

## Original Research Article

# Down-modulation of endoplasmic reticulum stress-initiated apoptosis by huperzine A in isoproterenol-provoked myocardial infarction rat model: Role of Nrf2/HO-1 signaling axis

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### Abstract

**Purpose:** To investigate the myocardial protective effect of huperzine A (HPA), a sesquiterpene alkaloid, in a rat model of isoproterenol (ISP)-provoked MI and ER stress.

**Methods:** Three groups of rats were used: control, ISP and ISP+HPA groups. The following indices were assayed using standard protocols: oxidative stress parameters, including NADPH oxidase 4 (NOX4), reactive oxygen species (ROS), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1); indices of calcium homeostasis, namely, sarcoplasmic and endoplasmic reticulum calcium ATPase isoform 2a (SERCA2a); ER stress parameters, viz, protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), glucose-regulated protein 78 (GRP78), and C/EBP homologous protein (CHOP); and indices of apoptosis, i.e., B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase-12.

**Results:** Oxidative/ER stress and cardiomyocyte apoptosis were up-modulated ( $p < 0.05$ ), while SERCA2a, a key calcium handling channel, was downmodulated in the ISP group ( $p < 0.05$ ). In contrast, HPA treatment ameliorated these ISP-induced myocardial aberrations. ( $p < 0.05$ ).

**Conclusion:** These results indicate that HPA might be a potential therapeutic candidate for MI and associated cardiac problems.

**Keywords:** Caspase-12, ER stress, Huperzine A, Myocardial infarction, SERCA2a

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## INTRODUCTION

Cardiovascular diseases (CVDs) account for about 40 % of the deaths in China, a country with the highest CVD burden due to aging population and lifestyle-related factors [1,2]. Myocardial infarction (MI), a form of coronary heart disease

(CHD), arises from the death of cardiomyocytes heralded by extended ischemic coronary illness. Isoproterenol (ISP), a non-selective  $\beta$ -adrenoceptor agonist, is a well-documented non-surgical actuator of MI and myocardial remodelling-associated repercussions in rodent models [3, 4]. It is worthy of note that

supramaximal ISP doses elicit aberrant pathological changes in rat heart which are similar to those of heart failure (HF) in humans [5].

Several studies have reported that in MI, oxygen deprivation triggers endoplasmic reticulum (ER) stress-initiated apoptosis in cardiomyocytes, leading to myocardial injury, hypertrophy and heart failure [6, 7]. Apart from the conventional mitochondria-centred (intrinsic) and Fas/FasL-mediated (extrinsic) cell death pathways, ER stress-mediated apoptosis has been recognized as a pivotal pathway in myocardial cell death. Accretion of unfolded and misfolded proteins in the ER triggers apoptosis of cardiomyocytes. If there is any functional deficit in the unfolded protein response (UPR), a cascade of cellular stress signaling responsible for ER stress mitigation and homeostatic reinstatement occurs.

Aberrant shift in the oxidant/antioxidant equilibrium towards the oxidant overload, and dysregulated calcium handling system in ER, culminate in ER stress in cardiomyocytes [7]. Perturbations in the synchronized shuttling of  $Ca^{2+}$  within and across the cardiomyocyte lead to cardiac dysfunction. Various regulatory members of the calcium handling system in the heart, including sarco(endo)plasmic reticulum  $Ca^{2+}$ -ATPase (SERCA2a), phospholemman (PLM), phospholamban (PLN), sodium-calcium exchange (NCX), FK506-binding protein (FKBP12.6), and ryanodine receptor type 2 (RyR2) are known to be altered in MI, ventricular hypertrophy and cardiac failure [8, 9]. Calcium dyshomeostasis and oxidative stress are the products of flawed interplay between calcium and ROS signaling circuits. Nuclear factor erythroid-2 related factor 2 (Nrf2), a protein central to the regulation of antioxidant and detoxification functions, is negatively modulated by ER stress signals. Besides, Nrf2 accords resistance to myocardial injury and involves homeostatic myocardial remodelling against trauma and noxious insults [10].

Although extensive mechanistic studies on cardiac pathology/treatment are available, lack of robust and precise risk prediction models, and dearth of therapeutic strategies are pushing the horizon of drug discovery for MI and associated repercussions [2]. Hence, there is a dire necessity to discover novel therapeutics and appropriate biomarkers for MI. Bio-isolates with targeted actions are emerging as new facets of herbal renaissance in the treatment of various ailments, including cardiac dysfunction [3,11]. Thus, huperzine A (HPA), a sesquiterpene alkaloidal isolated from *Huperzia serrata* and

used in Chinese folklore medicine, has attracted lots of attention. In fact, an array of studies has reported the beneficial effects of HPA against ischemia/infarction in heart, brain and liver [12-14]. However, earlier studies did not investigate the effect of HPA on ISP-provoked MI from the perspective of ER stress-mediated myocardial injury. This was the focus of the present investigation

## EXPERIMENTAL

### Animals and treatment regimen

Forty Sprague-Dawley rats (210-250 g) were randomly divided into control, ISP and ISP+HPA groups. Rats in control group were injected with physiological saline (100  $\mu$ l/100 g, i.p.) for seven consecutive days, while those in ISP group received isoproterenol (85 mg/kg, s.c.) for 2 successive days (24 h inter-dose interval). Rats in HPA group received huperzine A at a dose of 500  $\mu$ g/kg, i.p. for 7 consecutive days, along with isoproterenol, as in ISP group. The rats were kept in a temperature- and humidity-regulated setting (21–23 °C and 60 – 70 % relative humidity) in an environment with 12-h light/12-h dark cycle. All rats received standard murine chow and tap water *ad libitum*. The experimental protocols used in this study were approved by the Ethics Committee of our institution, and the procedures were conducted in harmony with the NIH ethical guidelines. Heart tissues from control, ISP and HPA-treated groups were excised and rinsed in normal saline (ice-cold) before snap-freezing in liquid nitrogen, and kept at –80 °C for further investigation.

### Evaluation of ROS

Estimation of cardiac tissue ROS was performed using ROS assay kits (Jiancheng Bioengineering, Nanjing, China). The supernatant (90  $\mu$ L) from the tissue homogenate was slightly agitated with about 10  $\mu$ L of DCFH-DA solution, and the resultant solution was poured into a 96-well microplate. Then, the microplate was incubated at 37°C for 30 min. Thereafter, the fluorescence intensity of DCF was measured in a fluorescence microplate reader (HITACHI, Japan).

### Histological investigations

For the histological analysis, the myocardial tissues were fixed in 10 % neutral buffered formalin. The middle myocardial segment was cut into 5-mm thick sections and subjected to Masson trichrome staining.

## Immunohistochemical measurement of caspase-12

In line with the protocol given in immunohistochemical staining kit, myocardial paraffin sections were treated with H<sub>2</sub>O<sub>2</sub> (3 % v/v) and then subjected to microwave oven heating with 10-mM citrate buffer for 10 min. Primary antibody against caspase-12 (1:50 dilution, Beijing Biosynthesis Biotechnology Co. Ltd., China), was added, and the tissue sections were incubated overnight at 4°C. Then, the secondary antibody was added, followed by incubation for 30 min and staining with 3,3'-diaminobenzidine, a chromogen. The stained slides were analysed using Image-Pro Plus analysis system (Media Cybernetics, USA).

## Western blot analysis

Heart sections were cut and homogenized in RIPA ice bath, using an electric homogenizer. The homogenate was centrifuged at 16,000 g at 4°C for 20 min to obtain the supernatant containing the proteins. The proteins were subjected to separation using SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes. The membranes were treated with blocking buffer. After washing, the membranes were incubated overnight at 4°C with specific primary antibodies for NOX4, Nrf2, HO-1, SERCA2a, PERK, GRP78, CHOP, Bcl2 and Bax (Cell Signaling Technology, USA). Thereafter, the membranes were rinsed and subsequently incubated with HRP (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., CA) at room temperature. Post-incubation, quantitative analysis of the antibodies was done using gel image processing system.

## Statistical analysis

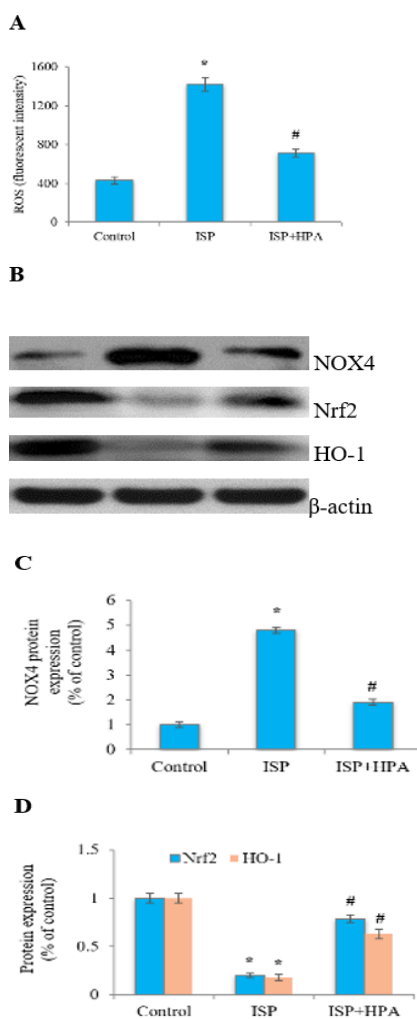
The SPSS software (V13.0; SPSS, Inc., USA) was used for statistical analysis. One-way analysis of variance (ANOVA) was applied, along with Tukey's post-hoc test for comparisons among different animal groups. Significant level was kept at  $p < 0.05$ .

## RESULTS

### Huperzine A reversed oxidative stress in ISP-provoked MI rats

Orchestration of ROS generation by NOX4 is a major signal in cardiac dysfunction, including MI. Hence, the ROS and NOX4 levels were assayed in all the animal groups. The results are shown in

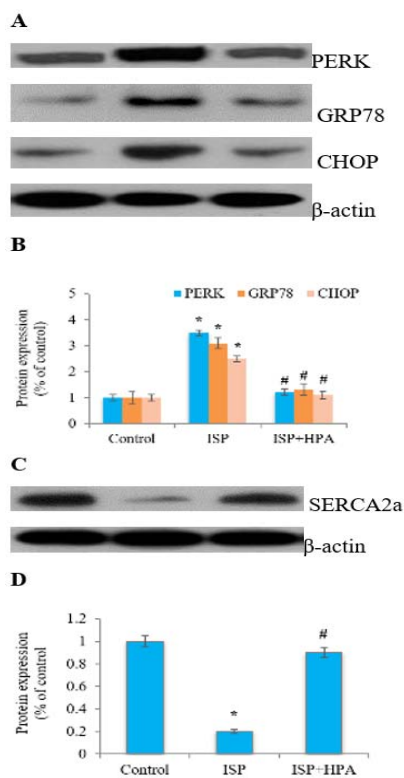
Figure 1. The cardiac tissue of ISP-challenged rats had markedly ( $p < 0.05$ ) increased ROS (3.3-fold vs control) and NOX4 protein expression (4.8-fold vs control) levels. Besides, the Nrf2/HO-1 antioxidant/anti-apoptotic signaling pathway was also downregulated, as indicated by about 80 and 82 % reductions in the cardiac protein expressions of Nrf2 and HO-1, respectively, when compared to corresponding expression levels in the control. Thus, HPA functioned as an effective antioxidant against ISP-provoked oxidant stress and myocardial infarction.



**Figure 1:** Effect of HPA on oxidative stress parameters in ISP-provoked MI rats. A: ROS levels in the myocardial tissues of the animal groups; B: Representative bands of NOX4, Nrf2 and HO-1 expressions, with β-actin internal control; C: protein expressions of NOX4, and D: Nrf2 and HO-1 represented as % of that of internal control. Data are indicated as mean ± SD. \* $P < 0.05$ , ISP vs control; # $p < 0.05$ ; ISP+HPA vs ISP

## Huperzine A ameliorated calcium dyshomeostasis-associated ER stress in ISP-provoked MI rats

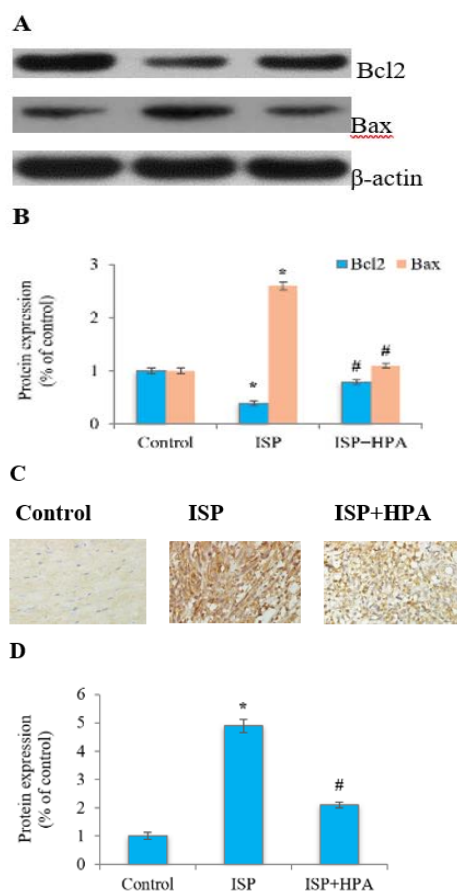
Dysregulation calcium handling circuitry due to oxidant load in the cardiomyocyte elicits ER stress. The PERK, GRP78 and CHOP, key biomarkers ER stress level, were assayed in all the rat groups. The results are shown in Figure 2A and Figure 2B). It was observed that ISP-challenged cardiac tissues expressed significantly higher protein levels of PERK (3.5-fold), GRP78 (3.1-fold) and CHOP (2.5-fold), when compared to control. On the other hand, the ISP challenge significantly reduced the protein expression levels of SERCA2a, a pivotal conduit for maintaining calcium shuttling, to about 80 % ( $p < 0.05$ ; Figures 2C-2D). Surprisingly, HPA reduced ER stress, as indicated by the improved SERCA2a and normalized ER-stress biomarkers.



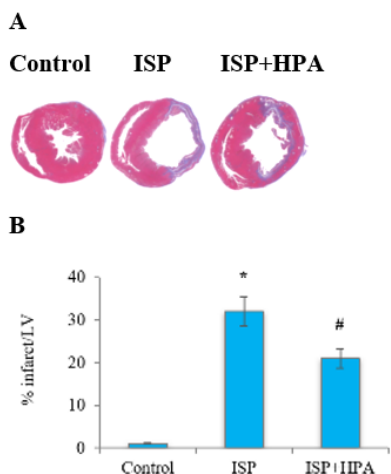
**Figure 2:** Effect of HPA on calcium dyshomeostasis-associated ER stress in ISP-provoked MI rats. A: Representative bands of PERK, GRP78 and CHOP expressions, with  $\beta$ -actin as internal control; B: protein expressions of PERK, GRP78 and CHOP represented as % of that of internal control; C: Representative band of SERCA2a expression, with  $\beta$ -actin as internal control; D: protein expression of SERCA2a presented as percentage of that of internal control. Data are mean  $\pm$  S.D. \* $P < 0.05$ , ISP vs control; # $p < 0.05$ ; ISP+HPA vs ISP.

## Huperzine A attenuated apoptosis in ISP-provoked MI rats

Apoptosis of cardiomyocytes after ISP-provoked MI leads to cardiac hypertrophy and heart failure. In this study, Bcl2 protein expression was significantly ( $p < 0.05$ ) reduced to 39 %. The expressions of Bax (assayed with western blot) and caspase-12 (assayed immunohistochemically) were significantly ( $p < 0.05$ ) upregulated (2.6- and 4.9-fold, respectively), due to oxidative assault and ER stress provoked by ISP administration. These results are depicted in Figure 3 A - D. Besides there, was consequential and massive increase in myocardial infarcted area in the ISP-challenged rats (Figures 4 A and B). These results indicate that HPA may be used as a prospective cardioprotective agent.



**Figure 3:** Effect of HPA on apoptosis in ISP-provoked MI rats. A: Representative bands of Bcl-2 and Bax expressions, with  $\beta$ -actin internal control; B: protein expressions of Bcl2 and Bax represented as % of that of internal control; C: Representative immunohistochemical stained section showing caspase-12 expression; D: Expression level of caspase-12 represented as % of that of control



**Figure 4:** Effect of HPA on apoptosis in ISP-provoked MI rats. A: Representation slide of infarcted area stained using Masson trichrome, and B: percentage of infarcted area/left ventricle (LV). Data are indicated as mean  $\pm$  SD. \* $P < 0.05$ , ISP vs control; # $p < 0.05$ ; ISP+HPA vs ISP

## DISCUSSION

The present study provides evidence showing that HPA downmodulates ER stress-mediated apoptosis through regulation of oxidative stress and calcium homeostasis, and also protects rats against ISP-provoked myocardial infarction. Owing to MI, heart maladapt itself through a sequence of events known as “cardiac remodelling” (CM) during which anatomical and functional changes occur in the cardiomyocytes and also in the extra-myocyte matrix. These changes are phenomenal, leading to serious cardiac aberrations, including dysregulated cardiac mechano-energetics, and ultimately cardiac failure. A plethora of robust evidence support the involvement of ROS in the maladaptive cardiac remodelling process [6,10,11].

Although distinct ROS sources are involved in the MI-CM nexus, the NADPH oxidase family (NOXs) is the pivotal ROS generator. It has been reported that NOX4, a prime isoform in cardiac myocytes, is present chiefly in the membrane of cardiomyocyte organelles including nucleus, endoplasmic reticulum and mitochondria [15]. A critical upstream redox switch, NOX4 regulates the expression of Nrf2 in cardiomyocytes. The upregulation of NOX4 and repression of Nrf2/HO-1 axis are used in various models of cardiac dysfunction [7,16,17]. Consistent with the evidence, it was observed that ROS level and NOX4 expression were increased, while Nrf2/HO-1 expressions were decreased in the ISP-provoked MI rats. Nevertheless, HPA

treatment reversed these abnormalities and produced near-normal values similar to those in control rats. In fact, it has been reported that the protective effect of HPA, is at least in part, mediated through its antioxidant activity via the Nrf2/ARE signaling loop [18]. This implies that HPA could preserve cardiomyocyte integrity and myocardial function by reversing ISP-provoked oxidative stress-mediated pathology.

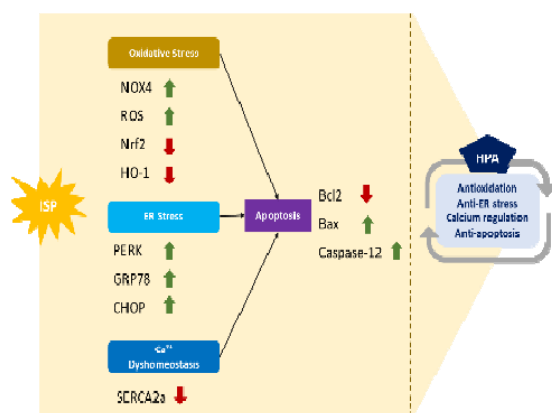
Activated NOX4 signaling has been reported to trigger ER stress in cardiomyocytes via Xbp1s-mediated RIPK1/NF- $\kappa$ B signaling axis, leading to cardiac hypertrophy [15]. An elegant study by Cullinan *et al.* [19] showed that protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), a biomarker of ER stress, elicited dissociation of Nrf2/Keap1 complex and promoted the shuttling of Nrf2 from cytoplasm to the nucleus. It is worthy of note that the kick-off of the Nrf2/HO-1 adaptogenic cascade was hindered by PERK-mediated ER stress, leading to myocardial apoptosis and maladaptive cardiac remodelling [11,20]. Moreover, glucose-regulated protein 78 (GRP78), a member of heat shock protein (HSP) family of molecular chaperones, and C/EBP homologous protein (CHOP), a DNA damage inducible protein, are known to be implicated in the ER-stress mediated apoptosis [7].

In this study, the protein expressions of PERK, GRP78 and CHOP were elevated in the ISP-provoked MI rats. In contrast, HPA administration effectively mitigated the ER stress, as portrayed by the downregulation of protein expressions of PERK, CHOP, and GRP78.

Targeting ER stress-linked myocardial apoptosis using the strategies that reverse calcium dyshomeostasis is gaining interest in the management of cardiac dysfunction. It is known that SERCA2a, a versatile  $\text{Ca}^{2+}$  shuttling unit, is a key regulator of  $\text{Ca}^{2+}$  homeostasis in myocardial tissues. An interesting study by Xin *et al.* demonstrated that SERCA2a gene transfer alleviated ER stress-initiated myocardial apoptosis [21]. Another study reported that cardiomyocyte-specific genetic ablation of SERCA2 triggered ER stress-mediated apoptosis [21]. In harmony with these results, it was observed that SERCA2a expression was downmodulated in the ISP-challenged myocardial tissue, while HAP treatment re-established the normal SERCA2a level.

Endoplasmic reticulum (ER) stress provokes the pro-apoptotic family protein, Bax, which in turn elicits m-calpain (a calcium-dependent protease)-mediated caspase-12 activation and cardiac

apoptosis [22]. Moreover, CHOP (GADD153) might sensitize myocardial cells to ER stress through specialized molecular signaling mechanisms that entail Bcl-2 downmodulation and oxidative assault [23]. With the upregulation of CHOP and other ER stress markers along with upmodulated Bax and caspase-12, concomitant Bcl2 repression in ISP-challenge was observed in the current study. Specifically, the role of caspase-12 as a crucial ER resident caspase in the induction of apoptosis [24] is clearly evidenced in the myocardial tissue of ISP-challenged rats in this study. On the other hand, treatment with HPA, in line with documented anti-apoptotic effects [12,14], produced elevated Bcl2 expression and downregulated Bax and caspase-12 expressions in the myocardial injury provoked by ISP. These findings indicate that HPA represses ER stress-mediated apoptosis in ISP-provoked MI. A schematic representation of the protective effect of HPA on ISP-provoked myocardial infarction is shown in Figure 5.



**Figure 5:** Scheme depicting the effect of HPA on ISP-provoked myocardial infarction and the key biomarkers/molecular signals involved in cardiac pathology

## CONCLUSION

The findings from this study suggest that HPA ameliorates ISP-provoked myocardial infarction and cardiac dysfunction via mitigation/abrogation of ER stress, oxidative stress and calcium dyshomeostasis. These outcomes suggest that HPA is a potential therapeutic candidate for the management of cardiomyopathy.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

## Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors equally contributed to the study design, conduction animal studies and manuscript writing. All authors read and approved the final manuscript for publication.

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