Journal of Molecular Medicine
https://doi.org/10.1007/s00109-023-02295-7

ORIGINAL ARTICLE

Never in mitosis gene A-related kinase-6 deficiency deteriorates diabetic cardiomyopathy via regulating heat shock protein 72

Shuangyin Shao[1] · Lili Xiao[2] · Meng Jia[3] · Chuyang Zhang[4] · Guojun Zhao[2] · Rui Yao[2] · Xiaofang Wang[2] · Lu Gao[2]

Received: 21 June 2022 / Revised: 1 February 2023 / Accepted: 6 February 2023

Abstract

NIMA (never in mitosis, gene A)-related kinase-6 (NEK6), a cell cycle regulatory gene, was found to regulate cardiac hypertrophy. However, its role in diabetes-induced cardiomyopathy has not been fully elucidated. This research was designed to illustrate the effect of NEK6 involved in diabetic cardiomyopathy. Here we used a streptozotocin (STZ)-induced mice diabetic cardiomyopathy model and NEK6 knockout mice to explore the role and mechanism of NEK6 in diabetic-induced cardiomyopathy. NEK6 knockout mice and wild-type littermates were subjected to STZ injection (50 mg/kg/day for 5 days) to induce a diabetic cardiomyopathy model. As a result, 4 months after final STZ injection, DCM mice revealed cardiac hypertrophy, fibrosis, and systolic and diastolic dysfunction. NEK6 deficiency causes deteriorated cardiac hypertrophy, fibrosis, and cardiac dysfunction. Furthermore, we observed inflammation and oxidative stress in the hearts of NEK6 deficiency mice under diabetic cardiomyopathy pathology. Adenovirus was used to upregulate NEK6 in neonatal rat cardiomyocytes, and it was found that NEK6 ameliorated high glucose-induced inflammation and oxidative stress. Our findings revealed that NEK6 increased the phosphorylation of heat shock protein 72 (HSP72) and increased the protein level of PGC-1α and NRF2. Co-IP assay experiment confirmed that NEK6 interacted with HSP72. When HSP72 was silenced, the anti-inflammation and anti-oxidative stress effects of NEK6 were blurred. In summary, NEK6 may protect diabetic-induced cardiomyopathy by interacting with HSP72 and promoting the HSP72/PGC-1α/NRF2 signaling.

Key messages

- NEK6 knockout deteriorated cardiac dysfunction, cardiac hypertrophy, fibrosis as well as inflammation response, and oxidative stress.
- NEK6 overexpression attenuated high glucose induced inflammation and oxidative stress.
- The underlying mechanisms of the protective role of NEK6 in the development of diabetic cardiomyopathy seem to involve the regulation of HSP72-NRF2- PGC-1α pathway.
- NEK6 may become a new therapeutic target for diabetic cardiomyopathy.

Keywords NEK6 · Diabetic-induced cardiomyopathy · HSP72 · PGC-1α · NRF2

Shuangyin Shao, Lili Xiao, and Meng Jia are co-first authors.

✉ Xiaofang Wang
xfwang066@163.com

✉ Lu Gao
gaomei1215@163.com

[1] Department of Cardiovascular Surgery, Henan Provincial Chest Hospital, Zhengzhou University, Zhengzhou 450000, China

[2] Department of Cardiology, The First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Zhengzhou 450052, China

[3] Department of Thyroid Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

[4] Department of Education, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Published online: 03 March 2023

Introduction

Diabetic cardiomyopathy is a unique form of heart disease that occurs independently of other heart diseases, such as coronary artery disease and hypertension [1]. The main characteristics of diabetic cardiomyopathy are heart tissue insulin resistance, compensatory hyperinsulinemia, and hyperglycemia, which promote cardiac remodeling and cardiac function deterioration [2]. An increase of 1% in HbA1c results in a 30% increased risk of heart failure in patients with type 1 diabetes, regardless of hypertension, smoking, and obesity [3]. In the early stage of diabetic cardiomyopathy, metabolic disorders contribute to compensatory cardiac structure and function changes. However, persistent metabolic disorders can promote cardiac hypertrophy, myocardial fibrosis, and diastolic cardiac dysfunction, which eventually leads to systolic dysfunction and heart failure [4]. Pathophysiological changes include impaired autophagy, increased cardiomyocyte death, activation of renin–angiotensin–aldosterone system (RAAS), and oxidative stress [5]. For decades, hypoglycemic drugs have reduced the mortality of diabetic patients, but heart complications caused by diabetes are gradually increasing. Most hypoglycemic drugs have been proven to increase the risk of cardiovascular disease. Therefore, it is necessary to further explore the development mechanism of diabetic cardiomyopathy and find therapeutic targets.

Oxidative stress plays an essential role in diabetic cardiomyopathy. Abnormal metabolism of diabetes leads to excessive mitochondrial superoxide production in the myocardium [6]. It leads to an increase in the formation of advanced glycosylation end products [7]. Additionally, ROS activates many pro-inflammatory pathways and leads to long-term epigenetic changes, resulting in a series of pathological changes [7]. Nuclear factor erythrocyte-related factor 2 (NRF2) is an important transcription factor regulating cell redox in vivo [8]. It maintains the oxidative balance by regulating the transcription of downstream antioxidants [9].

Additionally, many studies have found that increasing NRF2 expression could inhibit the development of diabetes cardiomyopathy [8, 10]. Inhibition of NRF2 accelerated the development of diabetic cardiomyopathy in many models [8]. Therefore, NRF2 can be used as a promising drug target to prevent the development of diabetic cardiomyopathy.

NIMA-related kinase family is a mitotic protein found by researchers through genetic screening of cell division cycle mutants in the filamentous fungus *Aspergillus nidus* [11]. Nek6, a serine/threonine kinase, is a family member closely related to cell cycle, cell division, and apoptosis. Xu has found that NEK6 was associated with cardiac collagen volume fraction (CCVF) in hypertrophic cardiomyopathy [12]. However, Bian found that NEK6 could attenuate pressure overload-induced cardiac hypertrophy by inhibiting AKT signaling [13]. These indicate that NEK6 may participate in the pathological process of diabetic cardiomyopathy. In this study, we used NEK6 knockout mice to elucidate the functional role of NEK6 in diabetic cardiomyopathy. We found that NEK6 may protect against diabetic-induced cardiac hypertrophy, fibrosis, and dysfunction by promoting HSP72 activation.

Methods

Animals

NEK6 knockout mice came from the European Mouse Mutant Archive (EMMA: 02,372) and were raised at the SPF laboratory animal center of Zhengzhou University. NEK6 knockout mice and their wild-type littermates (aged 8–10 weeks, 23.5–27.5 mg) were injected with streptozotocin (STZ, intraperitoneal injection, ip.50 mg/kg for five consecutive days) as the previous study described [14]. One week after the final injection, mice were subjected to the fast blood glucose (FBG) test. Diabetes was defined as FBG ≥ 16.6 mmol/L. Mice were sacrificed 16 weeks after the final STZ injection, and the hearts were collected. Control mice received the same volume of solution (0.1 mol/L citrate buffer, pH 4.5). All animal experiments were approved by the institutional animal care and use committee of Zhengzhou University (Zhengzhou, China). To overexpress NEK6 (or knockdown HSP72) in the heart, mice were subjected to AAV9-NEK6 or AAV9-shHSP72 injection 10 weeks after the final STZ injection. Consequently, 16 weeks after the final STZ injection, the mice were sacrificed, and their hearts were removed.

AAV9 construction and viral delivery

AAV9-NEK6 and control AAV9-NC were purchased from Vigene Bioscience Company (Jinan, China). AAV9-shHSP72 and the scramble RNA (AAV9-shRNA) were constructed by Vigene Bioscience (Shanghai, China). Ten weeks after STZ injection, both diabetic cardiomyopathy mice and sham mice were randomly assigned to receive either 60–80 μL AAV9-NEK6/ AAV9-shHSP72 ($n = 12$) or AAV9-NC/ AAV9-shRNA ($n = 12$) at $5.0–6.5 \times 10^{13}$ GC/mL in sterile PBS at 37 °C by injection into the retroorbital venous plexus as described in a previous study [14].

Echocardiographic evaluation

Transthoracic echocardiography was performed as previously described [15, 16]. Isoflurane (1.5%) was used to

anesthetize the mice, and echocardiography was performed with a 10-MHz linear-array ultrasound transducer to obtain M-mode echocardiography data. Left ventricle (LV) end-diastolic dimension (LVEDd) and LV end-systolic dimension (LVESd) were obtained, and the LV ejection fraction (LVEF) and LV fractional shortening (LVFS) values were calculated. Ten mice from each group were subjected to transthoracic echocardiography. More than ten beats per heart were analyzed per heart.

For hemodynamic measurements, a microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle of mice anesthetized with 1.5% isoflurane. After stabilization for 15 min, pressure, volume signals, and heart rate were continuously recorded using a Millar Pressure–Volume System (MPVS-400, Millar Instruments, Houston, TX, USA). Results were analyzed with Chart 5.0 software.

Hematoxylin & eosin (HE) staining, PSR staining

Hematoxylin & eosin (HE) staining evaluated the cross-section area as previously described [13]. Image-Pro Plus 6.0 analyzed ten sections from each heart and six hearts from each group. PSR staining showed the collagen volume. Image-Pro Plus 6.0 was used for the fibrosis area calculation to analyze six sections from each heart and six hearts from each group.

Assessment of oxidative stress

Activities of manganese superoxide dismutase, superoxide dismutase 2 (MnSOD), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and malondialdehyde (MDA) in heart tissues and cardiomyocytes were detected by the corresponding kits purchased from Beyotime (Shanghai, China) following the manufacturer's instructions. SOD activity was quantified by the inhibition of formazan formation from nitroblue tetrazolium, which was converted by superoxide anion yielded from a xanthine-xanthine oxidase reaction system. The reactive oxygen species (ROS) level was measured according to a previous study [17] using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and an ELISA plate reader (Synergy HT, BioTek, Vermont, USA). Briefly, tissue and cell homogenate in PBS was subjected to a 10 μM DCFH-DA probe. After a 30-min incubation at 37 °C in the dark, fluorescence intensity was measured using a SpectraMax Gemini-EM microplate reader with excitation and emission wavelengths of 488 and 525 nm, respectively.

Cardiomyocyte isolation and culture

Neonatal rat cardiomyocyte (NRCM) culture was performed as previously described [15, 16]. Briefly, hearts of Sprague–Dawley rats (1–3 days old) were quickly removed, and ventricles were preserved and digested with 0.125% trypsin–EDTA (Gibco) four times for 15 min each time. Digestion was halted with DMEM-F12 supplemented with 15% fetal bovine serum (FBS, Gibco, USA). After five digestion reactions, the cells were collected and incubated in a 100-mm dish with DMEM-F12 supplemented with 15% FBS. After 90 min, cell culture medium was collected. NRCMs in the upper layer of cell medium were removed and seeded onto a 6-well plate to exclude noncardiac myocytes adhered to the bottom of a 100-mm dish. NRCMs were identified by α-actin staining. Samples in one experiment were collected in one independent cell culture run. All experiments were repeated three separate times.

Cells were transfected with adenovirus (Ad-) for 12 h to upregulate NEK6 (Ad-NEK6, MOI = 50, Vigene Bioscience, Jinan China). Consequently, the cells were stimulated with 33 mmol/L glucose for 48 h using 5.5 mmol/L normal glucose with 27.5 mM mannitol (to control osmolarity) as a control. Cells were transfected with NRF2 siRNA to knock down the NRF2 (Santa Cruz, sc-156128). Cells were also transfected with HSP72 for 12 h to knock down HSP72.

ELISA detection of inflammatory cytokines

Tumor necrosis factor α (TNFα), interleukin (IL)-1 from mouse hearts (left ventricular tissue), and cardiomyocytes were detected with ELISA purchased from BioLegend (430901, 432604). ELISA (Synergy HT, BioTek, USA) was used to measure the absorbance according to the manufacturer's instructions.

Western blot and qPCR

Total protein was isolated from left ventricular heart tissues, and then, NRCMs were subjected to SDS-PAGE (50 μg per sample). After transfer to Immobilon membranes (Millipore, Billerica, MA, USA), proteins were incubated overnight at 4 °C with primary antibodies against PGC-1α which were purchased from Abcam (1:1000 dilution); NRF2 (1:1000 dilution) and GAPDH (1:1000 dilution) were purchased from (Cell Signaling Technology (1:1000 dilution); HSP72 (1:1000 dilution) and phosphorylated (P)-HSP72 (1:1000 dilution) were purchased from Enzo Life Sciences. Blots

were developed with enhanced chemiluminescence (ECL) reagents (Bio-Rad, Hercules, CA, USA) and captured by a ChemiDoc MP Imaging System (Bio-Rad). GAPDH served as an internal reference protein.

Total RNA (2 μg per sample) from frozen mouse heart tissue (left ventricular tissue) and cardiomyocytes was reverse transcribed into cDNA using the oligonucleotide (DT) primer and the transcript first strand cDNA synthesis kit (Roche). Subsequently, a light Cycler 480 instrument (software version 1.5, Roche) and the SYBR green PCR master mix (Roche) were used to perform RT-PCR. Analysis of relative gene expression was performed using the $2^{-\Delta\Delta Cq}$ method. All genes were normalized to GAPDH.

Co-immunoprecipitation assays

Cultured NRCMs were lysed in immunoprecipitation buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40 supplemented with protease inhibitor cocktail) for 20 min and then centrifuged to remove cell debris. For each immunoprecipitation, 500 μL of the sample was incubated with 10 μL Protein A/G-agarose beads and 1 μg antibody on a rocking platform (overnight at 4 °C), according to the manufacturer's recommendations. Finally, immunoprecipitates were washed 5–6 times with cold immunoprecipitation buffer before adding 1 × loading buffer. Finally, eluted proteins were immunoblotted using the indicated primary antibodies.

Statistical analysis

All data are expressed as mean ± SD. Differences between groups were analyzed using a two-way analysis of variance followed by Tukey's post hoc test. An unpaired Student's *t*-test analyzed comparisons between the two groups. *P*-values less than 0.05 indicated statistical significance.

Results

The expression level of NEK6 in diabetic cardiomyopathy

We explore the expression level of NEK6 during the diabetic cardiomyopathy process. As a result, we found that protein level NEK6 was downregulated in heart tissue 2 months after the final STZ injection (Fig. 1A). The mRNA level of NEK6 was also reduced four months after the final STZ injection (Fig. 1B). We also detected the protein level of NEK6 in cardiomyocytes stimulated with high glucose (HG). A decrease in the level of NEK6 expression was observed in cardiomyocytes 24 h after exposure to HG (Fig. 1C). The mRNA level of NEK6 was also reduced in cardiomyocytes at 48 h post HG exposure (Fig. 1D).

NEK6 knockout deteriorates cardiac hypertrophy and fibrosis during diabetic cardiomyopathy

Consequently, NKE6 knockout mice were used to explore the functional role of NEK6 in diabetic cardiomyopathy. Figure 2A shows that the level of NEK6 was abolished in the hearts of NEK6 knockout mice. Fast blood glucose increased in the two groups of diabetic cardiomyopathy compared to the corresponding control group, while body weight decreased in the two groups of diabetic cardiomyopathy (Fig. 2B). No significant difference was observed in

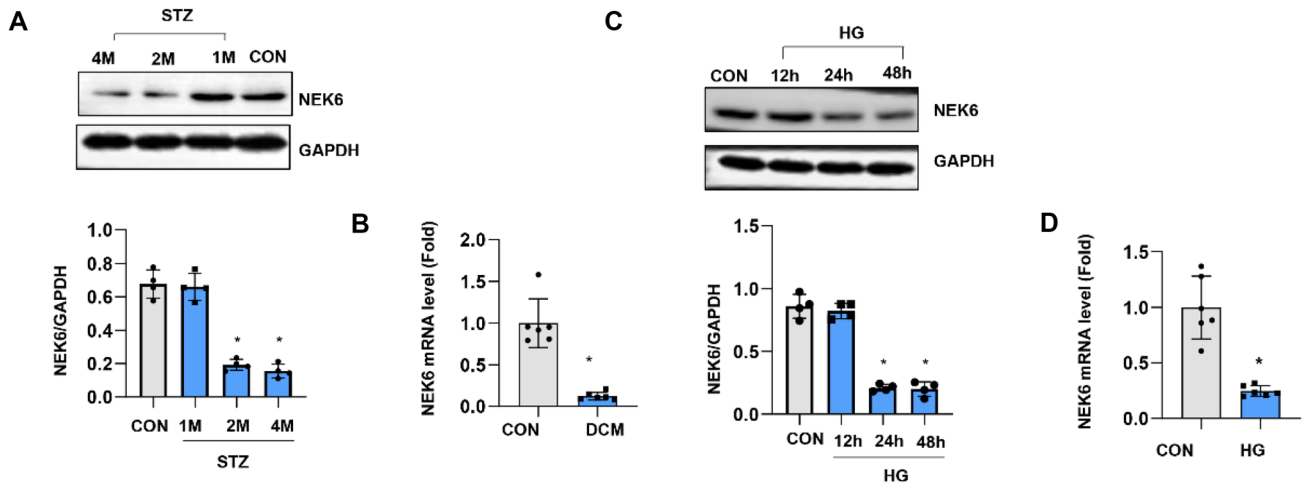

Fig. 1 Expression level of NEK6 in diabetic cardiomyopathy. **A** Protein level of NEK6 in diabetic cardiomyopathy mice heart ($n=4$); **B** mRNA level of NEK6 in DCM mice hearts ($n=6$); **C** protein level of NEK6 in cardiomyocytes exposed to high glucose (HG) ($n=4$); **D** NEK6 mRNA level in cardiomyocytes exposed to HG ($n=6$). $^*P<0.05$ vs. CON group. DCM diabetic cardiomyopathy

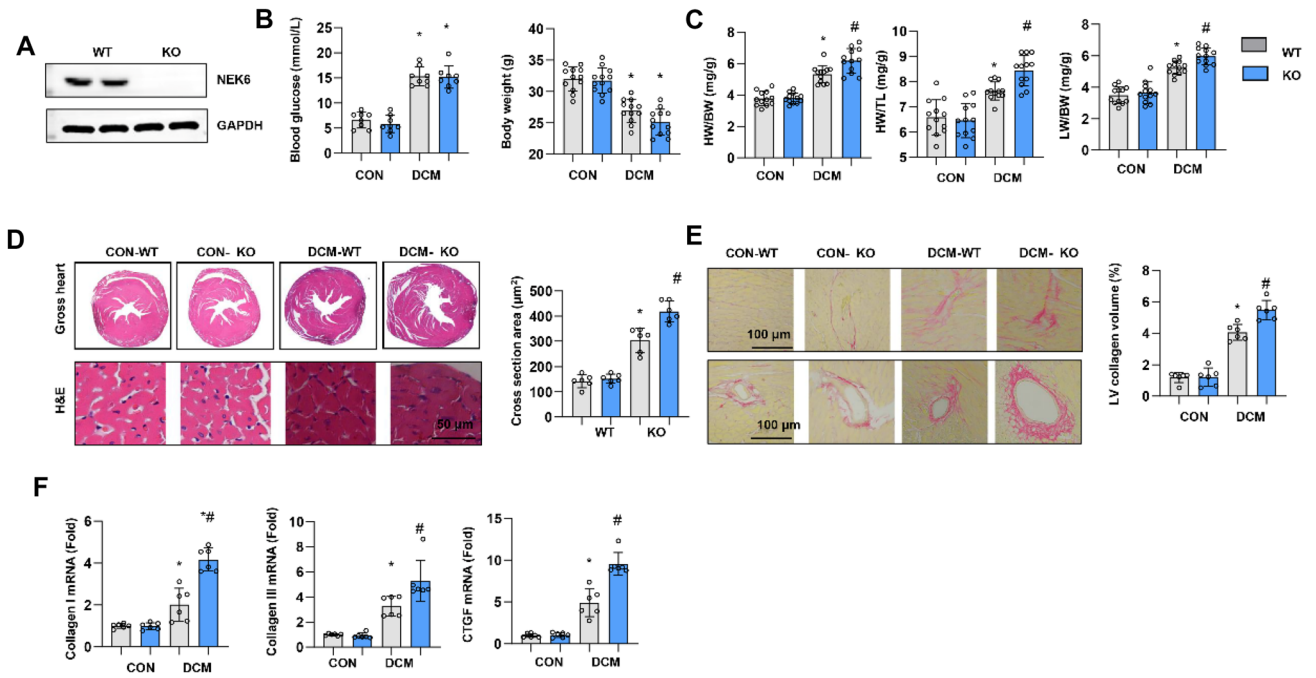

Fig. 2 NEK6 knockout deteriorates cardiac hypertrophy and fibrosis during DCM. **A** Protein level of NEK6 in mice hearts in each group ($n=4$); **B** blood glucose and body weight after four months of final STZ injection ($n=8$); **C** heart weight to body weight ratio (HW/BW), HW/tibia length (HW/TL), lung weight to body weight ratio (LW/BW) ($n=12$); **D** H&E staining and cross-sectional area in heart tissue ($n=6$); **E** PSR staining and LV collagen volume in heart tissue ($n=6$); F. mRNA level of fibrosis markers ($n=6$). $^{*}P<0.05$ vs. WT-CON group; #P < 0.05 vs. WT-DCM group

fast blood glucose and body weight between WT and KO diabetic cardiomyopathy- mice. Four months after STZ injection, the hearts were removed. Figure 2C shows that heart weight to body weight ratio (HW/BW), HW/tibia length (HW/TL), and lung weight to body weight ratio (LW/BW) were increased in the diabetic cardiomyopathy group; NEK6 knockout increased HW/BW, LW/BW ratios when compared to WT-diabetic cardiomyopathy mice. H&E staining detected the cross-section area (CSA) of cardiomyocytes. Figure 2D illustrates that CSA increased in diabetic cardiomyopathy mice, while NEK6 deficiency increased this change in diabetic cardiomyopathy mice. Cardiac collagen volume was evaluated by PSR staining; diabetic cardiomyopathy induced an increased left ventricular (LF) collagen volume, while NEK6 deficiency deteriorated collagen deposition (Fig. 2E). We also detected the transcription level of those fibrosis markers: collagen I, collagen III, and connective tissue growth factor (CTGF), which was shown to further increase in NEK6 knockout mice during diabetic cardiomyopathy (Fig. 2F). As for the death rate, we observed three mice among 15 (20% death rate) were dead before final observation at the NEK6-KO-diabetic cardiomyopathy group. No death was observed in the WT-diabetic cardiomyopathy group.

NEK6 deficiency aggressive diabetic cardiomyopathy-induced cardiac dysfunction

Cardiac function was assessed by echocardiography four months after the final STZ injection, and the LV end-diastolic diameter (LVEDd) and systolic diameter (LVESd) were increased in two diabetic cardiomyopathy groups. LV ejection fraction (LVEF) and fractional shortening (LVFS) decreased compared to the control group. These indicate that our diabetic cardiomyopathy model was successfully established with impaired cardiac systolic and diastolic function. NEK6 knockout induced increased LVEDd and dropped LVEF, and LVFS, indicating deteriorating cardiac dysfunction (Fig. 3A–C). Pressure volume loop result also showed that the E/A ratio and maximum rate of left ventricular pressure rise/decay (dp/dt max, dp/dt min) were decreased in diabetic cardiomyopathy heart, while NEK6 knockout further enhanced this dysfunction (Fig. 3D).

NEK6 knockout increased cardiac inflammation and oxidative stress

Furthermore, inflammation and oxidative stress activities were evaluated, as these two factors are main features of the

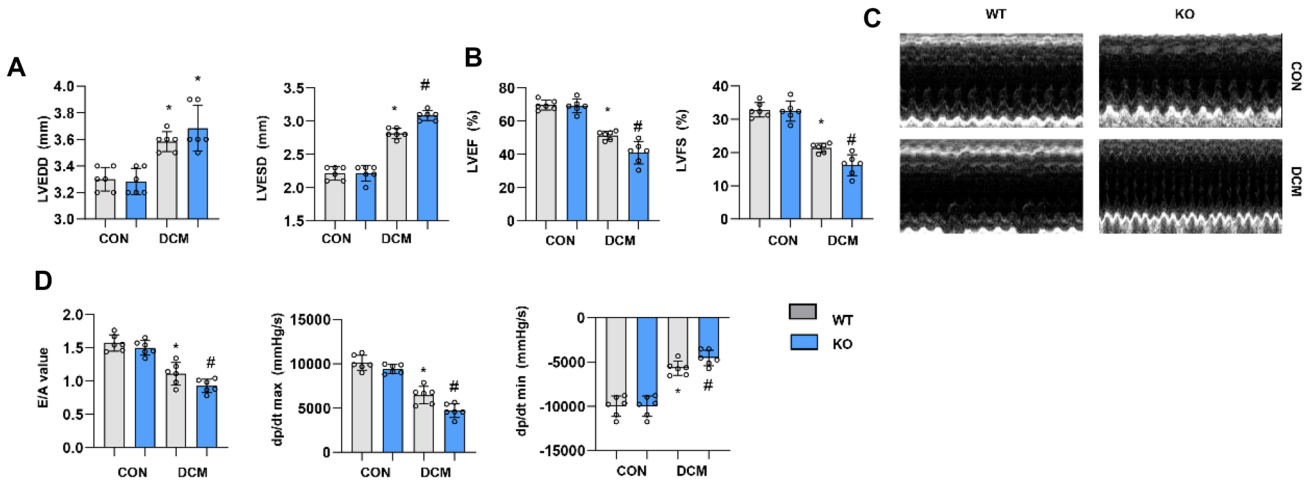

Fig. 3 NEK6 deficiency aggressive DCM-induced cardiac dysfunction. **A**–**C** echocardiography data after four months of final STZ injection ($n=6$); **D** pressure loop volume data after 4 months of final STZ injection ($n=6$). *$P<0.05$ vs. WT-CON group; #$P<0.05$ vs. WT-DCM group LV end-diastolic diameter (LVEDd), LV systolic diameter (LVESd), LV ejection fraction (LVEF), LV fractional shortening (LVFS), E/A ratio, maximum rate of left ventricular pressure rise/decay (dp/dt max, dp/dt min)

pathology of diabetic cardiomyopathy. The pro-inflammatory factors, including TNFα, IL-1, and IL-6, increased in mice hearts with diabetic cardiomyopathy, while NEK6 deficiency increased the level of these pro-inflammatory factors in the heart tissue (Fig. 4A). The ROS level was also increased in diabetic cardiomyopathy mice hearts with a remarkable increase in NEK6 deficiency heart tissue. Malondialdehyde (MDA), an intermediate product of lipid metabolism, was also elevated, while the activity of antioxidase, such as SOD2 and Gpx, was reduced in heart tissue of diabetic cardiomyopathy. These changes deteriorated NEK6 deficiency heart tissue (Fig. 4B, C).

NEK6 overexpress in cardiomyocytes attenuates high glucose-induced inflammation and oxidative stress

Cardiomyocytes were transfected with NEK6 to upregulate NEK6 (Fig. 5A). Cells were also exposed to HG. We

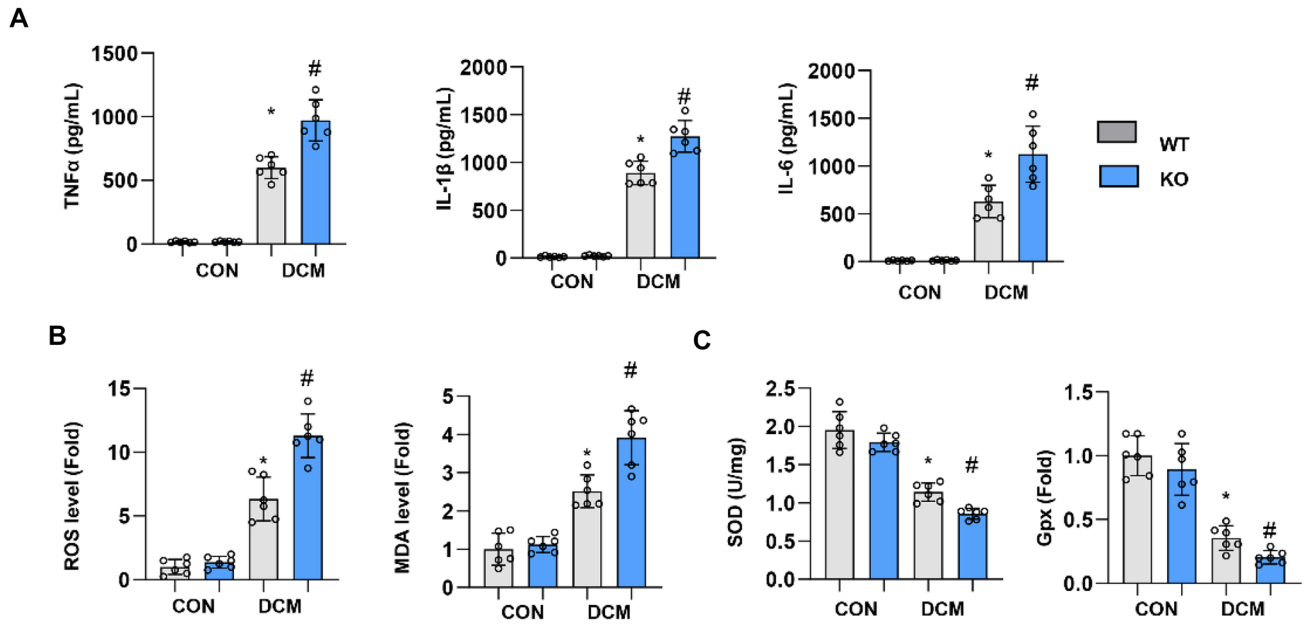

Fig. 4 NEK6 knockout increased cardiac inflammation and oxidative stress. **A** Pro-inflammatory factors in heart tissue after four months of final STZ injection ($n=6$); **B** ROS level and MDA level in heart tissue after 4 months of final STZ injection ($n=6$); **C** SOD2 and Gpx activity in heart tissue after 4 months of final STZ injection ($n=6$). *$P<0.05$ vs. WT-CON group; #$P<0.05$ vs. WT-DCM group

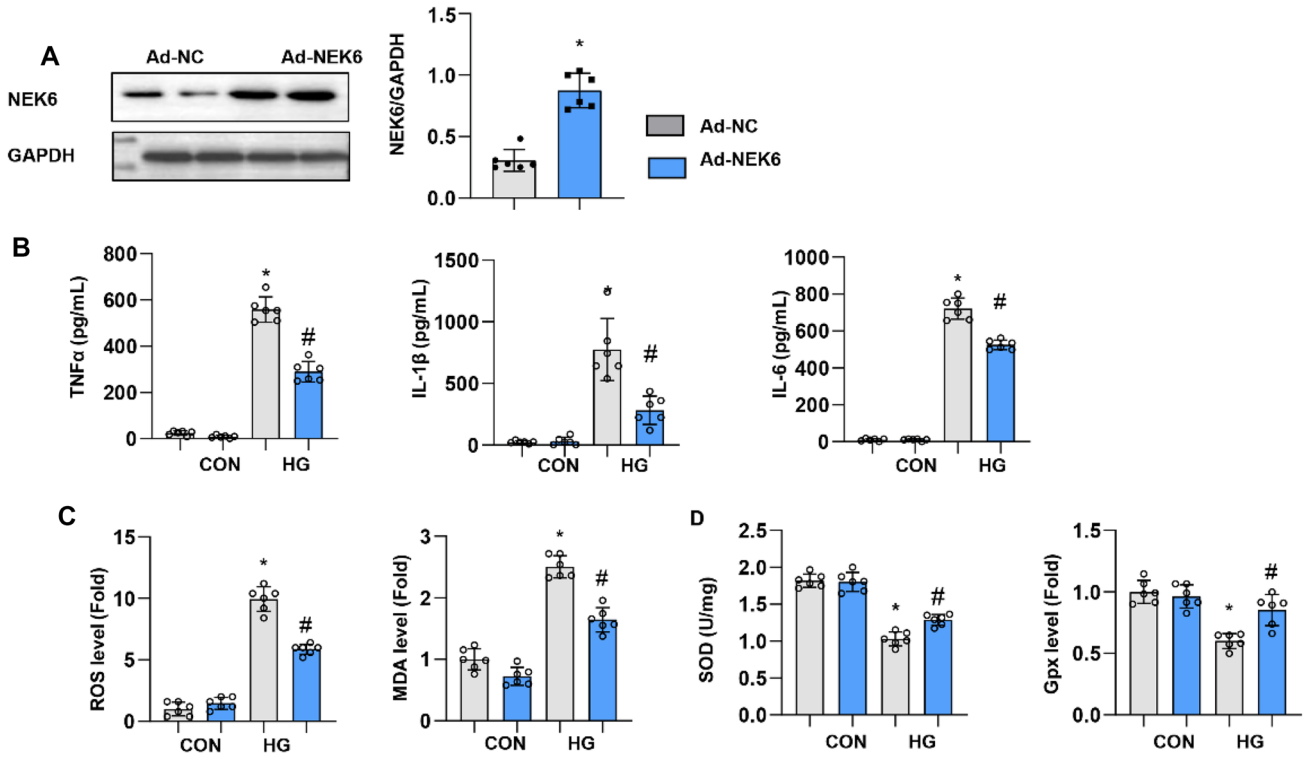

Fig. 5 NEK6 overexpress in cardiomyocytes attenuates high glucose-induced inflammation and oxidative stress. Cardiomyocytes were transfected with Ad-NEK6. **A** Protein level of NEK6 after transfection ($n=6$); **B** pro-inflammatory factors in cardiomyocytes after exposure to high glucose ($n=6$); **C** ROS level and MDA level in cardiomyocytes ($n=6$); **D** SOD2 and Gpx activity in cardiomyocytes ($n=6$). *$P<0.05$ vs. Ad-NC-CON group; #$P<0.05$ vs. Ad-NC-HG group

evaluated the role of NEK6 in cardiomyocyte inflammation and oxidative stress under HG stimulation. As expected, cells in the HG group showed increased release of TNFα and IL-1, and IL-6, while NEK6 overexpression could improve the release of this pro-inflammatory factor (Fig. 5B). The ROS level in cardiomyocytes was increased, and the MDA level after HG stimulation, while the activity of anti-oxidases, such as SOD2 and Gpx, was reduced. NEK6 overexpression may reduce cell ROS and MDA levels and increase SOD and Gpx activity (Fig. 5C, D).

NEK6 interacts with HSP72 and increases HSP72 phosphorylation

A previous study has found that NEK6 could affect HSP72 [11]. Levels of HSP72 in diabetic cardiomyopathy heart tissue and cardiomyocytes were assessed. Figure 6A shows that the total level of HSP72 was unchanged in the two hearts of mice with diabetic cardiomyopathy. However, phosphorylated HSP72 was downregulated in hearts of diabetic cardiomyopathy mice, while NEK6 knockout further decreased HSP72 phosphorylation (Fig. 6A). Downstream targets NRF2 and PGC1-α were also detected. As expected, the level of PGC1-α and nuclear NRF2 was downregulated in diabetic cardiomyopathy hearts while further reduced in NEK6 –KO mice hearts (Fig. 6A, B). In NEK6 overexpressed cardiomyocytes, cells in the HG group showed reduced levels of phosphorylated HSP72, NRF2, and PGC1-α. NEK6 overexpression attenuated these alterations. Nuclear NRF2 has also been upregulated in NEK6 overexpressed cardiomyocytes (Fig. 6C, D). We performed a co-IP assay to assess whether NEK6 could interact with HSP72. Figure 6E shows that NEK6 had an interaction role with HSP72. These data indicate that NEK6 may interact with HSP72 promoting its activation and then regulating the downstream antioxidant pathway.

HSP72 silence abrogates the anti-inflammation and anti-oxidative stress effects of NEK6

To confirm the mechanism of NEK6 on HSP72, we generated an HSP72 siRNA to knock down HSP72 (Fig. 7A–B). Cardiomyocytes were transfected with both Ad-NEK6 and HSP72 siRNA and exposed to HG. HG-induced remarkable pro-inflammatory factors release (TNFα, IL-1, IL-6) and oxidative stress (increased ROS, MDA, decreased SOD2, Gpx activity). HSP72 silence caused a deteriorated inflammation response and oxidative stress. However, Ad-NEK6

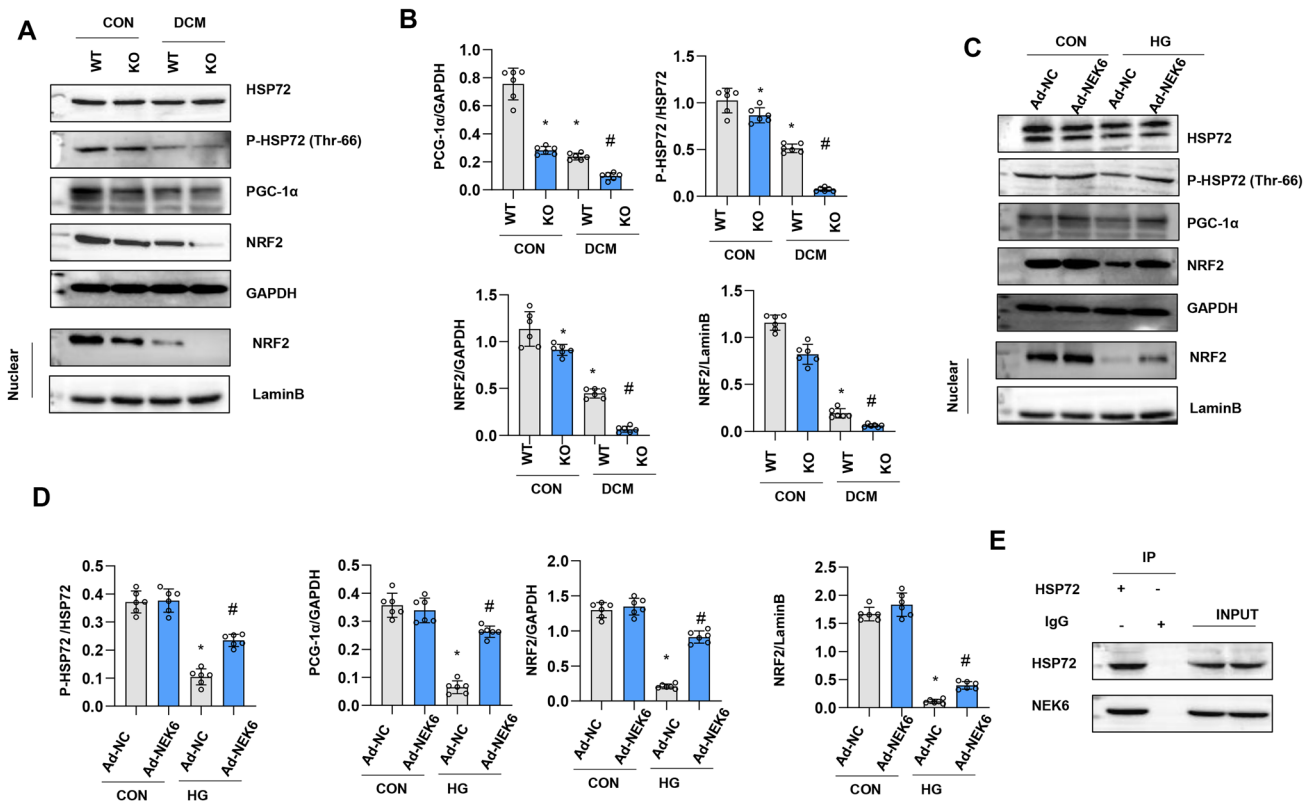

Fig. 6 NEK6 interacts with HSP72 and increases HSP72 phosphorylation. **A**, **B** protein level of total HSP72, P-HSP72, PGC-1α, NRF2, and nuclear NRF2 level in heart tissue after four months of final STZ injection ($n=6$). *$P<0.05$ vs. WT-CON group; #$P<0.05$ vs. WT-DCM group; **C**, **D** protein level of total HSP72, P-HSP72, PGC-1α, NRF2, and nuclear NRF2 level in cardiomyocytes transfected with Ad-NEK6 ($n=6$). *$P<0.05$ vs. Ad-NC-CON group; #$P<0.05$ vs. Ad-NC-HG group; **E** Co-IP assay about HSP72 and NEK6 in cardiomyocytes

and HSP72 siRNA cells revealed the same extent of inflammation response and oxidative stress as the cells in the HSP72 siRNA group (Fig. 7C–E). These data indicate that NEK6 could not protect cardiomyocytes without HSP72.

NEK6 overexpression in mice hearts inhibits cardiac hypertrophy and fibrosis, but HSP72 knockout blurs these effects

To confirm the mechanism of NEK6 on HSP72 in vivo, mice were injected with AAV9-NEK6 to overexpress NEK6 in mice hearts and mice injected with AAV9-shHSP72 to knockdown HSP72 in mice hearts (Fig. 8A). NEK6 overexpress led to an increase in HSP72 phosphorylation, while HSP72 suppression reduced the enhanced HSP72 phosphorylation level induced by NEK6 overexpress (Fig. 8A). Fast blood glucose increased in three groups of diabetic cardiomyopathy, and body weight decreased in three groups of diabetic cardiomyopathy (Fig. 8B). No significant difference was observed in fast blood glucose and body weight among these three diabetic cardiomyopathy groups. Four months after STZ injection, HW/BW and LW/BW ratios were increased in diabetic cardiomyopathy groups; NEK6 overexpressing reduced HW/BW and LW/BW ratios compared to the diabetic cardiomyopathy group, while HSP72 knockdown blurred this result. CSA and cardiac collagen volume were increased in diabetic cardiomyopathy mice. NEK6 overexpression decreased these changes in diabetic cardiomyopathy mice, while HSP72 knockdown counteracted these results by NEK6 overexpression (Fig. 8D, E).

NEK6 overexpress in mice improves cardiac function in diabetic cardiomyopathy, while HSP72 knockout blurs these effects

Cardiac function was assessed by echocardiography four months after the final STZ injection. LVEDd increased, while LVEF and LVFS decreased in three groups of diabetic cardiomyopathy compared to the control group. NEK6 overexpression induced an increase in LVEF and LVFS expression levels, indicating improved cardiac function (Fig. 9A). Pressure volume loop result showed that E/A ratio, dp/dt max, and dp/dt min were increased NEK6 overexpressing diabetic cardiomyopathy heart, while HSP72 knockdown counteracted these results

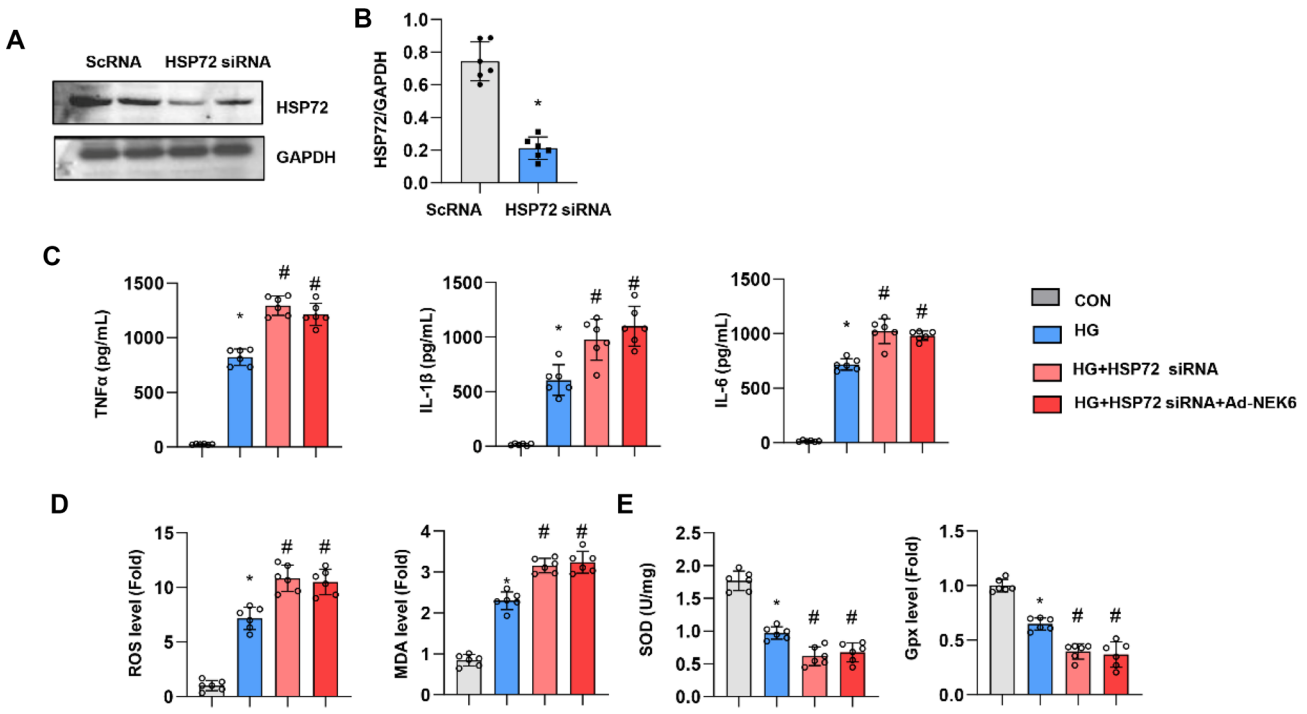

Fig. 7 HSP72 silence abrogates the anti-inflammation and anti-oxidative stress effects of NEK6. Cardiomyocytes were transfected with Ad-NEK6 and HSP72 siRNA. **A**, **B** Protein level of NEK6 after HSP72 siRNA transfection ($n=6$). *$P<0.05$ vs. ScRNA group; **C** pro-inflammatory factors in cardiomyocytes after exposure to high glucose ($n=6$); **D** ROS level and MDA level in cardiomyocytes ($n=6$); **E** SOD2 and Gpx activity in cardiomyocytes ($n=6$). *$P<0.05$ vs. CON group; #$P<0.05$ vs. HG group

induced by NEK6 overexpress (Fig. 9A, B). In addition, we evaluated inflammation and oxidative stress. Pro-inflammatory factors were inhibited in mice with the injection of AAV9-NEK6, while suppression of HSP72 increased the level of those pro-inflammatory factors in heart tissue (Fig. 9C). The MDA level was also increased in diabetic cardiomyopathy mice hearts with a remarkable decrease in NEK6 overexpressing heart tissue. The anti-oxidase, such as SOD2, Gpx activity was increased in NEK6 overexpressing diabetic cardiomyopathy heart tissue. However, these changes deteriorated in mice with AAV9-shHSP72 injection (Fig. 9D). All these data suggest that HSP72 knockdown counteracts the protective effects of NEK6 upregulation in the heart.

Discussion

Diabetes mellitus is an epidemic that affects 463 million people worldwide, and its incidence rate has increased annually [18]. Recently, despite the great progress in treating diabetes, diabetes-related cardiovascular diseases have become the main causes of disability and death [18]. Additionally, diabetes increases the incidence rate and severity of coronary artery disease and myocardial infarction, increasing the development of heart failure. The study found that the risk of cardiovascular disease-related death or hospitalization due to heart failure increased by 75% in diabetic patients [19]. The risk of HF in people with diabetes is significantly higher than that of non-diabetic patients [19]. Diabetic cardiomyopathy occurs independently of other heart diseases, such as coronary artery disease and hypertension [1]. This study found that NEK6 was downregulated in T1DM-induced cardiomyopathy heart tissue and cardiomyocytes. NEK6 knockout deteriorated cardiac dysfunction, cardiac hypertrophy, fibrosis, inflammation response, and oxidative stress. Additionally, we found that NEK6 overexpression could attenuate high glucose-induced inflammation and oxidative stress. NEK6 may be a new therapeutic target for diabetic cardiomyopathy.

Oxidative stress is a characteristic pathological feature of diabetic cardiomyopathy. Occurrence of cardiac dysfunction in the T1DM animal model was significantly correlated with the increase of ROS level in cardiomyocytes [6]. STZ-induced diabetic cardiomyopathy is characterized by increased lipid peroxidation, increased nitrotyrosine and carbonyl protein content, and reduced glutathione [1, 6, 7]. The increase in oxidative stress in the STZ model is considered to be related to the decrease in electron transfer chain enzyme activity [7]. Other animal models of diabetics, including transgenic OVE26 mice, insulin-dependent spontaneous diabetes mellitus Wistar rats, Akita diabetic mice, and four oxazine-induced diabetic mice, showed a marked

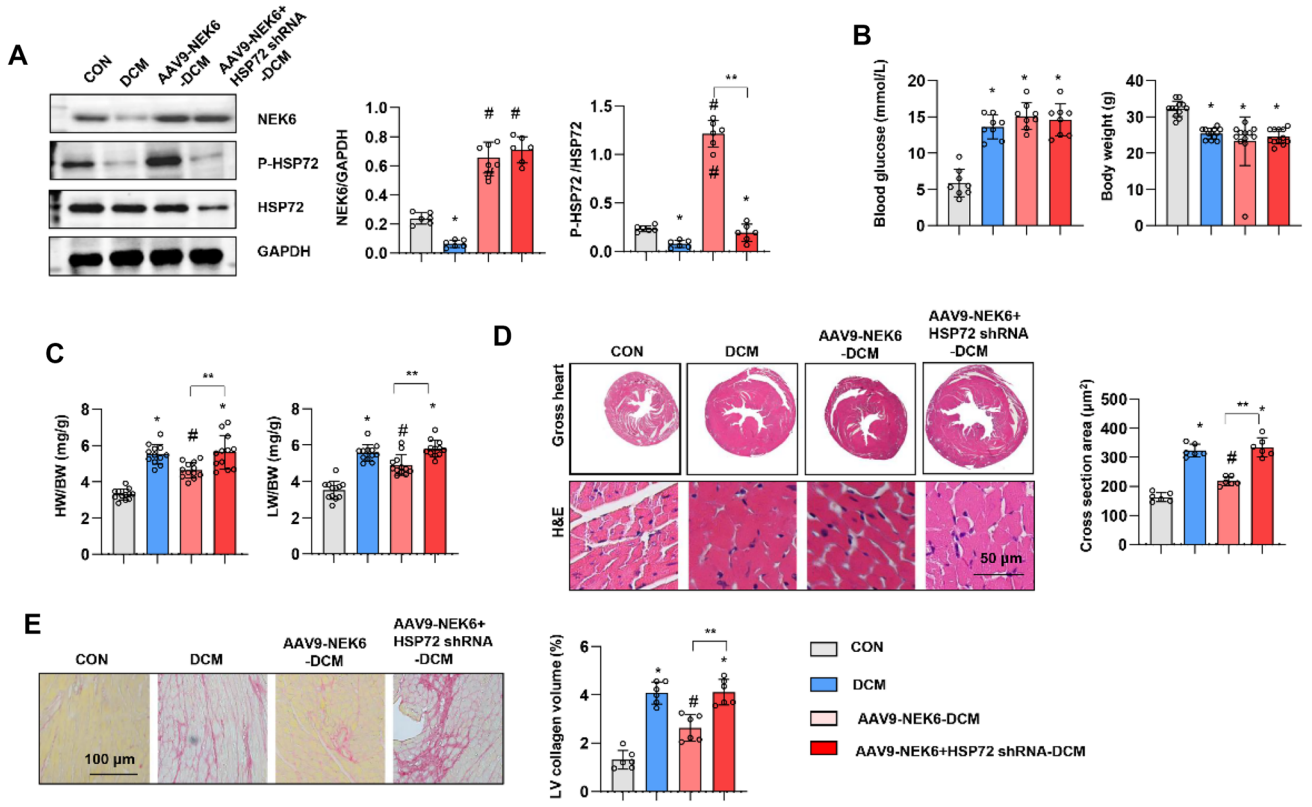

Fig. 8 NEK6 overexpress in mice hearts inhibits cardiac hypertrophy and fibrosis, but HSP72 knockout blurs these effects. **A** Protein level of NEK6 and phosphorylated-HSP72, total HSP72 in mice hearts injected with AAV9-NEK6 and HSP72 shRNA ($n=6$); **B** blood glucose and body weight after 4 months of final STZ injection ($n=8$). **C** Heart weight to body weight ratio (HW/BW), lung weight to body weight ratio (LW/BW) ($n=12$); **D** H&E staining and cross-section area in heart tissue ($n=6$); **E** PSR staining and LV collagen volume in heart tissue ($n=6$). $^*P<0.05$ vs. CON group; $\#P<0.05$ vs. DCM group

increase in lipid peroxidation, tyrosine nitration, and protein carbonylation [6].

Additionally, redox imbalance was significantly associated with impaired mitochondrial respiration [20]. In this study, we found that ROS was increased in STZ-induced diabetic cardiomyopathy; antioxidases were reduced in the hearts of mice hearts, which indicates a redox imbalance. This study found that NEK6 deficiency further increases this redox imbalance. Previous studies found that NEK6 could inhibit ROS generation in human cancer cells by targeting P53 [21]. We also found that NEK6 restored the redox balance in cardiomyocytes exposed to HG. Thus, NEK6 may exert protection in diabetic cardiomyopathy via a balancing redox system.

Heat shock protein 72 (HSP72) is an essential family member of the heat shock protein 70 family [11]. Its expression is low in healthy cells, but it increases under stress. Hsp72 is a stress-inducible Hsp70. Studies showed that HSP72 has an antioxidant effect and can regulate different signal molecules to participate in oxidative cell stress and apoptosis [22, 23]. Activating Hsp72 has been reported to increase the number of mitochondrial skeletal muscles and oxidative capacity [24]. Mice overexpressing Hsp72 have also been reported to be protected from oxidative stress and liver injury-induced hepatocellular death [25].

Moreover, Daolin Tang found that nuclear Hsp72 is a negative regulator of oxidative stress-induced inflammation [26]. These indicate the anti-oxidative stress effects of Hsp72. Laura O'Regan reported that NEK6 could activate Hsp72 and increase mitotic progress [11]. However, whether NEK6 affects Hsp72 in cardiomyocytes was largely unknown. In this study, we found that NEK6 increased the activation of Hsp72 phosphorylation. NEK6 also increased the anti-oxidative stress transcriptional factor NRF2 expression. Furthermore, we found that NEK6 could interact with HSP72, which may promote its activation. Nrf2 normally translocates into the nucleus in the cytoplasm under cell stress. It binds directly to the gene regulatory region of the antioxidant response elements, including many antioxidant and detoxifying enzymes, ECT transporters, and mitochondrial biogenesis proteins [8]. Paula reported that after high-intensity interval training, intramuscular expresses high

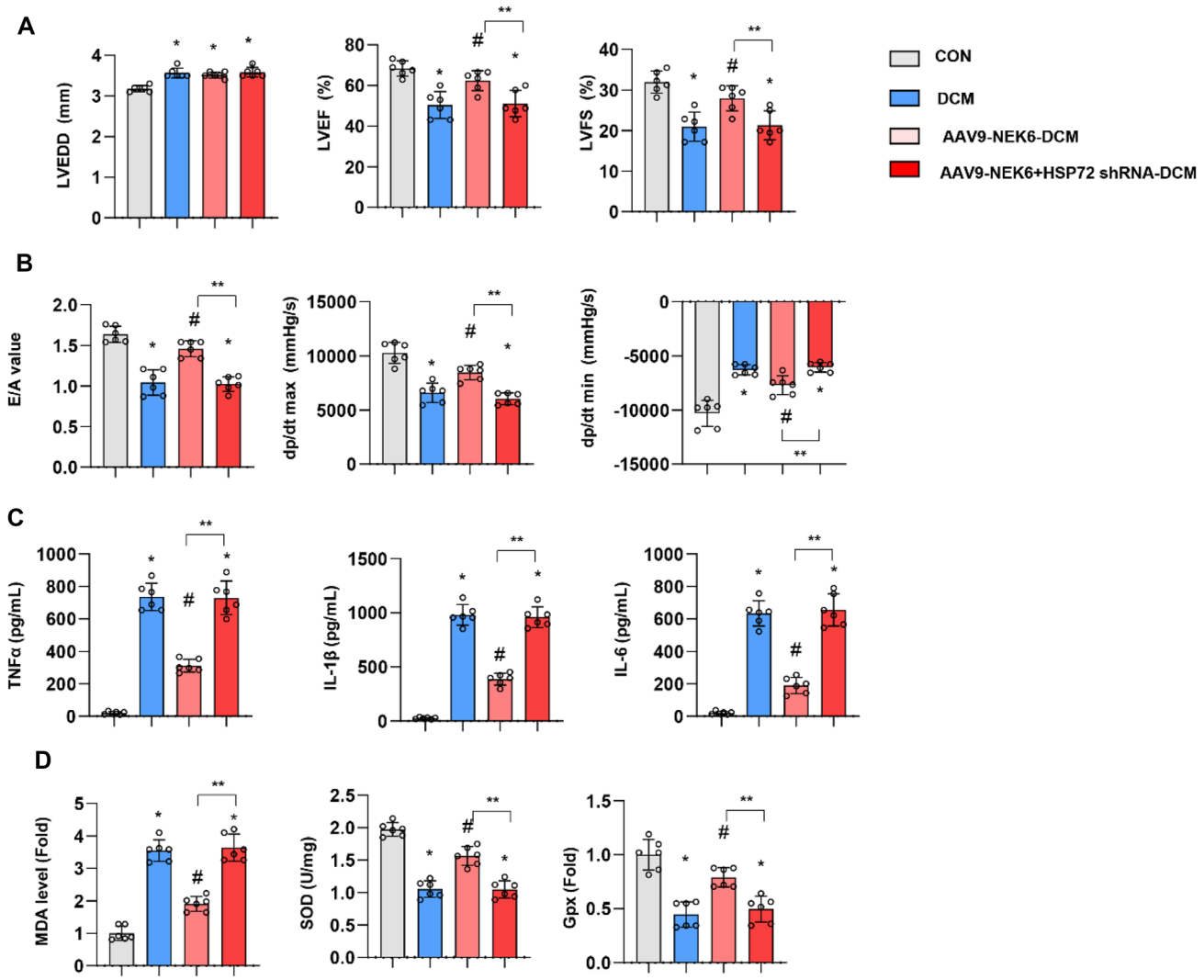

Fig. 9 NEK6 overexpress in mice improves cardiac function in DCM, while HSP72 knockout blurs these effects. **A** Echocardiography data after 4 months of final STZ injection ($n=6$); **B** pressure loop volume data after 4 months of final STZ injection ($n=6$); **C** pro-inflammatory factors in heart tissue after 4 months of final STZ injection ($n=6$); **D** MDA level, SOD2, and Gpx activity in heart tissue after 4 months of final STZ injection ($n=6$). $^{*}P<0.05$ vs. CON group; $^{\#}P<0.05$ vs. DCM group

levels of HSP72 and PGC-1α, associated with high mitochondrial biogenesis [27]. In this study, we also found that NEK6 upregulated the level of PGC-1α. Thus, we imply that NEK6 may interact with HSP72 and promote its activation. The activation of Hsp72 caused anti-oxidative stress in cardiomyocytes, which activated the NRF2/PGC-1α pathway. In the reversion experiments, HSP72 knockdown abolished the protective effects of NEK6 overexpression in vivo and in vitro, confirming our hypothesis. Does an increase in NRF2 a secondary effect of Hsp72 activation? Or could Hsp72 directly activate NRF2?

Further studies are needed to elucidate how Hsp72 activates NRF2 in cardiomyocytes. Herein, we used an STZ-induced type 1 diabetic mice model, and cardiomyopathy was shown by cardiac remodeling and dysfunction. Further studies are needed to elucidate whether NEK6 exerts the same effects on cardiomyopathy induced by type 2 diabetes.

In conclusion, NEK6 knockout deteriorated cardiac dysfunction, cardiac hypertrophy, fibrosis, inflammation response, and oxidative stress. NEK6 overexpression could attenuate high glucose-induced inflammation and oxidative stress. By regulating the HSP72-NRF2 pathway, NEK6 may become a new therapeutic target for diabetic cardiomyopathy.

Acknowledgements We thank Jun Gong for excellent technical assistance.

Author contribution Shuangyin Shao and Lu Gao had the idea for this review. Lu Gao, Lili Xiao, and Meng Jia provided advice on experimental design and contributed critical suggestions for revision and

finalization of the manuscript. Lili Xiao, Meng Jia, Rui Yao, and Xiaofang Wang contributed to the literature search and critical appraisal of the studies. Chuyang Zhang and Guojun Zhao contributed suggestions for data analyses part. Lu Gao and Shuangyin Shao revised and finalized the manuscript. All authors have given critical suggestions for the final version of the manuscript.

Funding This study was supported by the Outstanding Youth Science Fund of Henan Province (Grant No. 212300410076), the National Natural Science Foundation of China (Grant Nos. 81970201, 82070284), the Key Scientific Research Project of Henan Province College(21A320044).

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate All experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

Consent for publication All authors consent to the publication of the article in Journal of Molecular Medicine.

Competing interests The authors declare no competing interests.

References

1. Dillmann WH (2019) Diabetic cardiomyopathy. Circ Res 124(8): 1160–1162
2. Jia G, Whaley-Connell A, Sowers JR (2018) Diabetic cardiomyopathy: a hyperglycaemia- and insulin-resistance-induced heart disease. Diabetologia 61(1):21–28
3. Lind M et al (2011) Glycaemic control and incidence of heart failure in 20,985 patients with type 1 diabetes: an observational study. Lancet 378(9786):140–146
4. Jia G, Hill MA, Sowers JR (2018) Diabetic cardiomyopathy: an update of mechanisms contributing to this clinical entity. Circ Res 122(4):624–638
5. Jia G, DeMarco VG, Sowers JR (2016) Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. Nat Rev Endocrinol 12(3):144–153
6. Byrne NJ et al (2021) Therapeutic potential of targeting oxidative stress in diabetic cardiomyopathy. Free Radic Biol Med 169:317–342
7. Faria A, Persaud SJ (2017) Cardiac oxidative stress in diabetes: Mechanisms and therapeutic potential. Pharmacol Ther 172:50–62
8. Vashi R, Patel BM (2021) NRF2 in cardiovascular diseases: a ray of hope! J Cardiovasc Transl Res 14(3):573–586
9. Wang X et al (2022) Ferroptosis is essential for diabetic cardiomyopathy and is prevented by sulforaphane via AMPK/NRF2 pathways. Acta Pharm Sin B 12(2):708–722
10. Gu J et al (2017) Metallothionein is downstream of Nrf2 and partially mediates sulforaphane prevention of diabetic cardiomyopathy. Diabetes 66(2):529–542
11. O'Regan L et al (2015) Hsp72 is targeted to the mitotic spindle by Nek6 to promote K-fiber assembly and mitotic progression. J Cell Biol 209(3):349–358
12. Xu F et al (2021) Characterizing modifier genes of cardiac fibrosis phenotype in hypertrophic cardiomyopathy. Int J Cardiol 330:135–141
13. Bian Z et al (2014) Never in mitosis gene A related kinase-6 attenuates pressure overload-induced activation of the protein kinase B pathway and cardiac hypertrophy. PLoS ONE 9(4):e96095
14. Gao L et al (2018) LAZ3 protects cardiac remodeling in diabetic cardiomyopathy via regulating miR-21/PPARa signaling. Biochim Biophys Acta Mol Basis Dis 1864(10):3322–3338
15. Zong J et al (2018) Nuclear localization leucine-rich-repeat protein 1 deficiency protects against cardiac hypertrophy by pressure overload. Cell Physiol Biochem 48(1):75–86
16. Gu Y et al (2021) CTRP1 aggravates cardiac dysfunction post myocardial infarction by modulating TLR4 in macrophages. Front Immunol 12:635267
17. West TM et al (2019) Phosphodiesterase 5 associates with beta2 adrenergic receptor to modulate cardiac function in type 2 diabetic hearts. J Am Heart Assoc 8(15):e012273
18. Saeedi P et al (2019) Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. Diabetes Res Clin Pract 157:107843
19. Kristensen SL et al (2017) Clinical and echocardiographic characteristics and cardiovascular outcomes according to diabetes status in patients with heart failure and preserved ejection fraction: a report from the I-Preserve Trial (Irbesartan in Heart Failure With Preserved Ejection Fraction). Circulation 135(8):724–735
20. Tan Y et al (2020) Mechanisms of diabetic cardiomyopathy and potential therapeutic strategies: preclinical and clinical evidence. Nat Rev Cardiol 17(9):585–607
21. Jee HJ et al (2010) Nek6 overexpression antagonizes p53-induced senescence in human cancer cells. Cell Cycle 9(23):4703–4710
22. Cong X et al (2017) Puerarin ameliorates heat stress-induced oxidative damage and apoptosis in bovine Sertoli cells by suppressing ROS production and upregulating Hsp72 expression. Theriogenology 88:215–227
23. Yamashima T (2012) Hsp70.1 and related lysosomal factors for necrotic neuronal death. J Neurochem 120(4):447–494
24. Henstridge DC et al (2014) Activating HSP72 in rodent skeletal muscle increases mitochondrial number and oxidative capacity and decreases insulin resistance. Diabetes 63(6):1881–1894
25. Levada K et al (2018) Hsp72 protects against liver injury via attenuation of hepatocellular death, oxidative stress, and JNK signaling. J Hepatol 68(5):996–1005
26. Tang D et al (2007) Nuclear heat shock protein 72 as a negative regulator of oxidative stress (hydrogen peroxide)-induced HMGB1 cytoplasmic translocation and release. J Immunol 178(11):7376–7384
27. Aguiar PF et al (2016) Post-exercise cold water immersion does not alter high intensity interval training-induced exercise performance and Hsp72 responses, but enhances mitochondrial markers. Cell Stress Chaperones 21(5):793–804