The potential protective effects of nintedanib against cardiac hypertrophy in vitro and in vivo

Keywords
cardiac hypertrophy, SIRT1, oxidative stress, cardiomyocytes, Nintedanib

Abstract

Introduction
Cardiac hypertrophy is an independent risk factor for heart failure. However, the underlying mechanisms of cardiac hypertrophy are still unclear. Nintedanib is a Food and Drug Administration (FDA) approved therapeutic agent for the treatment of progressive fibrosing lung diseases.

Material and methods
In this study, we examined the effects of nintedanib on cardiac hypertrophy using an in vivo murine model with the transverse aortic constriction (TAC) operation and an in vitro cardiomyocytes model stimulated with Ang II.

Results
Nintedanib has a protective effect on cardiac function in TAC mice with decreased heart rates, heart weight/body weight (HW/BW), and reduced plasma levels of creatine kinase-MB (CK-MB) and aspartate aminotransferase (AST). Wheat germ agglutinin (WGA) staining proved that the increased cardiomyocytes sizes in TAC mice were restored by nintedanib treatment. Nintedanib also reversed the decreased plasma levels of oxidative markers nuclear factor erythroid-2-related factor 2 (Nrf2), lipid peroxidation products thiobarbituric acid reactive substances (TBARS), and GSH, as well the increased homocysteine (Hcy) levels in TAC mice. In the in vitro cardiomyocytes model, cells were treated with nintedanib, followed by Ang II stimulation. Nintedanib improved Ang II induction-caused cell injury and oxidative stress in H9C2 cells, as shown by the decreased release of lactate dehydrogenase (LDH), and elevated mRNA levels of GPX1 and HO-1. Mechanistically, we prove that the protective effect of nintedanib is mediated by SIRT1.

Conclusions
In conclusion, this study demonstrates the protective effects of nintedanib on cardiac hypertrophy both in vivo and in vitro, which was attributed to its anti-oxidative activity through regulating SIRT1 expression.

Title: The potential protective effects of nintedanib against cardiac hypertrophy *in vitro* and *in vivo*

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Abstract

Cardiac hypertrophy is an independent risk factor for heart failure. However, the underlying mechanisms of cardiac hypertrophy are still unclear. Nintedanib is a Food and Drug Administration (FDA) approved therapeutic agent for the treatment of progressive fibrosing lung diseases. Recent studies have proved that nintedanib exerts therapeutic effects on cardiac fibrosis and dysfunction. In this study, we examined the effects of nintedanib on cardiac hypertrophy using an *in vivo* murine model with the transverse aortic constriction (TAC) operation and an *in vitro* cardiomyocytes model stimulated with Ang II. The results show that nintedanib has a protective effect on cardiac function in TAC mice with decreased heart rates, heart weight/body weight (HW/BW), and reduced plasma levels of creatine kinase-MB (CK-MB) and aspartate aminotransferase (AST). Wheat germ agglutinin (WGA) staining proved that the increased cardiomyocytes sizes in TAC mice were restored by nintedanib treatment. Nintedanib also reversed the decreased plasma levels of oxidative markers nuclear factor erythroid-2-related factor 2 (Nrf2), lipid peroxidation products thiobarbituric acid reactive substances (TBARS), and glutathione (GSH), as well the increased homocysteine (Hcy) levels in TAC mice. In the *in vitro* cardiomyocytes model, cells were treated with nintedanib, followed by Ang II stimulation. Nintedanib improved Ang II induction-caused cell injury and oxidative stress in H9C2 cells, as shown by the decreased release of lactate dehydrogenase (LDH), and elevated mRNA levels of glutathione peroxidase1 (GPX1) and heme oxygenase-1 (HO-1). Mechanistically, we prove that the protective effect of nintedanib is mediated by SIRT1. In conclusion, this study demonstrates the protective effects of nintedanib on cardiac hypertrophy both *in vivo* and *in vitro*, which was attributed to its anti-oxidative activity through regulating SIRT1 expression.

Key words: Nintedanib, Cardiac hypertrophy, Cardiomyocytes, Oxidative stress, SIRT1

1. Introduction

Cardiac hypertrophy is a pathological phenomenon due to an increase in the muscle mass of heart tissue when faced with detrimental stimuli [1]. The heart undergoes remodeling to preserve normal function, however, sustained cardiac hypertrophy can result in impaired contractile function [2]. The pathophysiology of cardiac hypertrophy is multifactorial, several molecular and cellular systems are involved in its development [3]. Experimental evidence has supported that oxidative stress may serve as a key mediator of cardiac hypertrophy (both in genetic and acquired forms) [4]. Mitochondrial dysfunction, metabolic disturbances, inflammation, and other dysregulated cellular processes may arise in response to intrinsic or external stress, which may result in oxidative stress in cardiomyocytes [4]. However, a better understanding of the pathogenesis of cardiac hypertrophy and investigation into essential therapeutic methods are urgently required.

Nintedanib, an intracellular inhibitor of tyrosine kinases, is an FDA-approved therapeutic agent for attenuating progressive fibrosing lung diseases. It has been documented that nintedanib exerts its roles due to its consistent anti-fibrotic and anti-inflammatory activities. Additionally, several studies have also highlighted the anti-oxidative property of nintedanib. Boxhammer *et al*. [6] reported that nintedanib prevents lung allograft dysfunction via its potential anti-oxidative effect through downregulation of reactive oxygen species (ROS). Fois *et al*. [7] found that the combination of pirfenidone with nintedanib has inhibitory effects on inflammation and systemic oxidative stress markers in idiopathic pulmonary fibrosis (IPF) patients. A recent study has shown that in a murine heart failure (HF) model, nintedanib exerts considerable therapeutic effects on cardiac fibrosis and dysfunction [8]. However, evidence of the therapeutic efficacy of nintedanib in other cardiac diseases is still insufficient. More direct evidence to support the cardioprotective effects of nintedanib is needed.

In this study, we examined the effects of nintedanib on cardiac hypertrophy using an *in vivo* murine model with the transverse aortic constriction (TAC) operation and an *in vitro* cardiomyocyte model stimulated with Ang II. We also explored the

involvement of SIRT1 in the cardioprotective effects of nintedanib and the related mechanisms.

2. Methods and materials

2.1 Animals and treatment

All of the C57BL/6J mice (n=45; 8-week-old male) were obtained from Shanghai Slac Laboratory Animal Co. LTD. They either underwent a sham operation (n=15) or a transverse aortic constriction (TAC) operation (n=30) as previously described [9]. The mice that underwent TAC operation were divided into two groups: the TAC operation group (n=15), which underwent TAC operation; the nintedanib treatment group (n=15), which underwent TAC operation and were administrated daily by oral gavage for 6 weeks (60 mg/kg dissolved in saline) [10, 11] after the TAC operation.

After 6 weeks, the heart rate (HR) was recorded by using Acknowledge 4.1 (BIOPAC Systems, CA, USA). Serum samples were isolated from the cardiac blood as per the modified protocol. At the end of the experiments, the mice underwent necropsy and the heart tissues were collected, weighted, and stored for further H&E staining. The animal studies were approved by the Ethics Review Committee of the "People's Hospital of Chongyi County".

2.2 Measurement of plasma biochemical parameters

Collected plasma was pipetted into separate tubes, and creatine kinase-MB (CK-MB) and aspartate aminotransferase (AST) levels were analyzed using a CK-MB ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) and AST kit (Jiancheng Bioengineering Institute) following the instructions in kits.

2.3 Wheat germ agglutinin (WGA) staining

To examine the morphological alterations of heart tissues, WGA staining was performed. Briefly, the tissues were fixed in 4% formaldehyde, embedded in paraffin, and cut into sections. Then the cross-sectional area of cardiomyocytes was measured using the WGA staining assay using fluorescein-conjugated WGA (5 μg/mL) (Emission/Excitation wavelength: 594/638 nM). After staining for 10 min, images were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.4 Measurement of systemic oxidative stress markers

Four systemic circulating markers of oxidative stress including transcription factor Nrf2, lipid peroxidation products, thiobarbituric acid reactive substances (TBARS), glutathione (GSH), and homocysteine (Hcy) in blood samples were determined as previously described [7].

2.5 Cardiomyocytes culture and treatment

The rat cardiomyocyte cell line H9C2 cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in DMEM medium (Gibco/BRL Life Technologies, Eggenstein, Germany), which was supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/mL penicillin, as well as 100 U/mL streptomycin. For the Ang II treatment group, H9C2 cells were induced with Ang II (300 nM; Aladdin Chemicals, Shanghai, China) for 48 h.

2.6 Cell death assay

Pyroptotic cell death of H9C2 cells was evaluated with lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara, Kusatsu, Japan). Briefly, after incubation with 100 μL of the reaction mixture for 30 min at room temperature, the absorbance was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA).

2.7 Western blot analysis

H9C2 cells were lysed with ice-cold radioimmunoprecipitation assay (RIPA) and the homogenates were centrifuged at 12,000 *g* for 20 min at 4°C. Protein concentration was measured using bicinchoninic acid (BCA) (Sigma-Aldrich, USA) assay. Protein samples were then separated by 8-12 % gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The targeted proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes to perform western blot [11] with primary antibodies against SIRT1 (1:3000, Abcam, Cambridge, MA, USA), STAT3 (1:3000, Sigma-Aldrich, St. Louis, MO, USA), p-STAT3 (1:1000, Sigma-Aldrich), p65 (1:5000, Thermo Fisher Scientific), p-p65 (1:1000, Thermo Fisher Scientific), β-actin (1:5000, Abcam) and following horseradish peroxidase-conjugated secondary antibody (1:1000, Abcam). Protein bands were

detected with enhanced chemiluminescence detection (Thermo Fisher Scientific). The optical density of each band was analyzed by Image J software.

2.8 RT-PCR analysis

The mRNA levels of SIRT1, superoxide dismutase1 (SOD1), GPX1, HO-1 were quantified using quantitative PCR. Total RNA was extracted from H9C2 cells using a Total RNA isolation kit (Tiangen Biotech, Beijing, China), and then, cDNA synthesis was performed using a PrimeScript II first strand cDNA Synthesis kit (TaKaRa Bio, Shiga, Japan). The quantitative PCR reaction was carried out using SYBR-Green premix (TaKaRa). The levels of target gene transcripts were normalized to GAPDH.

2.9 SiRNA transfection

The si-SIRT1 and negative control (si-NC) were synthesized in GenePharma (Suzhou, China). The H9C2 cells (1.5×10^5 cells/well) were seeded into 6-well plates and incubated overnight. The siRNAs transfection was performed as per the instructions. After the 48 h incubation, the transfection efficiency was determined using western blot and RT-PCR analysis.

2.10 Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 with Student's *t*-test complemented with one-way analysis of variance (ANOVA) with Tukey's post hoc test. All results were presented as mean value ± standard deviation (SD).

3. Results

3.1 Nintedanib supplementation restored the cardiac function in TAC mice

The elevated heart rate and heart weight/body weight (HW/BW) in the TAC mice were decreased after nintedanib treatment (Figures 1A and 1B). In TAC mice with nintedanib supplementation, the increased levels of CK-MB and AST were mitigated (Figures 1C and 1D).

3.2 Nintedanib supplementation attenuated cardiomyocyte size in TAC mice

The heart tissues were collected and used for the assessment of cardiomyocyte size by WGA staining. As shown in figure 2, in the TAC operation group, the mean cross-sectional area of the cardiomyocytes was significantly larger than that in the

sham operation group. However, nintedanib supplementation attenuated the increased cardiomyocyte size in TAC mice.

3.3 Nintedanib administration reduced the systemic oxidative stress markers in TAC mice

Compared to mice in the sham operation group, the TAC operation group mice had significantly lower levels of systemic oxidative stress markers, Nrf2 (115.8 ± 21.6 vs. 76.5 ± 10.5 pg/ml), TBARS (3.81 ± 0.57 vs. 3.02 ± 0.42 μmol/L), GSH (335.6 ± 39.3 vs. 275.6 ± 31.0 μmol/L), and higher levels of Hcy (25.6 ± 4.1 vs. 43.5 ± 6.3 μmol/L). Nintedanib treatment significantly restored the levels of Nrf2 (76.5 ± 10.5 vs. 103.2 ± 13.9 pg/ml), TBARS (3.02 ± 0.42 vs. 3.87 ± 0.51 μmol/L), Hcy (43.5 ± 6.3 vs. 23.1 ± 2.8 μmol/L), and GSH (275.6 ± 31.0 vs. 325.2 ± 38.8 μmol/L) (figure 3A-D).

3.4 Nintedanib improved Ang II induction-caused cell injury in cardiomyocytes

As illustrated in figure 4, the LDH release assay results show that the LDH release in the H9C2 cells culture medium was increased significantly after induction with Ang II. In contrast, nintedanib treatment alleviated the Ang II induction-caused increase in the release of LDH.

3.5 Nintedanib attenuated oxidative stress and induced the expression of SIRT1 in Ang II-induced cardiomyocytes

In figure 5A, the changes in SOD1 mRNA levels among the different groups were not obvious. However, significantly lower mRNA levels of GPX1 and HO-1 were observed in Ang II-induced H9C2 cells, compared to control H9C2 cells. After nintedanib treatment, the decreased mRNA levels of glutathione peroxidase1 (GPX1) and HO-1 were markedly elevated (Figures 5B and 5C). Furthermore, Ang II induction caused an apparent decrease in SIRT1 mRNA, which could be mitigated with nintedanib treatment (Figure 5D).

3.6 Nintedanib reduced the activation of p65 and STAT3 in Ang II-induced cardiomyocytes

To explore the downstream signaling, the activation of p65 and STAT3 were respectively determined by detecting the p65, p-p65, STAT3, and p-STAT3 expression levels. Western blot results in figure 6 show that the ratios of p-p65/p65

and p-STAT3/STAT3 were significantly increased in Ang II-induced H9C2 cells, while they were decreased by nintedanib.

3.7 Transfection with si-SIRT1 abolished the protective effect of nintedanib by inhibiting the activation of p65 and STAT3

Results of Western blot and RT-PCR show that SIRT1 expression was dramatically downregulated after transfection with si-SIRT1 (Figures 7A and 7B). The nintedanib-caused inhibitory effect on LDH release was abolished by si-SIRT1 (Figure 7C). The elevated mRNA levels of GPX1 and HO-1 in nintedanib-treated H9C2 cells were mitigated by transfection with si-SIRT1 (Figures 7D and 7E). In addition, the inhibition of p-p65/p65 and p-STAT3/STAT3 by nintedanib was also prevented by si-SIRT1 (Figure 7F).

4. Discussion

This study demonstrates that nintedanib has protective effects against cardiac hypertrophy using an *in vivo* murine model with decreased heart rate, HW/BW, plasma levels of CK-MB and AST, and cardiomyocyte size. Oxidative stress is a critical pathophysiological feature in patients with cardiac hypertrophy, evidenced by significantly increased plasma levels of oxidative stress markers [12-15]. In consideration of the anti-oxidative property of nintedanib, we speculated that it might regulate the oxidative stress levels in the cardiac hypertrophy mice model.

Nrf2 is an antioxidant transcription factor that regulates the expression of diverse antioxidant and detoxification enzymes in response to insults derived from oxidative stress [16]. The reduction of Nrf2 is considered to be an indicator of increased susceptibility to oxidative stress in both humans and animals [17]. TBARS are oxidative alteration products of polyunsaturated fatty acids, and good oxidative indicators used for reflecting lipid peroxidation levels in biological fluids [18]. GSH is a tripeptide thiol that reduces ROS, including the hydroxyl radical and superoxide. A decrease in GSH concentration often occurs during oxidative reactions since it can be converted into glutathione disulphide (GSSG) [19]. Elevated Hcy has been shown to inhibit mitochondrial respiration and result in ROS accumulation. Furthermore,

hyperhomocysteinemia is recognized as an independent risk factor during the development of cardiovascular diseases [20]. Thus, we investigated the effects of nintedanib on these oxidative stress markers and found that the decreased levels of Nrf2, TBARS, and GSH, as well as the increased Hcy levels in TAC mice were reversed by nintedanib treatment.

Inflammatory processes, Ca^{2+}-handling abnormalities, oxidative stress, and apoptosis in cardiomyocytes have been suggested to contribute to pathological hypertrophy due to their depression of contractile function [3]. In this study, we used Ang II to induce cardiomyocytes damage *in vitro* to further explore the protective effect of nintedanib. We demonstrate that nintedanib improved Ang II induction-caused cell injury and oxidative stress in cardiomyocytes, as shown by the decreased release of LDH, and elevated mRNA levels of glutathione peroxidase 1 (GPX1) and HO-1. These results highlight the protective effects of nintedanib on cardiomyocytes *in vitro* and support the involvement of the anti-oxidative activity of nintedanib in attenuating Ang II-caused cardiomyocytes damage.

Sirtuin 1 (SIRT1) is recognized as a nicotine adenine dinucleotide (NAD)-dependent deacetylase. It has been documented to play crucial roles in multiple biological processes, such as oxidative stress responses, inflammation, and apoptosis [21]. Increasing evidence has shown that SIRT1 has great therapeutic potential in the treatment of cardiovascular diseases. The pharmacological activation of SIRT1 plays great protective roles in the prevention of cardiac hypertrophy and fibrosis [22, 23]. Acacetin ameliorates cardiac hypertrophy by reducing oxidative and inflammatory responses and preventing apoptosis in cardiomyocytes via regulating the activation of SIRT1/AMPK/PGC-1α signaling [24]. Activation of SIRT1 attenuates isoproterenol-induced cardiac fibrosis with the involvement of endothelial-to-mesenchymal transition (EndMT) modulation [25]. More importantly, SIRT1 has been reported to inhibit oxidative stress in vascular endothelial cells [26]. Our investigations showed that Ang II reduced the SIRT1 expression in cardiomyocytes, which could be attenuated by nintedanib. Transfection with si-SIRT1 abolished the protective effects of nintedanib on Ang II -caused cell injury and

oxidative stress in cardiomyocytes. In addition, SIRT1 also regulated the activation of downstream signals including p65 and STAT3, which may contribute to its roles in modulating cardiomyocytes function.

In conclusion, this study reports on the protective effects of nintedanib on cardiac hypertrophy both *in vivo* and *in vitro*. The cardioprotective property of nintedanib is attributed to its anti-oxidative activity by regulating SIRT1 expression. These findings highlight nintedanib as a potential therapeutic approach for the treatment of cardiac hypertrophy.

Acknowledgement and Funding declaration

There is no specific funding received for this study, and this study was supported by the “People's Hospital of Chongyi County”.

Ethical statements

The animal studies were approved by the Ethics Review Committee of the “People's Hospital of Chongyi County.

Consent to publication

All the authors agreed to publish this article.

Data Availability Statement /Availability of data materials

Data of this study are available upon reasonable request to the corresponding authors.

Conflict of interest/Competing interests

The authors declare that they have no competing interests.

Author Contribution

Wenjin Yuan and Dandan Zhang designed the study and wrote the draft, Wenjin Yuan, Dandan Zhang, Shiming Liu, Yuanhong Hua, and Lunjian Zhu performed the experiments and prepared the figures, Wenfeng Li revised the manuscript. All authors reviewed the manuscript and approved the submission.

References

[1] M. Samak, J. Fatullayev, A. Sabashnikov, M. Zeriouh, B. Schmack, M. Farag, A.F. Popov, P.M. Dohmen, Y.H. Choi, T. Wahlers, A. Weymann, Cardiac Hypertrophy:

An Introduction to Molecular and Cellular Basis, Med Sci Monit Basic Res, 22 (2016) 75-79.

[2] G.M. Ellison, C.D. Waring, C. Vicinanza, D. Torella, Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms, Heart, 98 (2012) 5-10.

[3] C.J. Oldfield, T.A. Duhamel, N.S. Dhalla, Mechanisms for the transition from physiological to pathological cardiac hypertrophy, Can J Physiol Pharmacol, 98 (2020) 74-84.

[4] C.J.A. Ramachandra, S. Cong, X. Chan, E.P. Yap, F. Yu, D.J. Hausenloy, Oxidative stress in cardiac hypertrophy: From molecular mechanisms to novel therapeutic targets, Free Radic Biol Med, 166 (2021) 297-312.

[5] I. Koniari, D. Velissaris, N.G. Kounis, *et al*. Anti-Diabetic Therapy, Heart Failure and Oxidative Stress: An Update. J Clin Med, 11(2022):4660.

[6] E. Boxhammer, K. Lehle, C. Schmid, M. von Suesskind-Schwendi, Anti-oxidative effect of the tyrosine kinase inhibitor nintedanib: a potential therapy for chronic lung allograft dysfunction?, Exp Lung Res, 46 (2020) 128-145.

[7] A.G. Fois, E. Sotgiu, V. Scano, S. Negri, S. Mellino, E. Zinellu, P. Pirina, G. Pintus, C. Carru, A.A. Mangoni, A. Zinellu, Effects of Pirfenidone and Nintedanib on Markers of Systemic Oxidative Stress and Inflammation in Patients with Idiopathic Pulmonary Fibrosis: A Preliminary Report, Antioxidants (Basel), 9 (2020).

[8] P. Umbarkar, A.P. Singh, S. Tousif, Q. Zhang, P. Sethu, H. Lal, Repurposing Nintedanib for pathological cardiac remodeling and dysfunction, Pharmacol Res, 169 (2021) 105605.

[9] Z. Liu, Z. Ma, H. Zhang, B.S. Summah, H. Liu, D. An, Q. Zhan, W. Lai, Q. Zeng, H. Ren, D. Xu, Ferulic acid increases intestinal Lactobacillus and improves cardiac function in TAC mice, Biomed Pharmacother, 120 (2019) 109482.

[10]. J. Mauer, A. Kuckhahn, M. Ramsperger-Gleixner, *et al*. Nintedanib reduces alloimmune-induced chronic airway changes in murine tracheal allografts. Transpl Immunol, 2022;73:101608.

[11]. J. Kataria, J. Kerr, S.R. Lourenssen, M.G. Blennerhassett. Nintedanib regulates intestinal smooth muscle hyperplasia and phenotype in vitro and in TNBS colitis *in vivo*. Sci Rep, 2022;12(1):10275.

[12] S.K. Maulik, S. Kumar, Oxidative stress and cardiac hypertrophy: a review, Toxicol Mech Methods, 22 (2012) 359-366.

[13] B. Quintana-Villamandos, L. Pazo-Sayos, I. Gonzalez Del Pozo, P. Rodriguez-Rodriguez, J.M. Bellon, A. Pedraz-Prieto, A.G. Pinto, M.C. Gonzalez, OXY-SCORE: a new perspective for left ventricular hypertrophy diagnosis, Ther Adv Chronic Dis, 11 (2020) 2040622320936417.

[14] Z.M. Dimitrijevic, S.S. Salinger Martinovic, V.N. Nikolic, T.P. Cvetkovic, Protein Carbonyl Content Is a Predictive Biomarker of Eccentric Left Ventricular Hypertrophy in Hemodialysis Patients, Diagnostics (Basel), 9 (2019).

[15] D. Drozdz, P. Kwinta, K. Sztefko, Z. Kordon, T. Drozdz, M. Latka, M. Miklaszewska, K. Zachwieja, A. Rudzinski, J.A. Pietrzyk, Oxidative Stress Biomarkers and Left Ventricular Hypertrophy in Children with Chronic Kidney Disease, Oxid Med Cell Longev, 2016 (2016) 7520231.

[16] I. Bellezza, I. Giambanco, A. Minelli, R. Donato, Nrf2-Keap1 signaling in oxidative and reductive stress, Biochim Biophys Acta Mol Cell Res, 1865 (2018) 721-733.

[17] H. Zhang, K.J.A. Davies, H.J. Forman, Oxidative stress response and Nrf2 signaling in aging, Free Radic Biol Med, 88 (2015) 314-336.

[18] J. Aguilar Diaz De Leon, C.R. Borges, Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay, J Vis Exp, (2020).

[19] V.I. Lushchak, Glutathione homeostasis and functions: potential targets for medical interventions, J Amino Acids, 2012 (2012) 736837.

[20] A.F. Perna, D. Ingrosso, N.G. De Santo, Homocysteine and oxidative stress, Amino Acids, 25 (2003) 409-417.

[21] S.H. Lee, J.H. Lee, H.Y. Lee, K.J. Min, Sirtuin signaling in cellular senescence and aging, BMB Rep, 52 (2019) 24-34.

[22] Z. Hajializadeh, M. Khaksari, The protective effects of 17-beta estradiol and SIRT1 against cardiac hypertrophy: a review, Heart Fail Rev, 27 (2022) 725-738.

[23] J. Zhang, Q.Z. Wang, S.H. Zhao, X. Ji, J. Qiu, J. Wang, Y. Zhou, Q. Cai, J. Zhang, H.Q. Gao, Astaxanthin attenuated pressure overload-induced cardiac dysfunction and myocardial fibrosis: Partially by activating SIRT1, Biochim Biophys Acta Gen Subj, 1861 (2017) 1715-1728.

[24] Y.K. Cui, Y.X. Hong, W.Y. Wu, W.M. Han, Y. Wu, C. Wu, G.R. Li, Y. Wang, Acacetin ameliorates cardiac hypertrophy by activating Sirt1/AMPK/PGC-1alpha pathway, Eur J Pharmacol, 920 (2022) 174858.

[25] Z.H. Liu, Y. Zhang, X. Wang, X.F. Fan, Y. Zhang, X. Li, Y.S. Gong, L.P. Han, SIRT1 activation attenuates cardiac fibrosis by endothelial-to-mesenchymal transition, Biomed Pharmacother, 118 (2019) 109227.

[26] W. Zhang, Q. Huang, Z. Zeng, J. Wu, Y. Zhang, Z. Chen, Sirt1 Inhibits Oxidative Stress in Vascular Endothelial Cells, Oxid Med Cell Longev, 2017 (2017) 7543973.

Figure legends:

Figure 1. Nintedanib supplementation restored cardiac function in TAC mice. The he art rate (A) and heart weight/body weight (HW/BW) (B) in mice were examined. The levels of CK-MB (C) and AST (D) in plasma samples were determined. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. TAC group.

Figure 2. Nintedanib supplementation attenuated cardiomyocytes size in TAC mice. The assessment of cardiomyocytes size was examined by WGA staining. Scale bar, 50 μm ("^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. TAC group).

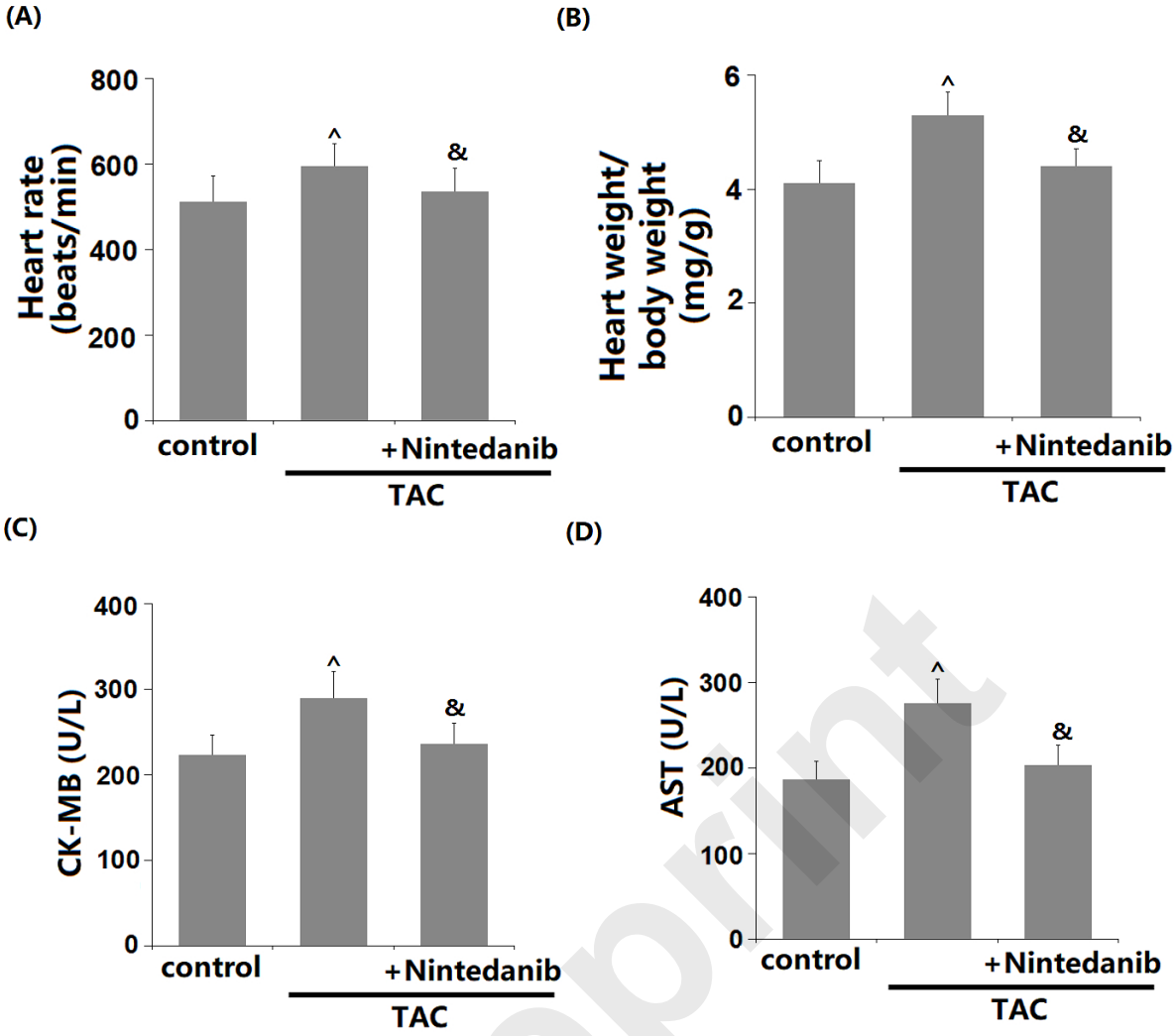
Figure 3. Nintedanib administration reduced the systemic oxidative stress markers in TAC mice. The levels of systemic oxidative stress markers, Nrf2 (A), TBARS (B), G SH (C), Hcy (D) in the plasma samples were determined. "^" indicates $p<0.05$ vs. con trol group; "&" indicates $p<0.05$ vs. TAC group.

Figure 4. Nintedanib improved Ang II induction-caused cell injury in cardiomyocytes. Pyroptotic cell death of H9C2 cells was evaluated by LDH release assay. The absorbance at 490 nm was recorded to indicate the LDH content. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

Figure 5. Nintedanib attenuated oxidative stress and induced the expression of SIRT1 in Ang II-induced cardiomyocytes. (A-C) The mRNA levels of SOD1, GPX1 and HO-1 were determined by qRT-PCR to reflect the oxidative stress status. (D) Changes of SIRT1 mRNA level in H9C2 cells among different groups. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

Figure 6. Nintedanib reduced the activation of p65 and STAT3 in Ang II-induced cardiomyocytes. The activation of p65 and STAT3 was respectively determined by detecting the p65, p-p65, STAT3, and p-STAT3 expression. The ratios of p-p65/p65 and p-STAT3/STAT3 were calculated. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

Figure 7. Transfection with si-SIRT1 abolished the protective effect of nintedanib by inhibiting the activation of p65 and STAT3. (A-B) Transfection efficiency of si-SIRT1 was confirmed by western blot and qRT-PCR. (C) LDH release assay was performed to indicate pyroptotic cell death of H9C2 cells. (D-E) The mRNA levels of GPX1 and HO-1 were detected by qRT-PCR. (F) Western blot was carried out to detect the p65, p-p65, STAT3, and p-STAT3 expressions. "^" indicates $p<0.05$ vs. control group; "^^" indicates $p<0.01$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group; "#" indicates $p<0.05$ vs. nintedanib treatment group; "##" indicates $p<0.01$ vs. nintedanib treatment group.



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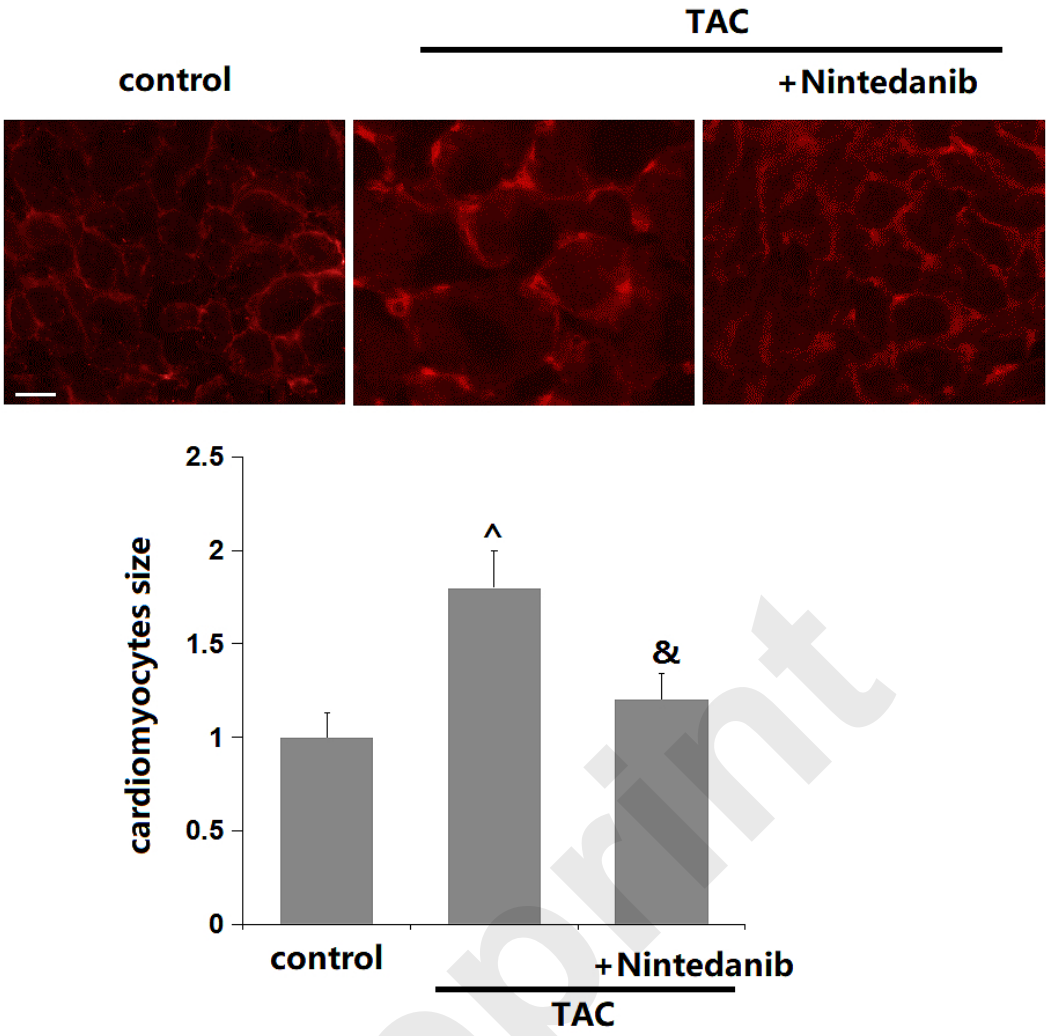

Figure 2. Nintedanib supplementation attenuated cardiomyocytes size in TAC mice. The assessment of cardiomyocytes size was examined by WGA staining. Scale bar, 50 μm ("^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. TAC group).

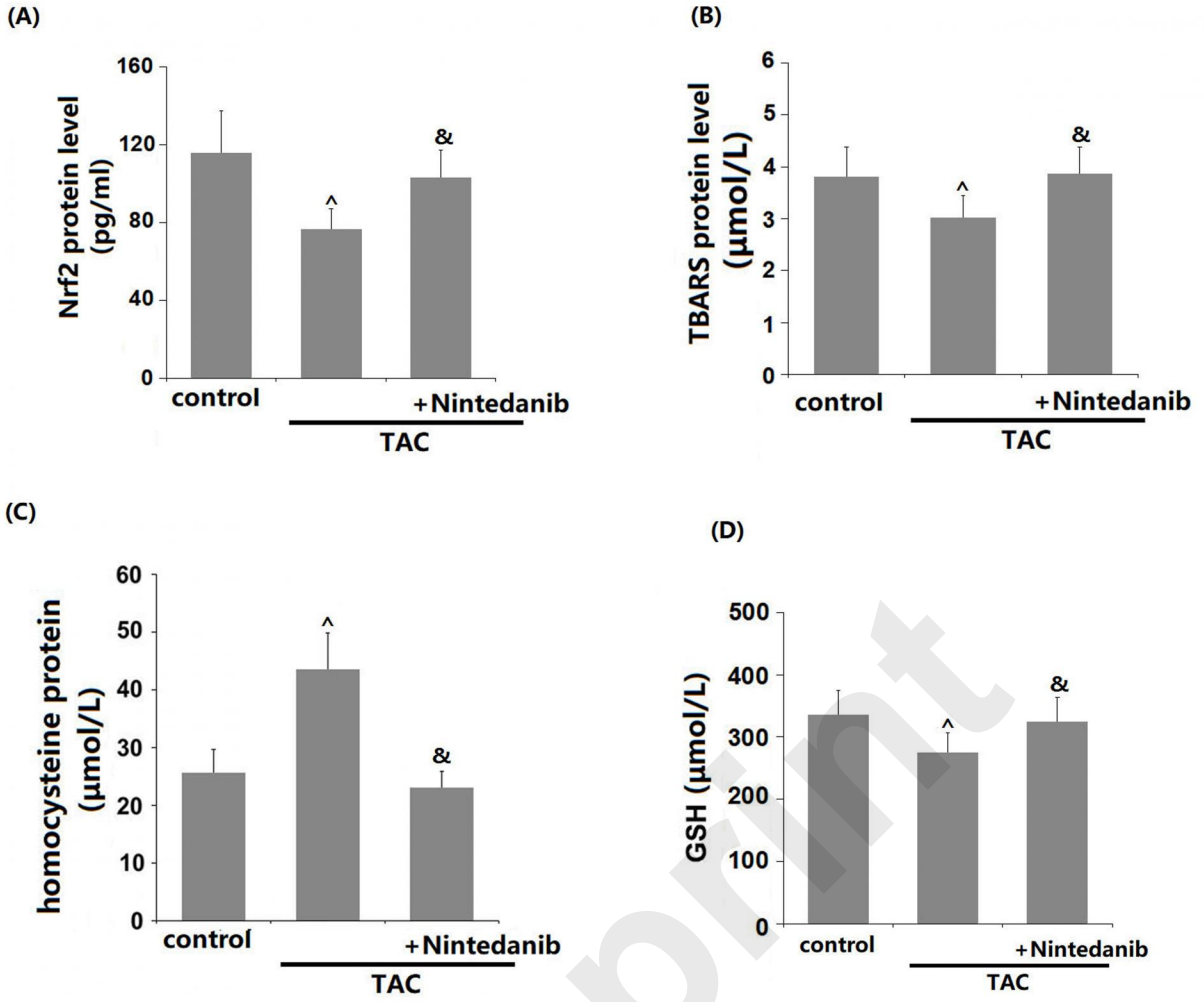

Figure3. Nintedanib administration reduced the systemic oxidative stress markers in TAC mice. The levels of systemic oxidative stress markers, Nrf2 (A), TBARS (B), GSH (C), Hcy (D) in the plasma samples were determined ("^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. TAC group).

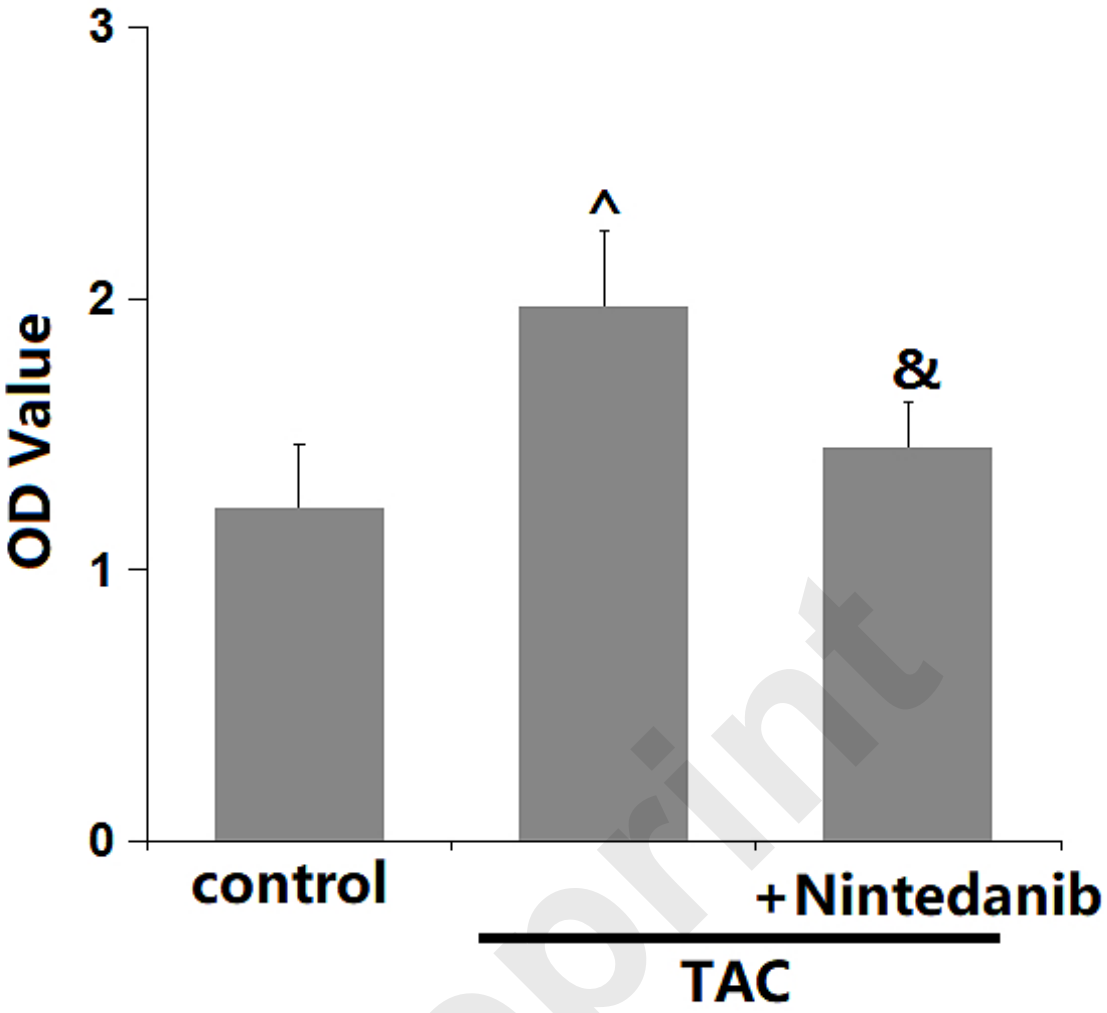

Figure 4. Nintedanib improved Ang II induction-caused cell injury in cardiomyocytes. Pyroptotic cell death of H9C2 cells was evaluated by LDH release assay. The absorbance at 490 nm was recorded to indicate the LDH content. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

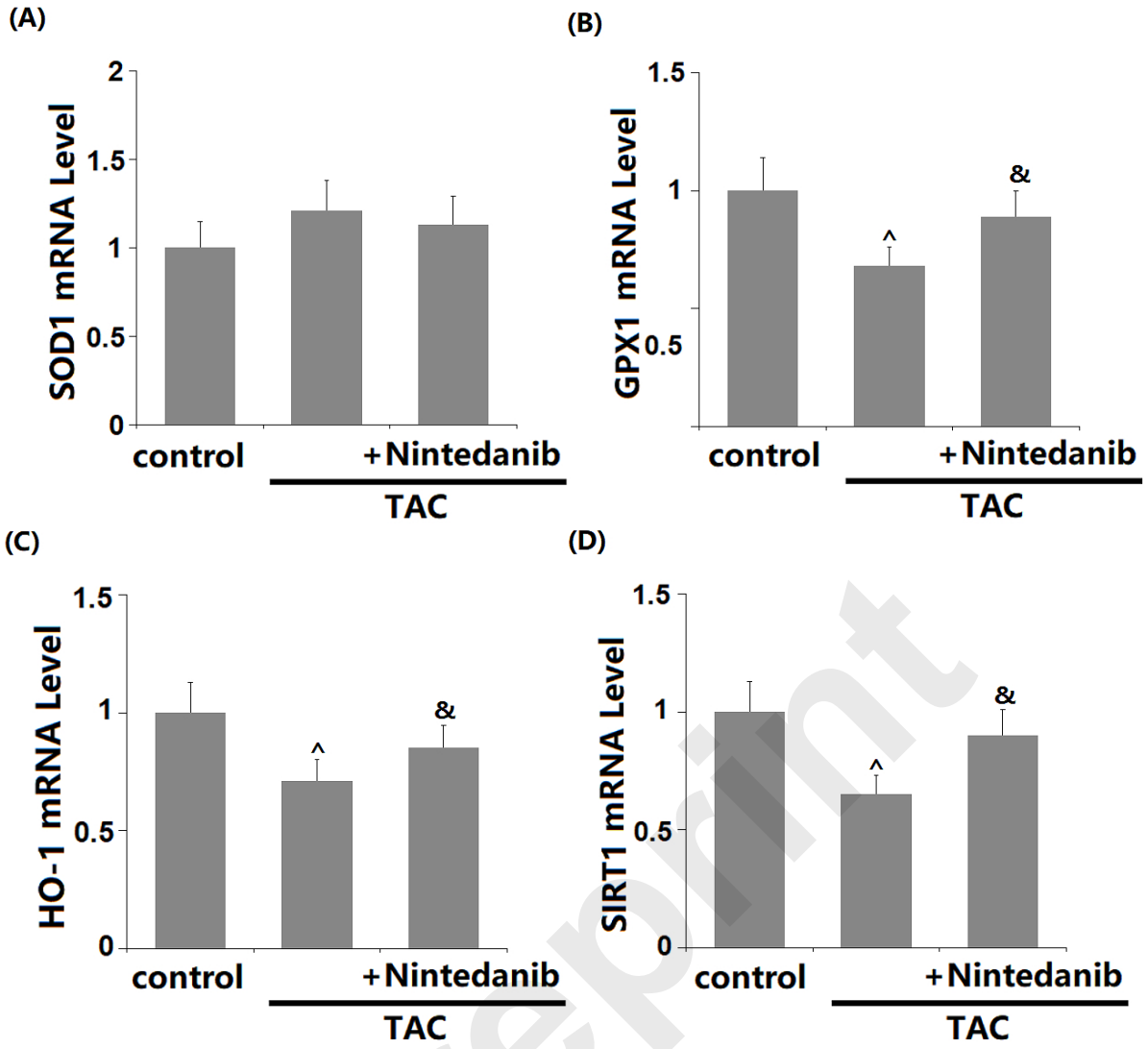

Figure 5. Nintedanib attenuated oxidative stress and induced the expression of SIRT1 in Ang II-induced cardiomyocytes. (A-C) The mRNA levels of SOD1, GPX1 and HO-1 were determined by qRT-PCR to reflect the oxidative stress status. (D) Changes of SIRT1 mRNA level in H9C2 cells among different groups. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

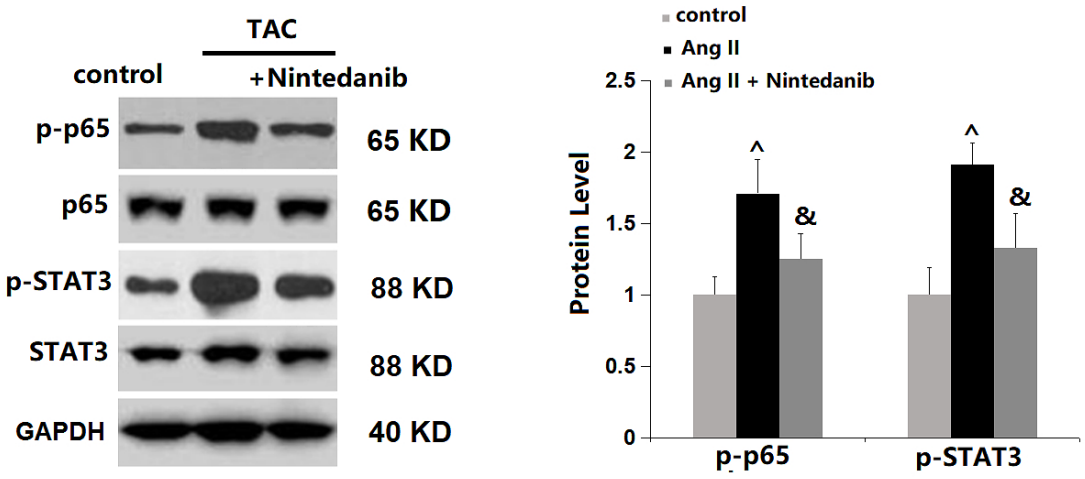

Figure 6. Nintedanib reduced the activation of p65 and STAT3 in Ang II-induced cardiomyocytes. The activation of p65 and STAT3 was respectively determined by detecting the p65, p-p65, STAT3, and p-STAT3 expression. The ratios of p-p65/p65 and p-STAT3/STAT3 were calculated. "^" indicates $p<0.05$ vs. control group; "&" indicates $p<0.05$ vs. Ang II induction group.

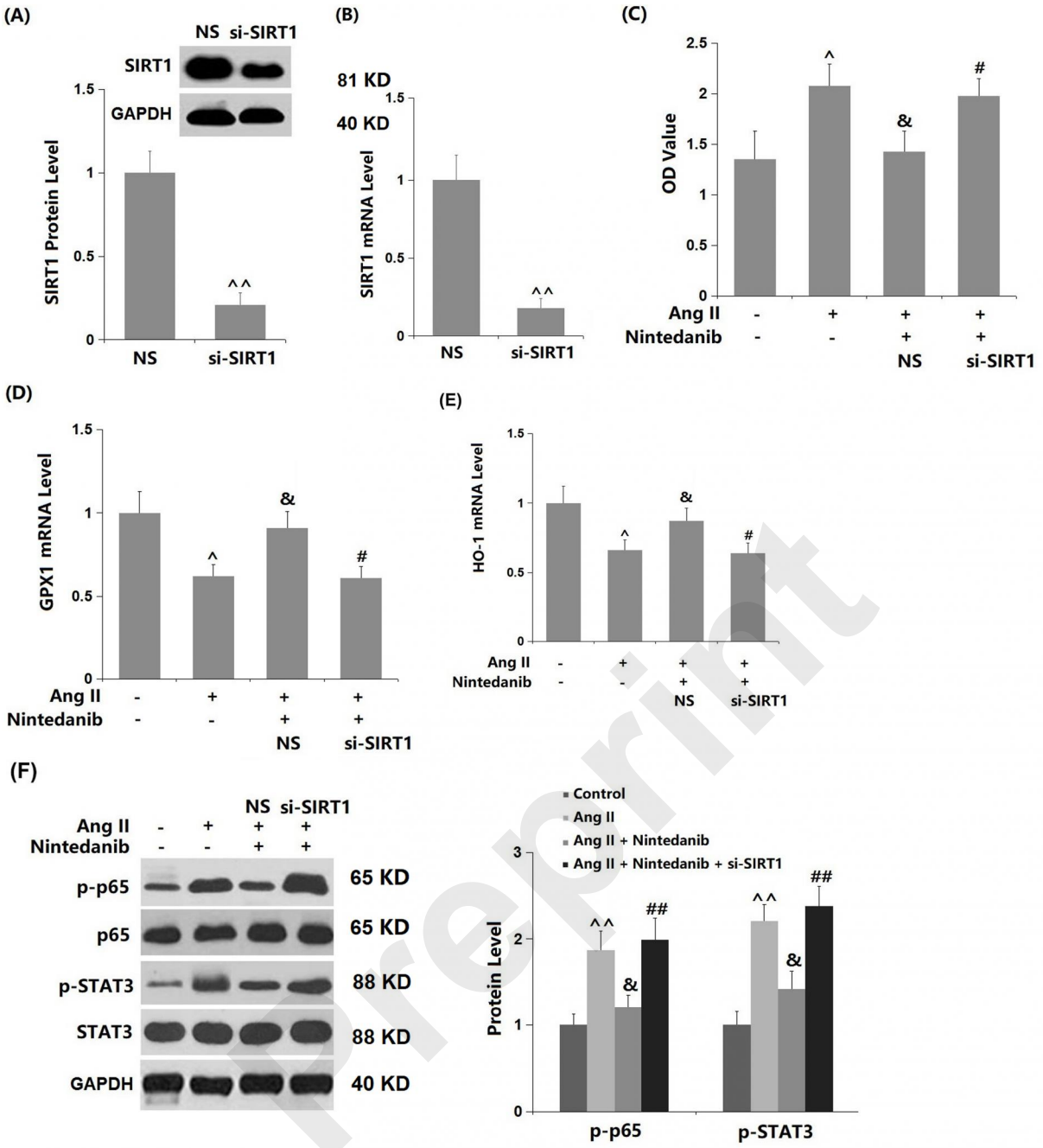

Figure 7. Transfection with si-SIRT1 abolished the protective effect of nintedanib by inhibiting the activation of p65 and STAT3. (A-B) Transfection efficiency of si-SIRT1 was confirmed by western blot and qRT-PCR. (C) LDH release assay was performed to indicate pyroptotic cell death of H9C2 cells. (D-E) The mRNA levels of GPX1 and HO-1 were detected by qRT-PCR. (F) Western blot was carried out to detect the p65, p-p65, STAT3, and p-STAT3 expressions. "^" indicates p<0.05 vs. control group; "^^" indicates p<0.01 vs. control group; "&" indicates p<0.05 vs. Ang II induction group; "#" indicates p<0.05 vs. nintedanib treatment group; "##" indicates p<0.01 vs. nintedanib treatment group.