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Inducible nitric oxide synthase contributes to insulin resistance and cardiac dysfunction after burn injury in mice

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ABSTRACT

Aims: Cardiac dysfunction is a major cause of multi-organ dysfunction in critical care units following severe burns. The purpose of this study was to investigate the role of inducible nitric oxide synthase (iNOS) in cardiac dysfunction in burned mice.

Materials and methods: Wild-type and iNOS-knockout mice were subjected to 30% total body surface area burns. Next, the expression of iNOS was measured at 1, 3 and 7 days post-burn. Cardiac function, insulin sensitivity, inflammation, oxidative stress, and apoptosis in the hearts of the mice were assessed at 3 days post-burn.

Key findings: Compared to control mice, iNOS expression was increased and reached a maximum in the heart of burned mice at 3 days post-burn. iNOS deficiency significantly alleviated the cardiac dysfunction and insulin resistance in burned mice. In addition, burn-induced inflammation, oxidative stress, and apoptosis in the heart were markedly reduced in iNOS-knockout burned mice when compared to corresponding values in wild-type burned mice.

Significance: Our study demonstrates that iNOS contributes to insulin resistance in the hearts of mice following burn injury, and iNOS deficiency protects cardiac function against burn injury in mice, suggesting iNOS as a potential therapeutic target to treat burn injuries.

1. Background

Cardiac dysfunction is a common clinical complication that is associated with high mortality rates in patients with severe burns. Delayed cardioprotective intervention often results in incurable multiple organ dysfunction syndrome, known as “cardiac shock” [1]. Therefore, finding potential effective cardioprotective strategies is very important for the management of burn patients.

Inducible nitric oxide synthase (iNOS) is an important inflammatory mediator in many types of cells. iNOS expression was induced by cytokines in the skeletal muscle of burn patients and mice [2,3], and excess expression resulted in metabolic dysfunction in many tissues [4–6]. It has also been reported that metabolic dysfunction can impair cardiac function and accelerate myocardial apoptosis at the early stage following burn injury [7]. Insulin resistance is a major manifestation of the metabolic dysfunction related to impaired cardiac function and is characterized by decreased levels of insulin-stimulated blood glucose and reduced insulin-stimulated Akt phosphorylation. However, weather iNOS plays a role in the development of cardiac dysfunction following burn injury has not been investigated. We hypothesized that excess iNOS following burn injury leads to impaired metabolism, increased apoptosis in the heart, and subsequent cardiac dysfunction. To test this hypothesis, we subjected iNOS-knockout and wild-type mice to burn injuries and explored the role of iNOS in the development of cardiac dysfunction after burn injury.

Abbreviations: Akt, Protein kinase B; eNOS, Endothelial nitric oxide synthase; EF, Ejection fraction; FS, Fraction shortening; iNOS, Inducible nitric oxide synthase; KO, Knockout; LDH, Lactate dehydrogenase; MDA, Malondialdehyde; nNOS, Neural nitric oxide synthase; NOS, Nitric oxide synthase; NO, Nitric oxide; NRCM, Neonatal rat cardiac myocytes; ROS, Reactive oxygen species; WT, Wild-tpye

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2. Materials and methods

2.1. Animals

Male, 8-week-old C57BL/6 wild-type and iNOS-knockout mice and male Sprague-Dawley (SD) rats (180–220 g) were used in this study. All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. The mice and rats were housed in a controlled facility (20 ± 22 °C; 12-h light/dark cycle) and provided standard rodent chow and water ad libitum. A full-thickness burn injury, comprising 30% of the total body surface area, was induced as described previously [8]. Briefly, the mice or rats were anesthetized with 2–3% isoflurane in 100% oxygen and burned by immersing the back of the trunk in 95 °C water for 10 s. Control mice or rats were immersed in warm water for 10 s. All animals received saline solution (4 ml/kg/%burn) by intraperitoneal injection.

2.2. Echocardiography

Cardiac function was assessed by echocardiography (Philips TIS 0.8) at 3 days post-burn. Mice were anesthetized with 2% isoflurane in 100% oxygen, and images were acquired in short-axis view to evaluate the ejection fraction and fraction shortening.

2.3. Glucose tolerance and insulin sensitivity testing

Mice were administered glucose (2 g/kg) by intraperitoneal injection or insulin (0.5 U/kg) by hypodermic injection as described previously [9]. Next, blood glucose levels were measured using tail clippings at 0, 15, 30, 60, 90, and 120 min after glucose injection for the glucose tolerance test and at 0, 15, 30, 60, and 90 min after insulin injection for the insulin sensitivity test.

2.4. Collection of blood serum from SD rats

Burned or sham rats were anesthetized with 3% isoflurane followed by pentobarbital sodium. Blood samples were collected from the abdominal aorta with a sterile tube and centrifuged at 3000 RPM for 10 min at room temperature as described previously [10]. The supernatant was collected for use as burn serum or sham serum.

2.5. Measurement of malonaldehyde (MDA) content and lactate dehydrogenase (LDH) levels

MDA content was measured in heart lysates as described previously [11], and LDH levels were measured in serum using a kit (Nanjing Jiancheng Reagents, China) according to the manufacturer's instructions.

2.6. Cardiomyocyte culture

Neonatal myocytes were isolated from SD rats at 1–3 days after birth as described previously [12]. Briefly, the hearts of neonatal rats were digested with collagenase II, and the obtained cells were cultured in M199 medium containing 10% FBS (Wisent, Canada) in a 5% CO_2 incubator.

2.7. Histology and staining

Heart tissues were embedded in paraffin and sectioned (5 μm thick). The sections were then dewaxed with xylene and stained with hematoxylin and eosin to visualize the myocardial structure. Cardiomyocyte apoptosis was assessed by TUNEL assays, and the TUNEL-positive cells were stained green. Mitochondrial reactive oxygen species (ROS) were detected after incubation with mitoSOX (5 μM) for 15 min. Eosinophilic infiltration was measured by myeloperoxidase (MPO) staining.

2.8. Real-time PCR

The mRNA expression levels of IL-1, IL-6, IL-8, TNF-α, IFN-α, MCP-1, IRE1, PERK, CHOP were measured using quantitative real-time PCR to assess inflammation in the hearts of burned mice. Total RNA was extracted from the hearts with TRIzol reagent as described previously [13]. Next, 1 μg of the extracted RNA was reverse transcribed. Real-time PCR amplification of the reverse transcribed cDNA was performed with SYBR green. The primers used in this study are shown in the supplementary data (supplementary Table 1).

2.9. Western blotting

Protein expression was assessed by western blotting as described previously [14]. In brief, protein samples were separated by 8–12% SDS-PAGE and transferred to PVDF membranes. Next, the membranes were blocked with 5% non-fat milk in PBST at room temperature for 1 h. The membranes were probed with primary antibodies against eNOS, nNOS, Akt, p-Akt (Ser473), Bax (Cell Signaling Technology), iNOS (BD for mice and Santa Cruz for NRCM), Bcl-2 (Santa Cruz), SOD2, and NOX4 (Proteintech) overnight at 4 °C, and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 60–90 min before detection.

2.10. Statistical analysis

All values are presented as the mean ± SEM. Data were analysed with one-way ANOVA followed by unpaired *t*-test. Differences were considered statistically significant at p values less than 0.05.

3. Results

3.1. Burn injury induced iNOS expression but did not affect the expression of eNOS and nNOS

To test whether burn injury induced iNOS in the myocardium, we measured iNOS levels in the hearts of mice at 1, 3, and 7 days post-burn (Fig. 1A). The immunoblot results showed that burn injury increased iNOS expression in a time-dependent manner, which reached a maximum at 3 days post-burn. In contrast, the expression levels of nNOS and eNOS were not affected by burn injury. To confirm the role of iNOS in burn-induced myocardial damage, we induced burn injuries in iNOS-knockout mice. iNOS knockout did not affect the protein expression of eNOS and nNOS, and there was no iNOS detected in the heart of $iNOS^{-/-}$ mice at 3 days post-burn (Fig. 1B).

3.2. iNOS deficiency protected cardiac function against burn injury

To investigate whether iNOS deficiency could ameliorate the cardiac dysfunction induced by burn injury, we assessed cardiac function by echocardiography at 3 days post-burn. The baseline cardiac function of the mice was comparable, as evidenced by echocardiography prior to burn injury on the first and third day (Figure S1). Compared to the control group, left ventricular ejection fraction (EF) and fraction shortening (FS) were significantly lower in burned mice, and iNOS deficiency increased EF and FS (Fig. 2A). Lactate dehydrogenase (LDH) increased with myocardium injury, and iNOS deficiency reduced serum LDH levels in burned mice (Fig. 2B). Hematoxylin and eosin staining was performed to visualize myocardial damage, which showed that iNOS deficiency alleviated the disorder in the myocardial fibre arrangement and the myocardial fibre degeneration induced by burn injury (Fig. 2C). These results indicate that iNOS deficiency exerted cardioprotective effects in burned mice.

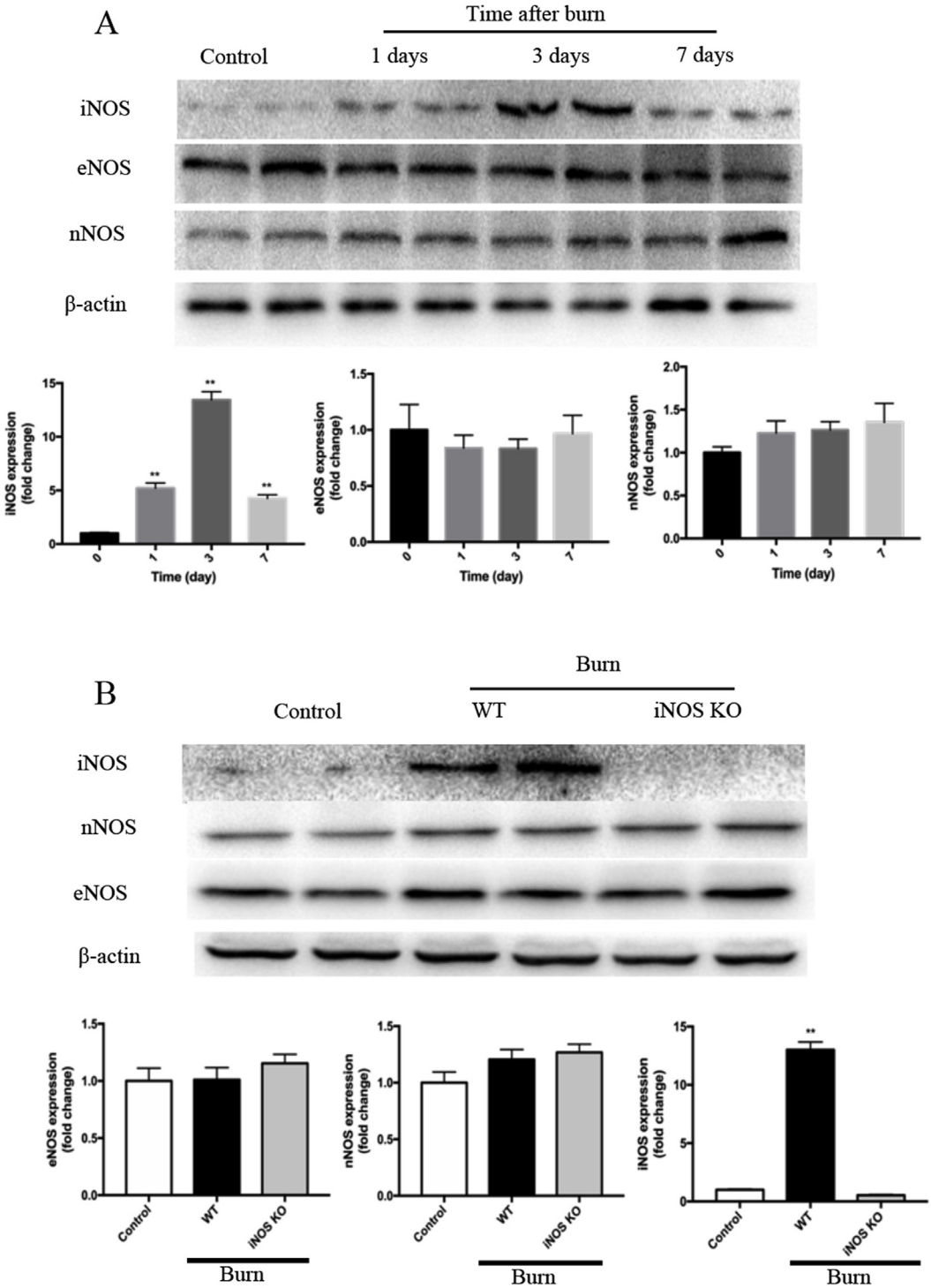

Fig. 1. Burn injury induced iNOS expression but did not affect the expression of eNOS and nNOS. (A) The expression levels of iNOS, nNOS, and eNOS were examined in the hearts of burned mice at 1, 3, and 7 days post-burn. iNOS protein expression was significantly increased at 3 days post-burn, and the quantified results are shown below. (B) The expression of iNOS, nNOS, and eNOS were examined in the hearts of burned iNOS-knockout mice at 3 days post-burn. iNOS knockout did not affect the expression of nNOS and eNOS, and the quantified results are shown. Values are the mean ± SEM. n = 6 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.3. iNOS deficiency improved myocardial insulin sensitivity in burned mice

Metabolic disorders, including insulin resistance, are major contributors to the multiple organ dysfunction that occurs after severe burns [15,16]. Thus, post-burn insulin resistance contributes to the adverse outcomes of burn injury [17,18]. To test the effect of iNOS deficiency on insulin sensitivity, we performed glucose tolerance and insulin sensitivity testing at 3 days post-burn. Blood glucose levels were significantly increased at 15, 30, 60, and 90 min after glucose injection, and iNOS deficiency reduced this hyperglycaemic response (Fig. 3A). Similarly, burn injury retarded the insulin response, but iNOS deficiency markedly improved insulin sensitivity as evidenced by the decrease in blood glucose (Fig. 3B). Consistent with these findings, insulin-stimulated phosphorylation of Akt was significantly decreased in burned mice, and iNOS deficiency reversed this effect (Fig. 3C). To verify whether this effect also occurs in cardiomyocytes, we exposed

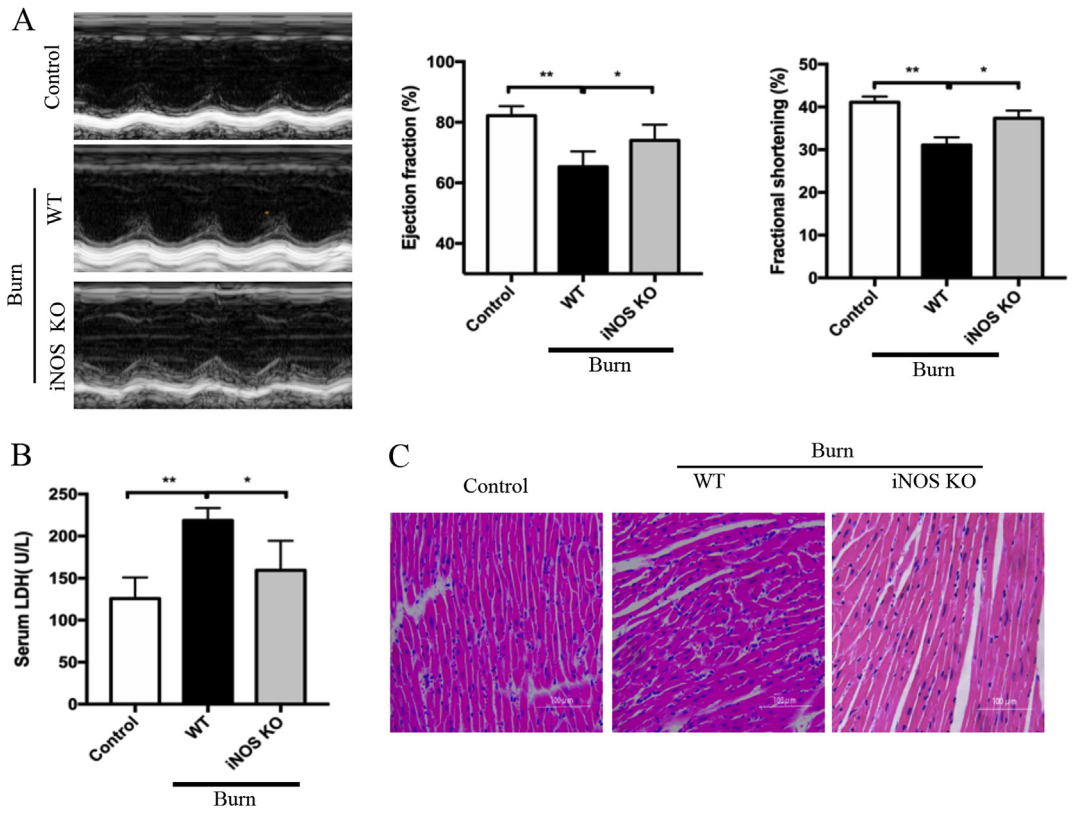

Fig. 2. iNOS deficiency protects the heart against burn-related injury. (A) iNOS deficiency ameliorated the cardiac dysfunction induced by burn injury. Representative echocardiography images are shown on the left. Left ventricular ejection fraction and fractional shortening are shown on the right. (B) iNOS deficiency reduced serum LDH levels in burned mice. (C) Heart tissue sections were stained with hematoxylin and eosin to visualize myocardial damage. Values are the mean ± SEM. n = 4–6 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

neonatal rat cardiomyocytes (NRCM) to serum from burned (burn serum) or sham (sham serum) SD rats to induce damage. To find a suitable serum concentration, we incubated the NRCM with a series of burn serum dilutions (20%, 10%, 5%, or sham serum) for 12 h and measuring cell viability by the CCK-8 assay to determine the best concentration. The results showed that sham serum and 5% burn serum had no effects on cell viability. In contrast, cell viability decreased to less than 50% after incubation with 20% burn serum (Figure S2). Therefore, we chose 10% serum for subsequent experiments. We found that incubation of isolated cardiomyocytes with burn serum decreased the insulin-stimulated phosphorylation of Akt (Fig. 3D), while treatment with the iNOS inhibitor AMG reversed this effect. These results suggest that iNOS deficiency improved systemic insulin sensitivity and ameliorated the impaired insulin signalling resulting from burn injury.

3.4. iNOS deficiency inhibited inflammation marker expression in the hearts of burned mice

Inflammation plays an vital role in burn-induced insulin resistance [19], and iNOS is a very important inflammatory mediator that is involved in various types of tissue damage that occurs in critical illnesses [20]. Therefore, we assessed the effects of iNOS deficiency on the mRNA expression of inflammatory markers in the hearts of burned mice at 3 days post-burn. Compared with the levels in control mice, the expression levels of these inflammatory markers were markedly increased in burned mice. iNOS deficiency reduced the mRNA expression levels of all tested markers except MCP-1, indicating that the inflammatory response induced by burn injury was inhibited in iNOS-knockout mice (Fig. 4A–F). MPO is a marker of neutrophil accumulation, and we examined the MPO content in the hearts of burned mice. Burn injury resulted in increased neutrophil infiltration in the heart, which was significantly decreased in iNOS-knockout mice (Fig. 4G). These findings suggest that inflammation may contribute to the development of cardiac dysfunction and that iNOS deficiency suppressed the inflammatory reactions in the hearts of burned mice.

3.5. iNOS deficiency reduced oxidative stress in the hearts of burned mice

It has been reported that burn injury induces mitochondrial dysfunction in the rectus abdominis muscle of burned mice [21]. ROS generated by damaged mitochondria leads to oxidative damage, which is involved in the pathology of burn injury. Thus, we measured mitochondrial ROS by mitoSOX. The results showed that mitochondrial ROS was significantly higher in the hearts of burned mice than in control (unburned) mice (Fig. 5A), suggesting that burn injury increased oxidative stress. The increased MDA and NOX4 contents and decreased SOD2 expression (Fig. 5B and C) in the myocardium also supported the notion that burn injury results in oxidative damage in the heart. The burn injury-induced increase in oxidative stress was significantly mitigated by iNOS deficiency (Fig. 5A–C). Similarly, treatment with an iNOS inhibitor ameliorated the oxidative stress induced by burn serum in isolated cardiomyocytes (Fig. 5D). We also assessed ER stress in the hearts of burned mice by measuring the mRNA expression of genes in the UPR signalling pathway. The results showed there were no significant differences in ER stress in burned mice. (Figure S3). These data indicate that iNOS deficiency suppressed the oxidative stress in the hearts of burned mice, which maybe the reason for the impaired cardiac systolic function in burned animals.

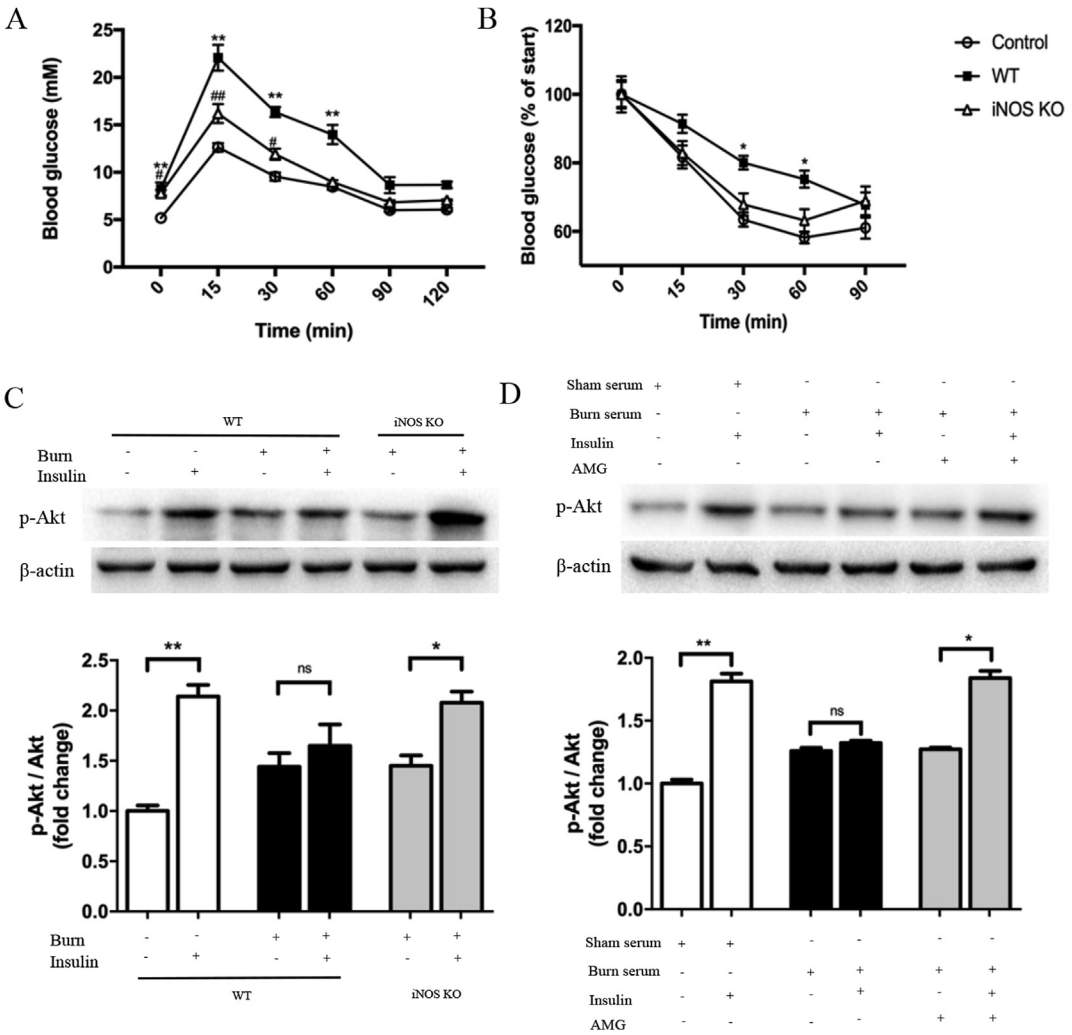

Fig. 3. iNOS deficiency improved myocardial insulin sensitivity in burned mice. An intraperitoneal glucose tolerance test (A) and insulin sensitivity test (B) were performed at 3 days post-burn injury. (C) iNOS deficiency increased insulin-stimulated p-Akt expression in the hearts of mice at 3 days post-burn injury. Representative images of western blots are shown above the quantified results (p-Akt/Akt). (D) Serum from burned SD rats suppressed insulin-stimulated p-Akt expression in neonatal rat cardiac myocytes, and the iNOS inhibitor AMG reversed this effect. Representative images of western blots are shown above the quantified results (p-Akt/Akt). Values are the mean ± SEM. n = 4–6 for each group. #, $P < 0.05$. ##, $P < 0.01$ versus control. *, $P < 0.05$. **, $P < 0.01$ versus burned WT mice.

3.6. iNOS deficiency protected cardiomyocytes from apoptotic death in burned mice

Our previous study showed that the insulin signalling pathway and oxidative stress contribute to apoptosis in the hearts of diabetic mice [22]. Thus, we examined the effects of iNOS on the burn-induced apoptotic changes in the myocardium. Burn injury increased apoptosis levels in the heart, as indicated by the increased ratio of Bcl-2/Bax (Fig. 6A) and TUNEL-positive index (Fig. 6B) compared with the values in control mice. These effects were blunted by iNOS deficiency (Fig. 6A and B). The Bcl-2/Bax ratio was decreased in isolated cardiomyocytes exposed to burn serum, and the iNOS inhibitor AMG increased the ratio to some degree (Fig. 6C). These results indicate that iNOS deficiency attenuated the apoptotic death of cardiomyocytes, which may contribute to the observed improvement in cardiac function described above.

4. Discussion

In this present study, we found that burn injury induced iNOS expression in the hearts of burned mice. iNOS deficiency improved systemic insulin sensitivity and suppressed inflammation, and thus ameliorated cardiac dysfunction in burned mice. These results, together with the finding that iNOS deficiency suppressed oxidative stress and protected cardiomyocytes against apoptotic death, indicate that iNOS plays a vital role in the cardiac dysfunction induced by burn injury in mice.

There are three distinct isoforms of NOS, neural NOS (nNOS), endothelial NOS (eNOS) and iNOS. eNOS and nNOS are calcium-dependent enzymes that produce small amounts of NO, whereas iNOS is a calcium-independent enzyme and produces excess NO when induced by cytokines [23]. Nitric oxide (NO) generated by NOS plays important roles in many physiological processes, including inflammation [24]. It has been reported that burn injury induces iNOS expression in the skeletal muscle of burned mice [25]; however, whether iNOS is involved in the myocardium is unknown. Our data showed that burn injury increased iNOS expression in a time-dependent manner. In contrast, the expression of nNOS and eNOS was not changed in burned WT or iNOS-knockout mice. These findings suggest that iNOS, but not nNOS or eNOS, is involved in cardiac dysfunction after burn injury.

It has been proposed that metabolic disorders are a common complication after severe burn injuries [26–28]. Insulin resistance, one of

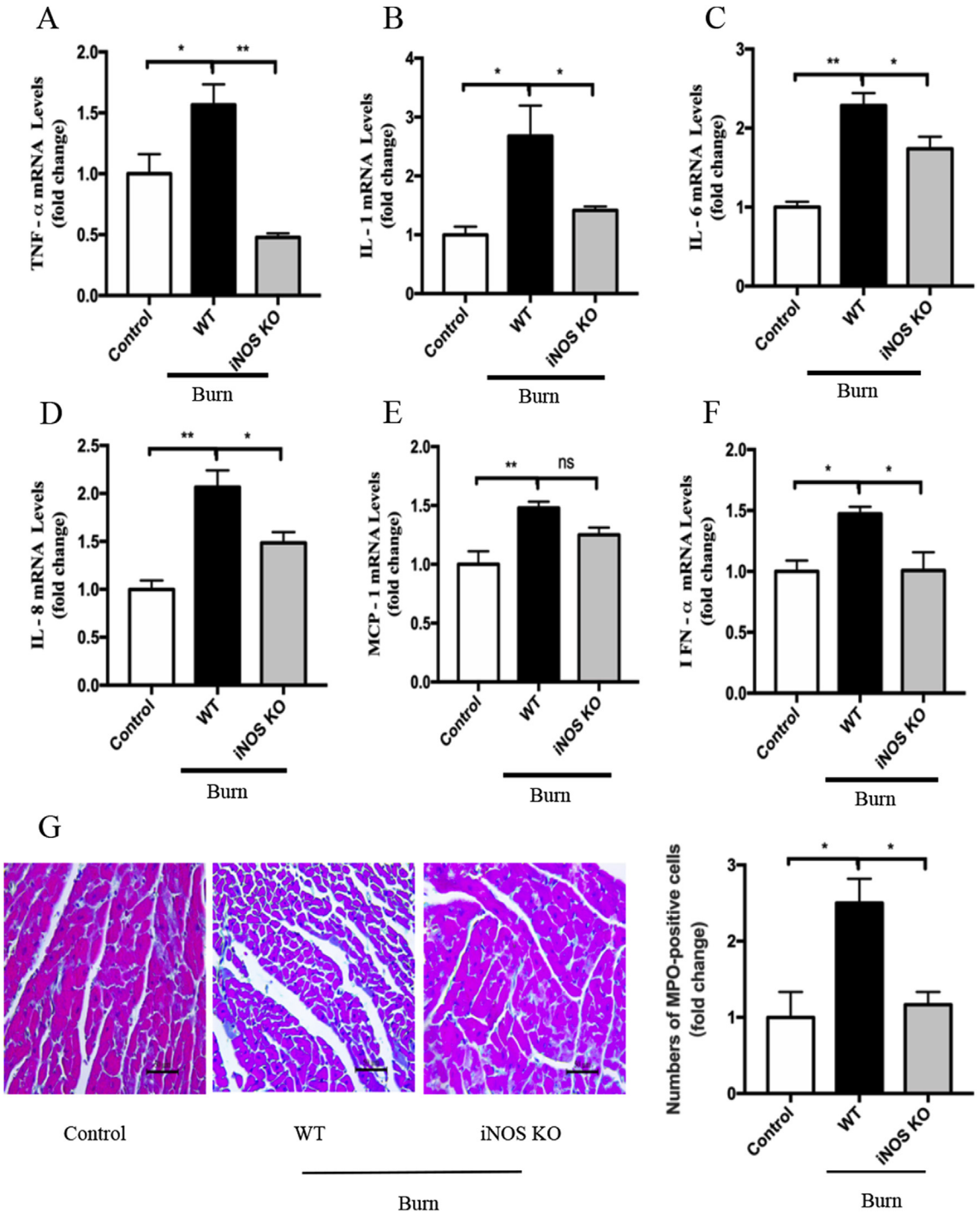

Fig. 4. iNOS deficiency inhibited inflammation marker expression in the hearts of burned mice. (A–F) The mRNA expression levels of TNF-α, IL-1, IL-6, IL-8, MCP-1, and IFN-α in the hearts of mice at 3 days post-burn as determined by q-PCR. (G) Heart sections were stained with myeloperoxidase to evaluate eosinophilic infiltration. The quantitative results are shown on the right. Values are the mean ± SEM. n = 4–6 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

the main manifestations of metabolic disorders, increases morbidity and mortality in patients with burn injuries [29]. In our study, we observed that burn injury increased p-Akt levels but decreased the insulin-stimulated phosphorylation of Akt, which suggested impaired insulin sensitivity. iNOS deficiency improved systemic insulin sensitivity and activated the insulin signalling pathway in the hearts of burned mice, as evidenced by the increased insulin-stimulated phosphorylation of Akt. More importantly, we observed that cardiac dysfunction induced by burn injury was significantly ameliorated in iNOS-knockout mice when compared to that in wild-type burned mice. These data indicate that iNOS deficiency moderates metabolic disorder and protects cardiac function in burned mice.

Although the mechanism underlying insulin resistance in burned mice is not well-understood, it is widely accepted that inflammation is involved in the pathogenesis of insulin resistance [30,31]. iNOS plays a critical role in inflammation and the activation of p65 NF-κB [32,33]; however, the relationship between iNOS and local inflammation in the hearts of burn mice has not been investigated. In the current study, we found that the increased inflammatory marker mRNA expression is markedly reduced in iNOS-knockout mice, suggesting that iNOS

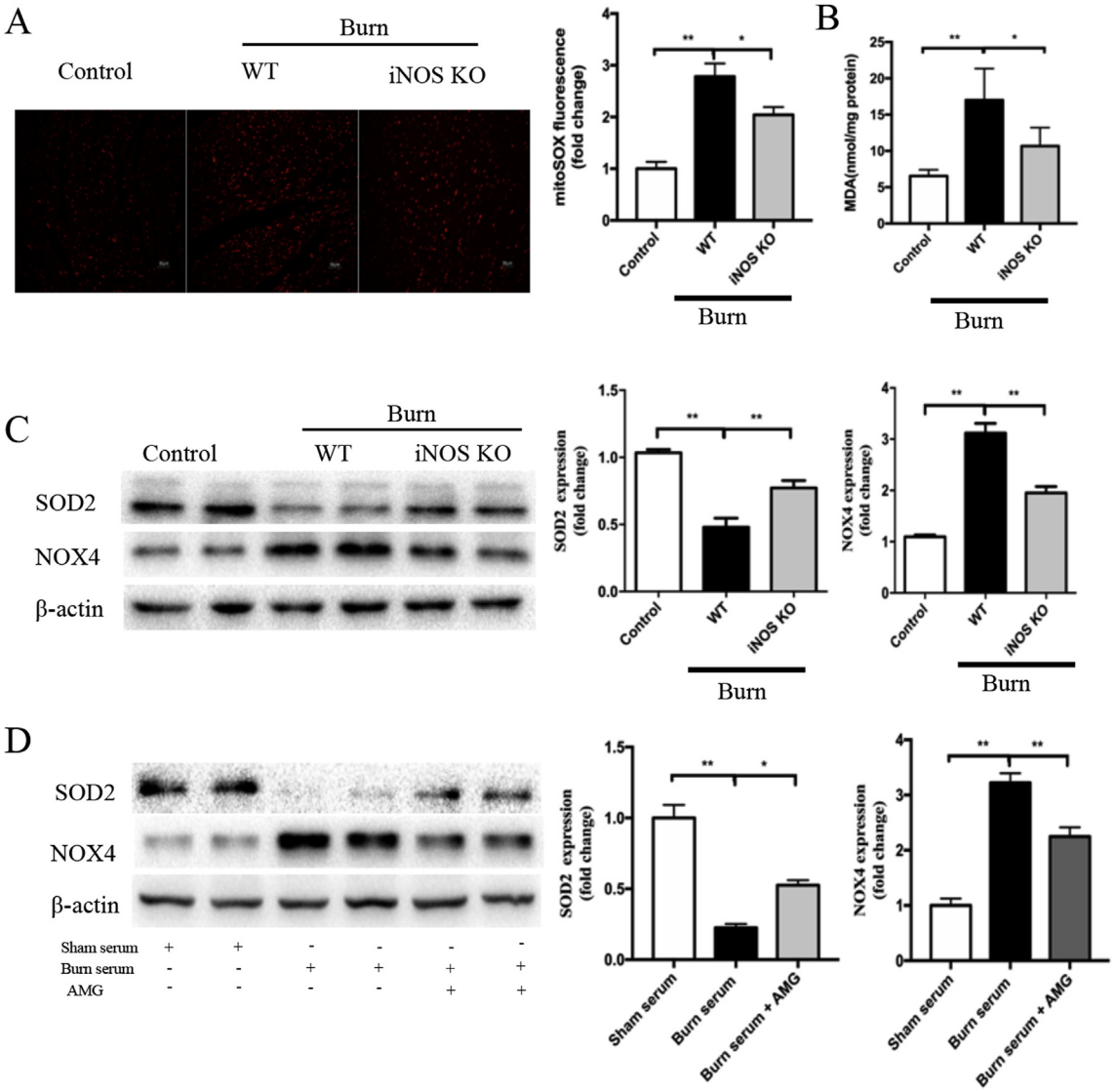

Fig. 5. iNOS deficiency reduced oxidative stress in the hearts of burned mice. (A) Mitochondrial ROS in heart sections were detected by mitoSOX. The statistical analysis of the results is shown on the right. (B) The malondialdehyde (MDA) contents in the hearts of burned mice. (C) SOD2 and NOX4 levels in the hearts of burned mice as measured by western blotting. The quantified SOD2 and NOX4 levels are shown on the right. (D) Neonatal rat cardiac myocytes (NRCM) were treated with serum from sham or burned SD rats, and the levels of SOD2 and NOX4 were measured. The quantified results are shown on the right. (E–G) The mRNA expression levels of IRE1, PERK, and CHOP. Values are the mean ± SEM. n = 4–6 for each group. *$P < 0.05$, **$P < 0.01$.

contributes to local inflammation in the heart after burn injury. However, whether this is the reason for insulin resistance in the myocardium requires further investigation.

As the cellular site of energy metabolism, mitochondria are closely related to metabolic disorders [34]. It has been reported that burn injury results in mitochondrial dysfunction in the rectus abdominis muscle of burned mice [21]. Dysfunctional mitochondria produce excess amounts of ROS, which causes oxidative damage in many tissues [35,36]. In our study, ROS generation was significantly increased in the myocardium, as evidenced by mitoSOX assay, and the MDA contents were higher in burned mice. iNOS deficiency reduced ROS production and increased the expression of the antioxidant kinase SOD2 in the hearts of burned mice. These data suggest that iNOS deficiency suppresses oxidative stress in burned mice, which maybe another important reason for the improved cardiac function described above.

It has been reported that apoptosis in cardiomyocytes contributes to burn-induced cardiac dysfunction [37]. Our previous study showed that increased phosphorylation of Akt protects against apoptosis in diabetic mice [22]. Here, we showed that iNOS deficiency increased insulin-stimulated phosphorylation of Akt. Furthermore, we found that the increased apoptotic death in the hearts of burned mice was markedly reversed in iNOS-knockout mice, as evidenced by decreased TUNEL-positive index and increased Bcl-2/Bax ratio. Additionally, we confirmed the effect in isolated cardiomyocytes exposed to burn serum by pharmacological inhibition of iNOS activity, suggesting that iNOS deficiency protects cardiomyocytes against apoptosis induced by burn injury.

This study has some limitations. Although we found that iNOS is involved in insulin resistance and apoptosis in the myocardium, the underlying mechanisms remain to be elucidated in future studies. In addition, a cardiac-specific iNOS-knockout mouse model would be more useful for elucidating the role of iNOS in the hearts of burned mice.

5. Conclusions

In summary, the present study clearly showed that iNOS plays an important role in the cardiac dysfunction induced by burn injury. iNOS

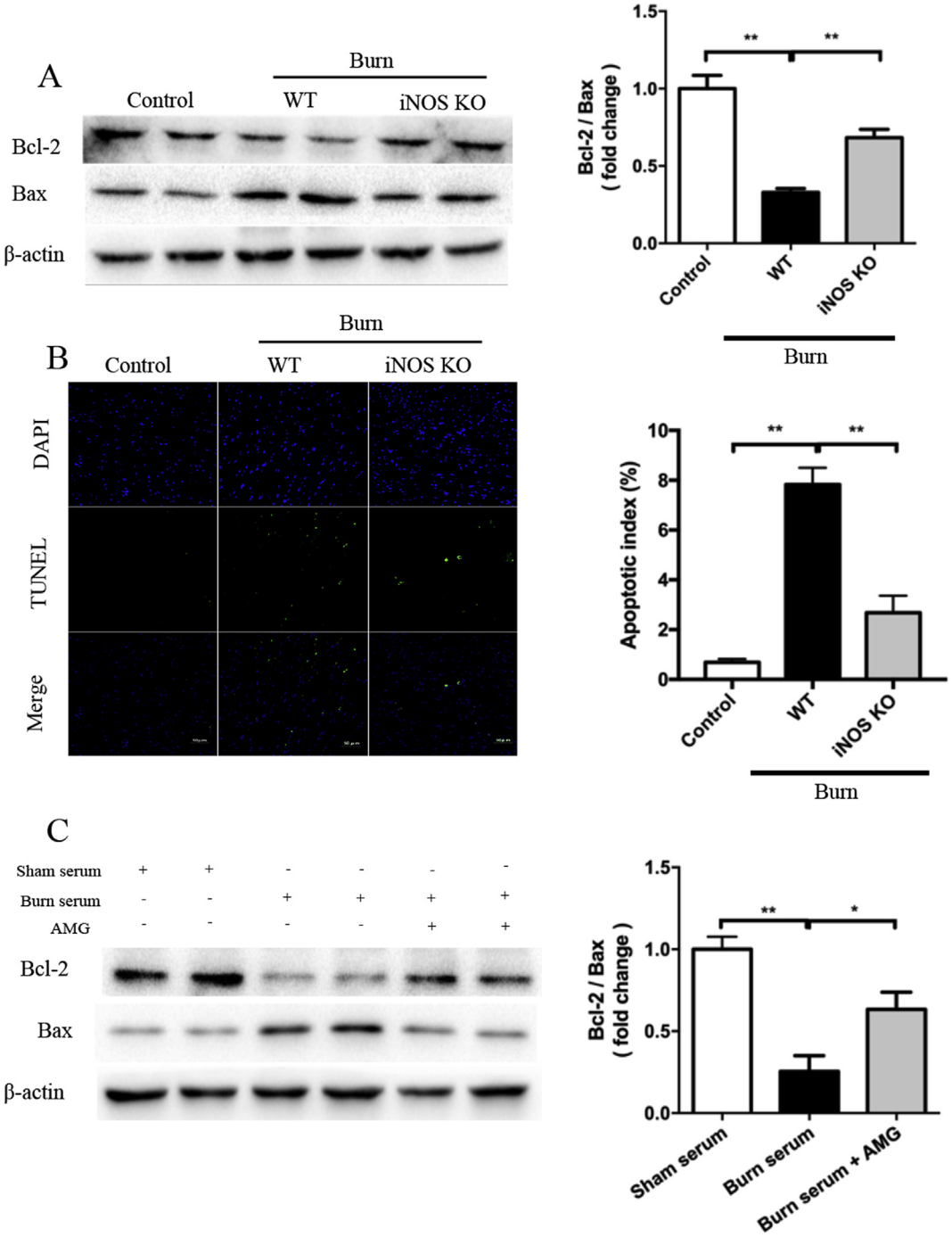

Fig. 6. iNOS deficiency protected cardiomyocytes from apoptotic death in burned mice. (A) iNOS deficiency increased the ratio of Bcl-2/Bax in the hearts of mice at 3 days post-burn. The quantitated Bcl-2/Bax ratios are shown on the right. (B) Representative TUNEL-stained (green fluorescence) and DAPI-stained (blue) sections of the heart from mice at 3 days post-burn. (D) Serum from burned SD rats reduced the Bcl-2/Bax ratio in neonatal rat cardiomyocytes, and the iNOS inhibitor AMG reversed this effect. The quantified results (Bcl-2/Bax) are shown on the right. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

deficiency improved cardiac function, at least in part, by ameliorating insulin resistance and suppressing apoptotic death in the hearts of burned mice. These findings suggest that inhibiting the activity of iNOS through genetic or pharmacological means may be a beneficial therapeutic strategy for treating burn injuries.

Declaration of competing interest

The authors have not disclosed any financial conflicts of interests relevant to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116912.

Authors' contributions

LSX, GYZ and YYH designed this study. YYH, GYZ, LDY, YJH and SFF performed experiments. GYZ wrote the manuscript. YJH, SFF, WXW and ZC helped to draft the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval

All animal protocols were approved by the Animal Research Committee at Chongqing medical university (Animal permit number SYXK2018-0003).

Consent for publication

N/A.

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