resist MIRI through improving myocardial energy metabolism, reducing calcium overload, inhibiting inflammatory response and decreasing myocardial apoptosis [6]. Previous studies also find that, after pretreated with zinc, the expression of the peroxiredoxins and phase 2 detoxify-

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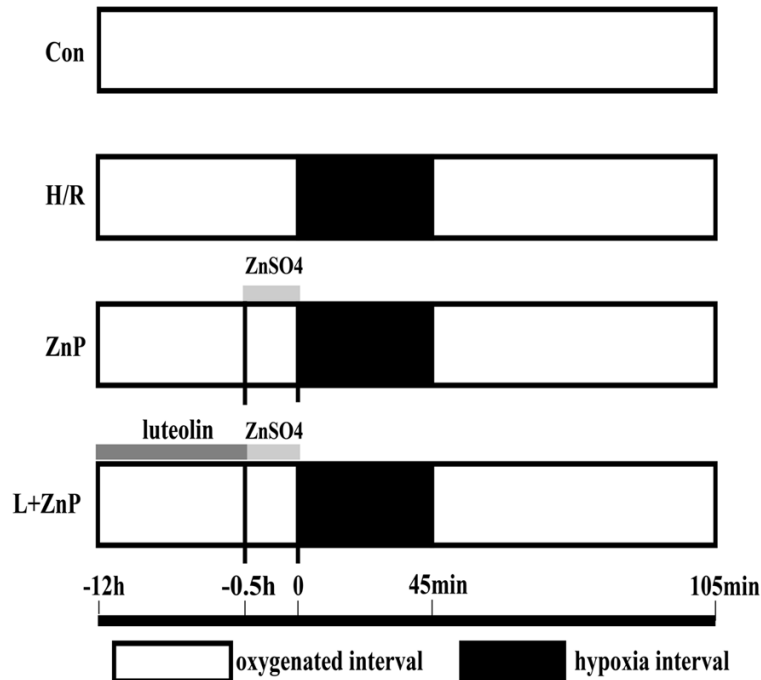


Figure 1. Treatment protocol to cardiomyocytes. After isolated from the SD rat heart, cardiomyocytes was randomly divided into 4 groups: Con, H/R, ZnP and L+ZnP. Con group: After 12 h regular cultivated with the oxygenated gas (95% air mixed with 5% CO₂), the cardiomyocytes was consistently cultivated with the same gas for 105 min. H/R group: After 12 h regular cultivated with the oxygenated gas, the cardiomyocytes was successively cultivated with the hypoxic gas (95% N₂ and 5% CO₂) for 45 min and the oxygenated gas for 60 min. ZnP group: On the basis of the H/R group, ZnSO₄ (10 μM) was added into the cell culture Medium 199 (M199) for 30 min before hypoxia. L+ZnP group: On the basis of the ZnP group, luteolin (10 μM) was added into the M199 for 11.5 h before ZnSO₄ preconditioning.

ing enzyme gene can be up-regulated by the activation of Nrf2 /ARE signaling pathway in the retinal pigment epithelial cells [7, 8]. Hence it is highly possible that the pretreatment of exogenous zinc would reduce MIRI through activating the Nrf2/ARE signaling pathway in cardiomyocytes.

Present study built the myocardial hypoxia re-oxygenation model in adult rat by using Langendorff perfusion system. We aimed to figure out the relationship between the myocardial protection effect of ZnSO₄ preconditioning and the Nrf2/ARE signaling pathway.

Materials and methods

Animal

Experimental animal was healthy male Sprague-Dawley (weight 250-300 g, 16 to 20 weeks

old). And before the beginning of the experiments, rats were housed in groups of three to four under a standard 12-hour light/dark cycle with accessing to food and water ad libitum for at least 1 week. Rats testing procedures and general handling complied with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication 88.23, revised 1996).

The culture of adult rat cardiomyocytes

The rats were intraperitoneal injected with 1% pentobarbital sodium (40 mg/kg) and heparin (250 u/kg). After anesthesia, thoracotomy and resection of the heart were performed rapidly, and 750 uM Ca²⁺ liquid (37°C) was used to wash out the blood in the isolated hearts. Aortic root was quickly fixed on the Langendorff perfusion system, and the flow rate was set at 9 mL/min/g. Under the temperature of 37°C, successively perfusion was

performed with the oxygenate Ca²⁺ liquid (750 uM, 2 min), EGTA (100 μM without Ca²⁺, 4 min) and type II collagenase solution (0.1%, 10~15 min). After the perfusion, the heart was cut from the cardiac base to apex. And then, type II collagenase solution was used to digest the cardiac tissue on a shaking water bath at 37°C for 5 min. The digested solution was filtered by nylon mesh and then the filtrate was naturally subsided in water bath (37°C). After repeating the procedure of digestion, filtration and sedimentation for 4 cycles, the collected precipitate was successively washed by the enzyme elution for 3 times and the Cell Culture Medium 199 (M199) for 3 times. In a 10⁴-10⁶/cm² density, the myocardial cells was covered on the six-wells plates, pretreated by laminin. In the end, the myocardial cells were cultivated with M199 and normal gas (95% air mixed with 5% CO₂) for 20 h.

ZnSO₄ activates Nrf2 in cardioprotection

The cardiomyocytes of each rat were randomly divided into the following four groups (**Figure 1**): Control group (Con, n=8), consistently cultured for 13.75 h; Hypoxia Reoxygenation group (H/R, n=8), 45 min hypoxia plus 60 min reoxygenation were performed after 12 h aerobic cultivation; ZnSO₄ preconditioning group (ZnP, n=8), 10 μM ZnSO₄ [9] was added into M199 for 30 min before hypoxia; Luteolin plus ZnSO₄ preconditioning group (L+ZnP, n=8), on the basis of the ZnP group, 10 μM luteolin [10] was added into M199 for 11.5 h before ZnSO₄ preconditioning. After treated in different groups, cardiomyocytes was collected for detection of genes/proteins and myocardial injury.

Transmission electron microscopy

The cardiomyocytes in each group was centrifuged at the speed of 1200 g under 4°C for 10 min, and then the supernatant was abandoned. After the 2.5% glutaraldehyde (4°C) was added into the centrifuge tube, the cardiomyocytes was cooled in refrigerator at 4°C for 1~1.5 h, then dehydrated by acetone in gradient concentration and imbedded with epoxy resin. Cut from each sample, ultrathin sections were double dyed by the uranyl acetate and lead citrate. At last, the dyed ultrathin sections were observed with a transmission electron microscopy (H7500, Hitachi Japan) and the myocardial mitochondria injury was evaluated by Flameng score criterion [11]. Flameng score criterion: 0, normal mitochondria was filled with granules; 1, mitochondria was nearly normal but granules were lost; 2, lost granules, lucid matrix and swollen mitochondria; 3, lost granules, lucid matrix, fractured mitochondrial cristae; 4, ruptured mitochondrial inner and external membranes, vacuolated mitochondria.

LDH leakage rate calculating

According to the LDH Elisa Kit (Jian Cheng biological company, China), the adherent cells with culture medium was centrifuged at the speed of 2500 g for 10 min in each group, and detected the supernatant LDH activity. The precipitate was continuously mixed with the PBS buffer and centrifuged at the speed of 1000 g for 3*10 min. At last, the cardiomyocytes was ultrasonic broken drastically in an ice-water bath, and the homogenization buffer was detected by the LDH Elisa Kit to calculated the cytoplasm LDH activity. The LDH leakage rate was calculated according to following formula: LDH leakage rate =

$$\frac{\text{supernatant LDH activity} \times 2}{\text{supernatant LDH activity} \times 2 + \text{cytoplasm LDH activity}} \times 100\%$$

RT-PCR

A RNAiso Plus Kit (TaKaRa company, China) was used to extract the total mRNA. Total mRNA concentration and purity of supernatant in each group was quantified by Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, USA). The reverse transcription of cDNA from total mRNA was conducted by a PrimeScript RT reagent Kit (TaKaRa company, China) and 2400 PCR instrument (Bio-Rad, USA) under the temperature profile: 37°C/15:00 min-85°C/0:05 min. With the help of primer, SYBR Premix Ex Taq II (TaKaRa, company, China), and CFX Connect instrument (Bio-Rad, USA), the amplification of the cDNA was performed under the following temperature profile: 95°C/3:00 min-[95°C/0:10 min-61.5°C/0:30 min] 40 cycles. The primer sequences were shown as below: Nrf2 (forward TTGGCAGACATTCC CATTGTGA3, reverse ATCAGTCATGGCCGTCTCCAG), HO-1 (forward AGGTGCACATCCGTGCAGAG, reverse TCCAGGGCCGT ATAGATATGGTACA), SOD1 (forward AATGTGTCCATTGAAGA TCGTGTGA, reverse GCTTCCAGCATTCCAGTCTTTGTA), NQO1 (forward TGGAAGCTGCAGACC TGGTG, reverse TTGTCATACATG GTGGCATACGTG), GAPDH (forward GGCACAGTCAAGGCTGA GAATG, reverse ATGGTGGTG AAGACGCCAGTA).

Western blot

We determined Nrf2, HO1, SOD1 and NQO1 by Western blot analysis. Equal amounts of total protein (40 μg) from cardiomyocytes was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes for immunoblotting. The primary antibodies were rabbit polyclonal to Nrf2, HO1, SOD1, NQO1 and GAPDH (Abcom, USA). Proteins expression was visualized with fluorescent secondary antibody (goat anti-rabbit, LI-COR, USA) and detected by the Odyssey Infrared Imaging System (LI-COR, USA).

Statistical analysis

Statistics were performed using GraphPad statistical software (Version 5.02 for Windows, GraphPad Software, USA). Multiple comparison between different groups were analyzed by one-way ANOVA or Dunnetts's T3 test. Variables are expressed as means ± SD, and statistical

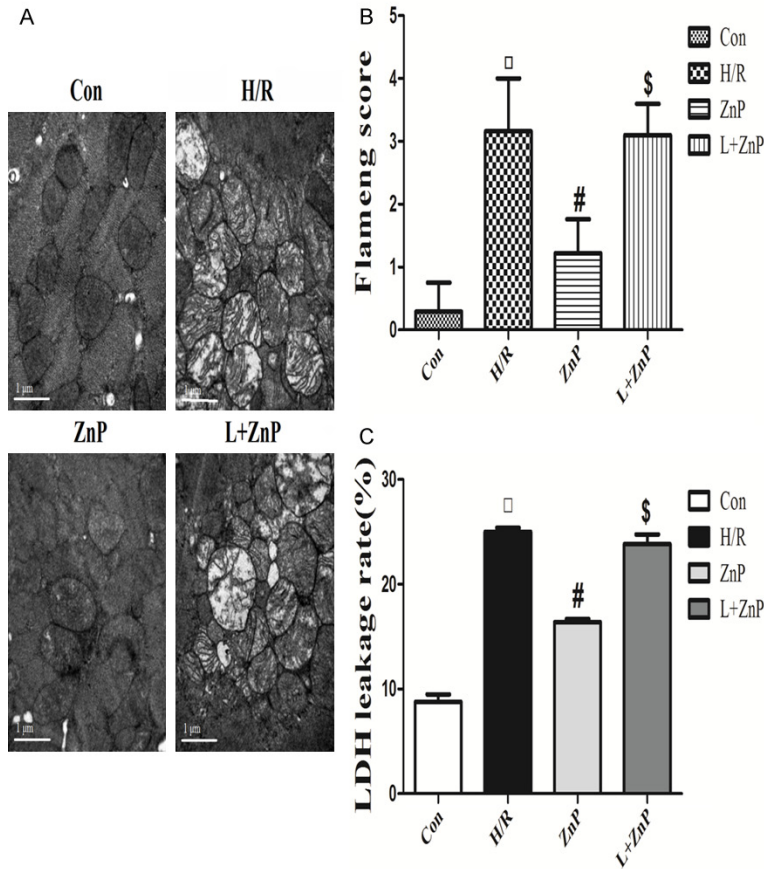


Figure 2. Morphologic and LDH leakage rate of myocardium. A. The myocardial ultrastructure of 4 groups were shown by transmission electron microscopy images (20,000× magnification). Myocardial ultrastructure were nearly normal in Con and ZnP group. But in H/R and L+ZnP group, myocardial ultrastructure were seriously damaged; mitochondrial swell and vesiculation were all over the cardiomyocytes. B. Compared with H/R group, the mitochondria Flameng score of ZnP group was significantly decreased. But compared with ZnP group, the mitochondria Flameng score of L+ZnP group was significantly increased. C. ZnSO₄ preconditioning significantly decreased the LDH leakage rate of cardiomyocytes suffered by hypoxia reoxygenation injury, but it was removed by luteolin. Data are presented as mean ± standard deviation. □P<0.05 versus Con group; #P<0.05 versus H/R group; \$P<0.05 versus ZnP group.

significance was reached at a two-sided *P* value ≤0.05.

Results

Morphologic of myocardium

As shown in **Figure 2A**, the H/R and L+ZnP group exhibited serious myocardial mitochondria damage, which was evident as excessive swelled mitochondria and disordered myocardial fibers. The average Flameng score of H/R group was significant higher than the Con group (3.16 vs. 0.29, *P*<0.05, **Figure 2B**). But in

ZnP group, the damage of myocardial mitochondria caused by hypoxia/reoxygenation was relieved, the membrane was intact, myocardial fibers was in order and myocomma was clear. The average Flameng score of ZnP group was significant lower than the H/R group (1.22 vs. 3.16, *P*<0.05, **Figure 2B**). However, the protective effect of ZnSO₄ preconditioning was eliminated by administration of luteolin in the L+ZnP group.

The LDH leakage rate of cardiomyocytes

As shown in **Figure 2C**, the LDH leakage rate of H/R group was significantly higher than Con group (25.014% vs. 8.750%, *P*<0.05); the LDH leakage rate of ZnP group was lower than H/R group (16.398% vs. 25.014%, *P*<0.05); the LDH leakage rate of L+ZnP group was significantly higher than the ZnP group (23.848% vs. 16.398%, *P*<0.05).

Expression of Nrf2-related proteins and genes

As shown in **Figure 3**, when compared with Con group, the expression level of Nrf2-related proteins and genes (Nrf2, HO-1, SOD1 and NQO1) in H/R group were significant-

ly increased (*P*<0.05). The expression level of Nrf2-related proteins and genes in the ZnP group were higher than H/R group (*P*<0.05). While compared with the ZnP group, the expression level of Nrf2-related proteins and genes in the L+ZnP group were significantly decreased (*P*<0.05).

Discussion

Present study adopted the adult rat cardiomyocytes to explore the role of ZnSO₄ preconditioning in the myocardial hypoxia reoxygenation injury *in vitro*. Compared with the Con group,

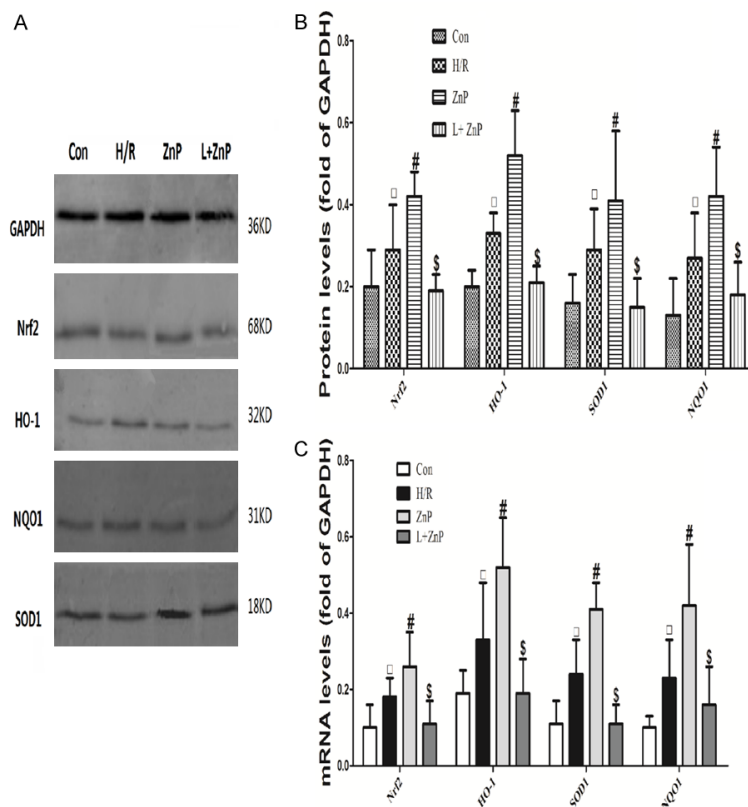


Figure 3. The expression of Nrf2-related proteins and genes in cardiomyocytes. A. Western Blot was performed to evaluate the level of Nrf2, HO-1, SOD1 and NQO1. B. Compared with H/R group, the Nrf2-related proteins (Nrf2, HO-1, SOD1 and NQO1) of ZnP group were upregulated. But compared with ZnP group, the Nrf2-related proteins of L+ZnP group were significantly decreased. C. Compared with H/R group, the Nrf2 and its target genes (HO-1, SOD1 and NQO1) of ZnP group were upregulated. But compared with ZnP group, the Nrf2 and its target genes of L+ZnP group were significantly decreased. Data are presented as mean ± standard deviation. □P<0.05 versus Con group; #P<0.05 versus H/R group; \$P<0.05 versus ZnP group.

the myocardial mitochondria score and myocardial LDH release in the H/R group were significantly increased. However, ZnSO₄ preconditioning significantly decreased the mitochondria Flameng score and LDH release. In addition, the level of Nrf2-related genes and proteins (Nrf2, HO1, NQO1 and SOD1) were increased after ZnSO₄ pretreatment. But the administration of luteolin, a blocker of Nrf2/ARE pathway, eliminated the protective effects of ZnSO₄ pretreatment.

ZnSO₄ preconditioning reduced myocardial hypoxia reoxygenation injury

Previous studies show that MIRI can break the homeostasis of zinc, and external zinc supplement exhibits a protective effect against MIRI.

Xu Z et al. [12] reported that zinc decreases in rat cardiomyocytes with MIRI, ischemia postconditioning can alleviate MIRI through increasing intracytoplasmic zinc and activating the RISK signaling pathway in the period of reperfusion. However, TPEN, a zinc chelating agent, can inhibit the myocardial protection of ischemia postconditioning. So, zinc might play an important role in the cardioprotection of ischemia postconditioning by activating RISK signaling pathway. Another experiment shows that, after the isolated rat heart suffered from ischemia-reperfusion injury, the concentration of zinc in the cardiomyocytes obviously decreased in the reperfusion period. And zinc chloride postconditioning can reduce cardiac infarction area caused by MIRI, which can also be inhibited by TPEN [13]. The signaling pathways of PI3K/Akt, ERK and PKC have also been proved to get involved in the zinc-induced alleviation of MIRI [14, 15].

Our data also show that ZnSO₄ preconditioning alleviates the hypoxia reoxygenation injury of cultured cardiomyocytes. ZnSO₄ preconditioning reduces the mitochondria Flameng score and the LDH release of cardiomyocytes in hypoxia reoxygenation injury.

The cardioprotection mechanism of ZnSO₄ preconditioning: against oxidative stress by activating Nrf2/ARE pathway

In the period of MIRI, the obstruction of energy metabolism restrains the reactive oxygen species (ROS) scavenging system, which increases the concentration of ROS in cardiomyocytes. ROS further causes intracellular calcium overload and cellular damage (biomembrane, protein, nucleic acid and mitochondria) [1, 16]. So, how to alleviate oxidative stress injury in MIRI is the key to cardioprotection.

ZnSO₄ activates Nrf2 in cardioprotection

As the key factor of anti-oxidative stress, Nrf2 can regulate its downstream genes to against peroxidation and plays an important role in cardioprotection [17, 18]. Under normal state, Nrf2 is anchored with the inhibitor Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and degraded by the ubiquitin-proteasome system [19, 20]. Under stressed condition, the ability of ubiquitin-proteasome system is inhibited, which result in the separation of Nrf2 from Keap1, which then translocates to the nucleus and forms heterodimers with Maf proteins. The Nrf2 antioxidant response element binds in promoter regions of downstream detoxifying enzymes, and increases the expression of anti-oxidant enzymes to maintain the body's oxidation-reduction equilibrium [20, 21].

Current researches show that, in a variety of organs, zinc can activate the Nrf2/ARE signaling pathway, which in turn generates the effect of oxidative stress resistance, and protects the organism from oxidative damage [22, 23]. As show in the study of human renal tubular cells HK11 and mice kidney with diabetes *in vivo*, Zinc supplementation can activate the Akt and increase the expression of Nrf2, which relieves the oxidative stress, inflammation and kidney fibrosis [24]. In the study of retinal epithelial cell ARPE-19, zinc also can activate the Nrf2/ARE signaling pathway to resist oxidation and detoxicate by increase glutathione; but after the Nrf2 knock-down by siRNA, this kind of anti-oxidant and detoxification function is suppressed [8].

As the control center of anti-oxidation stress, Nrf2 also exists in cardiovascular system. HO-1, NQO1 and SOD1 are the downstream genes of Nrf2/ARE signaling pathway, so the expression level of genes and proteins (Nrf2, HO-1, NQO1 and SOD1) can indirectly reflect the ability anti-oxidation stress in cardiomyocytes [25, 26]. Ours experiment show that, ZnSO₄ preconditioning significantly up-regulates the genes and proteins level of Nrf2, SOD1, HO1 and NQO1. This result indicates that ZnSO₄ preconditioning activates the transcription of Nrf2 gene, increases the accumulation of Nrf2 in the nucleus to combine with ARE, up-regulates downstream genes and proteins (antioxidant and detoxifying enzyme) expression to alleviate oxidative stress injury.

Furthermore, as a Nrf2/ARE signaling pathway blocker [10], luteolin can block the gene expres-

sion of redox reaction. In our study, after the administration of luteolin, the cardiac protective effects of ZnSO₄ preconditioning is eliminated, which indicates that luteolin can restrain the activation of Nrf2/ARE signaling pathway induced by ZnSO₄ preconditioning and reduce the downstream genes and proteins expression of Nrf2. So, this further confirms that the Nrf2/ARE signaling pathway plays an important role in relieving MIRI by ZnSO₄ preconditioning.

Base on our results, we can infer that, ZnSO₄ preconditioning is likely to activate Nrf2/ARE signaling pathway, thereby up-regulate the genes and proteins (Nrf2, HO-1, NQO1 and SOD1) expression of Nrf2. Therefore, it can improve the ability of cardiomyocytes to resist oxidative stress, alleviate oxidative damage and protect the cardiomyocyte from hypoxia reoxygenation injury. However, in order to confirm the accuracy of the mechanism, further work need to do on animal model *in vivo*.

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Disclosure of conflict of interest

None.

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