

Notopterol Protects against Heart Failure Post-myocardial Infarction via GSK3 β -dependent Pathway

Notopterol Protects against Heart Failure

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Abstract

Introduction: Heart failure (HF), which frequently results from myocardial infarction (MI), leads to death and morbidity to a great extent globally, commonly developed from. Notopterol (NOT) is an active monomer extract obtained from *Notopterygium incisum* Ting ex H.T. Chang (*N. incisum*), a traditional Chinese medicine, that is extensively used in the clinical practice of cardiovascular disease and provides definite curative effects. NOT has been proven to be the most abundant constituent enriched in patients' serum and *N. incisum* ethanol extract. However, the role of NOT and its molecular target in HF remain unknown.

Methods: In vivo, left anterior descending (LAD) ligation was conducted for MI induction and then for four weeks to induce HF. Mice were treated with NOT intragastrically for four weeks. Early (0.5 h post-MI) and delayed (12 h post-MI) pre-administration post-MI, as well as a therapeutic treatment after HF, were carried out. Echocardiography was used to assess heart function. H&E staining, wheat germ agglutinin (WGA), as well as Masson trichromatic heart stainings, were applied to evaluate cardiac remodeling, myocardial hypertrophy, and fibrosis, as well as the expression of molecules. In vitro, mouse HL1 cells were treated with NOT after Ang II-stimulation. We measured cardiomyocyte hypertrophy through α -actinin immunofluorescence staining and hypertrophy gene expression. Meanwhile, phosphorylation of GSK3 β is measured after NOT treatment. Then, GSK3 β inhibitor 1-AKP was utilized for investigating NOT underlying pathway.

Results: In vivo, early (0.5 h p-MI) and delayed (12 h p-MI) pretreatment of NOT alleviated cardiac dysfunction after MI and postponed the onset of HF. Intragastrical administration of NOT after HF prevented adverse cardiac remodeling and attenuated hypertrophy and fibrosis against cardiac injury, as well as inflammation. In vitro, NOT presented an anti-hypertrophy property in cultured HL1 cells subjected to Ang -stimulation. NOT served as an inhibitor of GSK3 β ; it upregulated the inhibitory phosphorylation of GSK3 β , and its beneficial action against heart hypertrophy is partially abolished by inhibiting GSK3 β .

Conclusion: Our studies show that NOT treatment prevented adverse cardiac remodeling, and attenuated hypertrophy and fibrosis, thereby effectively protecting against the development and progression of HF. GSK3 β pathways are involved in the anti-hypertrophy action of NOT.

Introduction

Heart failure (HF), the terminal heart disorder stage, leads to death and morbidity to a great extent globally [1–3]. Multiple risk factors trigger HF, including chronic pressure overload and ischemia-perfusion (I/R) injury [1]. Among them, HF developed from myocardial infarction (MI) still accounts for the largest proportion [1]. Its prevention and treatment are considered important strategic goals. Despite advances in HF clinical therapy, there are few viable therapeutic approaches. Chinese medicine and its active ingredients are believed to have great potential with wide research prospects [4].

Cardiac remodeling involves several impaired molecular events, including cardiac hypertrophy and fibrosis, which ultimately progresses to HF [1, 5]. Glycogen synthase kinase 3 β (GSK-3 β) is an essential negative cardiomyocyte hypertrophy and modulator of HF[6–8]. GSK-3 β , a ubiquitously expressed serine/threonine kinase, participates in the normal cardiac homeostasis that governs cardiac proliferation, hypertrophy, and fibrosis, with multifactorial roles in cardiac biology[6, 7]. A GSK3 β signaling pathway is activated to an excessive degree in response to hypertrophic stress, and inhibiting this activation reduces hypertrophy[6–8]. Active or elevated GSK-3 β levels (i.e., GSK-3 β mRNA overexpression or GSK-3 β dephosphorylation) induce myocardial injury by regulating mPTP opening[9] as well as apoptotic cascade and mitochondrial oxidative stress induction[10–13], and GSK-3 β - β -catenin[14], Akt-GSK-3 β -mTOR pathway [7], GSK-3 β -Nrf2[15, 16] were demonstrated involved. There are also contradictory results, with no adverse effects observed in heart disease[7, 17]. This contradictory data urges more evidence and a deeper understanding of GSK3 β .

Notopterol (NOT), a type of coumarin, is an active monomer extract obtained from *Notopterygium incisum* Ting ex H.T. Chang (N. incisum, Qianghuo), a traditional Chinese medicine. Current pharmacological research focuses on NOT's effectiveness in arthritis, tumor, and Alzheimer's disease[18–22]. In mice with rheumatoid arthritis (RA), NOT alleviated arthritis by inhibiting the JAK-STAT pathway and reducing inflammatory action [22]. NOT inhibit proliferation, enhance apoptosis and cell differentiation, and exhibit anti-proliferative effects on hepatoma and leukemia[19]. In APP/PS1 AD mice, NOT was reported to effectively improve cognitive impairment by directly inhibiting the activity of BACE1 and GSK3 β , then reducing A β and phosphorylated tau[20, 21]. However, NOT's contribution to heart disease is less studied. In monocrotaline (MCT) -induced pulmonary arterial hypertension (PAH) rats, NOT improved mortality rate and right ventricular function, eased pulmonary vascular remodeling, and attenuated inflammation of the lung[23]. NOT functions, as well as molecular targets in HF therapy, are still unclear. N. incisum is widely applied in the clinical practice of cardiovascular disease and provides definite curative effects, acting as anti-thrombosis, -ischemic, and -arrhythmic agents[24, 25]. The composition analysis of the patient's serum after administering the herb in vivo and the ethanol extract of N. incisum in vitro[20, 22, 25] reveals that NOT is the most abundant enriched constituent. Therefore, we speculated that NOT as a major bioactive ingredient extracted from N. incisum, may have a preventive or therapeutic effect against HF post-MI. Our aim is to validate NOT prevention and treatment against HF post-MI in vivo and in vitro, in addition to discovering the underlying pathway mediation of this cardioprotective influence.

Materials and Methods

Animals

Male C57 mice aged 8–9 weeks (23–25 g) were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. The animals were subjected to a 23–25°C, 12:12 L:D cycle with lights on from 8:00 AM to 8:00 PM and access to standard food and water freely, in addition to receiving humane care according to institutional guidelines of the 960th Hospital for the health and care of experimental animals.

Establishment of left anterior descending (LAD) model

Mice were subjected to 4-week LAD ligation for HF induction as described previously [3]; meanwhile, sham surgery was performed on control mice. Inhaled isoflurane was utilized for surgical anesthesia. The heart was exposed upon opening the chest with a left thoracotomy, where it was then closed following passing and ligating a 7–0 silk suture under LAD.

Echocardiography

Echocardiography was performed as described previously [3]. 5% isoflurane was utilized for anesthetizing the mice. At 14 and 28 days after NOT treatment, transthoracic echocardiography was performed in the supine or left lateral position. A MyLab twice high-resolution in vivo micro-imaging system (Esaote, Italy) with a 22 MHz center frequency SL3116 transducer was used to record echocardiographic images from the two-dimensional long-axis view. At the papillary muscle level, M-mode was utilized for measuring ejection fraction (EF), shortening fraction (FS), interventricular septum diameter (IVS.d), left ventricular (LV) mass (LV Mass), left ventricular end-diastolic diameter (LVID.d), left ventricular diastolic posterior wall thickness (LVPWd), left ventricular mass index (LVMI).

Drugs administration

NOT was purchased from Chengdu Prifa Technology Development Co., Ltd; After being dissolved in DMSO, saline was used to dilute NOT up to 1 mg/mL (DMSO < 0.1%) final concentration. NOT was mixed in 0.5% sodium carboxyl methyl cellulose (CMC-Na) to be intragastrically administrated; Fig. 1A presents the experiment scheme for NOT administration. Intragastric administration of NOT (10, 30, and 100 mg/kg) was administered at different times (first dose at 30 min and 12 h) post-MI. For early and delayed NOT pretreatment, mice were administrated orally gavaged NOT (10, 30, and 100 mg/kg/day) at the same time as the first day for 28 days. The sham and model groups received equivalent saline volume. After NOT administration, the mice were examined with echocardiography. Consequently, the mice were anesthetized and executed to obtain heart tissue for immunohistochemistry. The heart, liver, kidney, and lung weights were measured, and the organ weight ratio was calculated.

The second part, the therapeutic effect of NOT, was performed in the HF mice. The optimal dose of NOT was determined from the first part of the experiments, which was used in further study. NOT was intragastric administrated in HF mice. HF model was induced by LAD ligation for four weeks, and animals with the adequacy of HF, as evidenced by EF 45%, were determined by echocardiography[3]. Three groups of mice were given Not (optimum dose) or Captopril (CAP, positive control, 8.6 mg/kg) or vehicle for four weeks after the successful HF model was established.

After NOT administration for 14 and 28 days, the echocardiographic assessment was used for recording the LV cardiac function. Subsequently, the mice were anesthetized and executed to obtain heart tissue for immunohistochemistry.

Hematoxylin-Eosin (H&E), Masson, and WGA Stainings

After measuring and sampling hemodynamics, heart samples were collected for morphometry and histological analysis and were succeeded by dissecting, fixing for 24 h with 4% paraformaldehyde, paraffin-embedding, sectionalizing, and staining with H&E, wheat germ agglutinin (WGA), and Masson trichromatic stains to evaluate cardiac remodeling, myocardial hypertrophy, and fibrosis. Light microscopy (Carl Zeiss, Jena, Germany) was utilized for overall histological assessment and the deposition of collagen. Cardiomyocyte area and LV wall thickness were measured by sections with Image J.

Western blot analysis

Based on Guo et al.[26], protein extraction and western blotting were conducted. A lysis buffer containing a protease inhibitor mixture (Kangchen, Shanghai, China) was used to homogenize and lyse the heart tissue proteins, measuring protein concentrations with a BCA protein assay kit (Beyotime, Shanghai, China). After separating 30 µg proteins on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), they were transferred to a nitrocellulose membrane. Immunoblotting for α -actinin, GSK3 β , and GSK3 β -phosphor-Ser9 (p-GSK3 β -Ser9). Re-probing the blots for GAPDH verified equal sample loading. The appropriate horseradish peroxidase-bound secondary antibody (Cell Signaling Technology, Danvers, MA, United States) was then performed to treat the membrane, detecting the immune response bands using chemiluminescence reagent (ECL) (Thermo Fisher Scientific).

Antibodies used were anti-GSK3 β (BM3904; Cell Signaling Technology, China), anti-p-GSK3 β (Ser9) (BM4837; Cell Signaling Technology, China), α -actinin (AG1022; Beyotime Biotechnology, China), anti-rabbit IgG H&L (HRP; BA1054; BOSTER, China), and anti-mouse IgG H&L (HRP; BA1126; BOSTER, China).

Real-time PCR

TRIzol (Invitrogen) and PrimeScript RT Master Mix Perfect RealTime Kit (Takara, Otsu, Japan) were used to extract total RNA from cardiac tissues and cells and to be reversely transcribed into cDNA, respectively. Then, using suitable specific primers and the SYBR Green RT-PCR Kit (Takara, Japan), the mRNA levels were evaluated, using cDNA as the template. The primer pairs used (Takara, Japan) were: ANP sense (5'-CATCATGGGCTCCTTCTCCAT-3') and antisense (5'-TGYACACAGGATTTGGTCCAATATG-3'); BNP sense (5'-AAGTCCTAGCCAGTCTCCAGA-3') and antisense (5'-GAGCTGTCTCTGGGCCATTTC-3'); β -MHC sense (5'-ATGTGCCGGACCTTGGA-3') and antisense 5'-CCTCGGGTTAGCTGAGAGATCA-3'); TGF- β sense 5'-CGGGGCGACCTGGGCACCATCCATGAC-3') and antisense (5'-CTGCTCCACCTTGGGCTTGCGACCCAC-3'); COL1A1 sense (5'-GAGCGGAGAGTACTGGATCG-3') and antisense (5'-GCTTCTTTTCCTTGGGGTTC-3'); COL1A2 sense (5'-CCGTGCTTCTCAGAACATCA-3') and antisense (5'-GAGCAGCCATCGACTAGGAC-3'); COL5A2 sense (5'-CTCAGGGAATTGATGGAGA-3') and antisense (5'-AGAGCCAGGCATGAGTCCTA-3'); TNF- α sense (5'-CAGCCTCTTCTCATCCTGCTTGTG-3') and antisense (5'-CTGGAAGACTCCTCCCAGGTATAT-3'); IL-1 β sense (5'-CAAGGCCACAGGTATTTTGT-3') and antisense (5'-GAAATGCCACCTTTTGACAG-3'); and β -actin sense (5'-GGCTGTATTCCCCATCG-3') and antisense (5'-CCAGTTGGTAACAATATGT-3'). Normalizing the target-gene mRNA transcript expression levels was relative to β -actin controls.

Establishment of AngII-induced HL1 cell injury model

Mouse HL-1 atrial myocytes (Procell CL-0605, Wuhan Pinsai Life Technology Co., LTD) were cultured at 37°C, 5% CO₂ in complete MEM medium (C11095500BT, Gibco) containing 10% (vol/vol) FBS and 100 U/mL penicillin/streptomycin (P/S). To investigate Ang II effects, HL-1 cell seeding took place into pre-coated 100-mm dishes at 1×10⁵ cells/mL. After 24 h (at > 80% confluence), the medium was substituted with 5% FBS MEM medium. Subsequently, HL-1 cells followed treatment with Ang II (5 μM) treatment for 24 h, then harvested for further analysis.

Immunofluorescence staining

After incubating the cells and Not (10, 30, and 100 μmol/L) in MEM supplemented at 37°C for 24 h, AngII co-culture was performed for 24 h and was succeeded by fixing the cells with 4% paraformaldehyde (Beijing Pulilai Gene Technology Co., Ltd., China), permeabilizing with 0.5% TritonX-100 (Beijing BioDee Biotechnology Co., Ltd., China) and blocking by 1% BSA (Beijing BioDee Biotechnology Co., Ltd., China) for 1 h at room temperature. The 1:100 diluted rabbit polyclonal primary antibody against α-actinin (AG1022; Beyotime Biotechnology Co., Ltd., China) was used to resolve the cells at 4°C for a whole night and was followed by using goat anti-rabbit IgG polyclonal (ab15007; Abcam, United States) as the secondary antibody at darkness and 37°C for 1 h. Using 10 μL/mL DAPI, the cell nuclei were stained for 5 min, collecting images and processing them with an FV3000 laser confocal microscope (Olympus). For detecting cellular viability, CCK-8 was performed.

Statistical analysis

SPSS 20.0 was performed to statistically analyze the data, reporting the data as mean ± SD. Both one-way analysis of variance (ANOVA) analysis and Dunnett's test were utilized for comparing two or more groups. *P* < 0.05 indicated a significant difference. The random permutation table was conducted to assign the animals in a random manner.

Results

NOT pretreatment attenuated MI-induced cardiac dysfunction and delayed development of HF

MI (LAD ligation) was performed to induce chronic HF in mice. NOT was early (0.5h-pMI) pretreated with the first dose at 30 min post-MI surgery for 28 days. Figure 1B shows the three group-representative echocardiography images. EF and FS of LV were decreased in model mice and reversed after NOT (100 mg/kg, not 10 and 30 mg/kg) early pretreatment (Figs. 1C–D). The LVID.s were elevated in MI mice, which indicated the occurrence of cardiac dysfunction and structural modifications. Such increases were attenuated after pretreatment with NOT (100 mg/kg, not 10 and 30 mg/kg) in vivo (Fig. 1E). Not 30 mg/kg exhibited a protective latency. The results suggested that NOT early pre-administration significantly alleviated MI-induced cardiac dysfunction. In addition, 14 days after Not treatment, the

protective effect on cardiac insufficiency was observed, and it is slightly weaker than that at 28 days. Compared to early and delayed treatment, the percentage increase in EF and FS induced by Not 100 mg/kg was slightly higher in early than in delayed (EF 89.52% vs. 52.06%; FS 127.14% vs. 45.93%).

To further tease out whether the effect of Not on the heart is limited by the dosage time window, we applied a delayed administration of Not for 28 days with the first dose at 12 h post-MI. Delayed NOT administration exhibited similar results and prevented MI-induced cardiac dysfunction and pathological alteration of cardiac structure, evidenced by increased EF and FS and decreased LVIDs in NOT 100 mg/kg treated mice more than the vehicle-treated mice (Figs. 1B–E). These results suggested that NOT only early pretreatment but also delayed pretreatment, NOT significantly alleviate cardiac damage in MI mice. We used NOT 100 mg/kg in the subsequent experiments.

As EF was less than 45% as a standard of the HF model, the incidence of HF was calculated. At 28 days after MI surgery, model mice developed 100% HF, while none progressed to HF (EF < 45%) in mice with NOT (100 mg/kg) early and delayed pretreatment. The findings reveal that NOT significantly delayed the development of HF.

Table S1 lists the morphometric parameters. NOT administration had no effect on the body weight (BW) of HF mice. To assess the effect of NOT on body organs, organ weight and hepatic-renal function were measured. There were no obvious differences in the weight of heart, liver, lung, and kidney (**Table S1**), as well as hepatic-renal function indicators between groups (**Table S2**), including alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), and urea nitrogen (BUN). Heart weight (HW) and HW/BW ratio tended to increase in MI mice and tended to decrease after NOT treatment, unfortunately without significance.

NOT pretreatment alleviated MI-induced myocardial remodeling, cardiac hypertrophy, and fibrosis

MI-induced HF produced myocardial remodeling, cardiac hypertrophy, and fibrosis, as measured by immunoblotting staining and biomarker gene transcription. On the basis of H&E staining, the left ventricle cardiomyocytes exhibited an orderly arrangement in the sham group and a loose arrangement in the model group. NOT early pretreatment reversed the pathological changes significantly (Fig. 2A). It is suggested NOT to attenuate MI-induced myocardial remodeling. Masson staining was used to assess fibrosis, revealing blue heart collagen deposition, indicating fibrosis formation, and this deposition occurred more significantly in the MI group than in the sham group, as expected; in contrast, it was diminished in the NOT early pretreated group (Fig. 2A). Cardiac fibrosis biomarkers were detected. The mRNA of collagen IA1 (COL1A1), collagen IA2 (COL1A2), collagen 5A1 (COL5A1), collagen 5A2 (COL5A2), collagen 3A1 (COL3A1), and TGF- β were elevated in the MI mice heart and were inhibited through NOT therapy in vivo (Figs. 2C–G). Then, WGA staining was applied to detect cardiomyocyte hypertrophy. As expected, NOT 100 mg/kg/day pretreatment led to a significant reduction in MI-induced cardiomyocyte hypertrophy more than the vehicle group (Fig. 2A), which was parallel to significant suppression of hypertrophic expression of ANF and β -MHC hypertrophic gene transcription expressions (Figs. 2H–I) in

the NOT groups more than in the vehicle group, suggesting inhibited hypertrophic responses in vivo. The delayed pretreated NOT has a similar effect to the early treatment (Figs. 2B–H). In summary, NOT treatment significantly prevents HF in MI mice by inhibiting adverse myocardia remodeling, cardiac hypertrophy, and fibrosis. NOT exerted potential to suppress multiple pathological processes of HF.

NOT treatment attenuated cardiac injury in HF mice

Four weeks after MI surgery, animals with the adequacy of HF, as evidenced by $EF \leq 45\%$, were determined by echocardiography. HF mice were gavaged NOT (100 mg/kg) or CAP (positive control, 6.8 mg/kg) or vehicle for 28 continuous days. CAP of 6.8 mg/kg, as a positive control group, significantly attenuated cardiac dysfunction of HF mice, evidenced by increased EF and FS, as well as reduced LVID d (Figs. 3A–H). Intragastric administration of NOT exhibited a similar protective effect with CAP. After NOT therapy, EF and FS levels were significantly elevated, and IVS.d, LVID.d, LVPW.d, LV mass and LVMI were significantly reduced ($P < 0.05$) (Figs. 3A–H). It is worth noting that CAP exhibited an increase in EF at 14 days by 10.08%, which is more obvious at 28 days by 16.93%. While NOT's protective effect was observed only at 28 days by 12.61%, which is more stable than that at 14 days, taking an indicator of EF. These results indicate that NOT alleviates heart dysfunction of HF.

NOT attenuated myocardial remodeling, cardiac hypertrophy, and fibrosis in HF mice

NOT treatment effect on the heart after failure was measured by HW/BW, myocardia remodeling, cardiac hypertrophy, fibrosis, and the expression of corresponding gene transcription. As expected, the HF model elevated the HW and the HW/BW ratio of mice more significantly than the sham-surgery mice. NOT treatment decreased HW and HW/BW more than vehicle treatment in HF mice (Figs. 4A–C). Examining cardiomyocyte hypertrophy using H&E and WGA staining revealed that, as expected, the cardiomyocyte diameter of the HF group was greater than that of the sham group. The enlargement was attenuated in the NOT-treated groups, which is slightly weaker than that in the CAP-treated group (Figs. 4D–E). Consistent with the results, the increase in hypertrophy transcription factors (ANP, BNP, and β -MHC) were inhibited by NOT treatment (Figs. 4F–H). To access the excessive collagen composition and fibrosis induced by HF, HF induced the increase in fibrotic gene marker expressions COL1A1, COL1A2, and COL5A2, while upregulated expression was reversed in the group not treated more than in the group treated with vehicle (Figs. 4I–J). Additionally, NOT ameliorated inflammatory reaction of the failed heart, evidenced by the decrease of pro-inflammatory transcription factors, IL-1 β and TNF- α (Figs. 4K–L). To summarize, NOT treatment suppressed the pathological changes of failed hearts, including myocardial remodeling, cardiac hypertrophy, fibrosis, and inflammation, indicating multi-factor features of NOT.

Tables S3–4 list the morphometric parameters. NOT administration had no effect on the BW of HF mice (**Table S3**). To assess the effect of NOT on body organs, organ weight and hepatic-renal function were measured. HW decreases after NOT treatment, as expected. Moreover, there was no obvious difference in the weight of liver, lung, and kidney between the groups (**Table S4**).

NOT alleviated AngII-induced myocardial hypertrophy of HL1

We examined NOT effects further on cardiomyocyte hypertrophy with mouse HL1 cells. The Ang II-stimulated cell model was established as previously described[16]. Cells were treated with different doses of NOT (10, 30, and 100 μ M) or vehicle, followed by adding Ang II (5 μ M) to induce hypertrophic responses. For determining the degree to which NOT inhibited these responses, cardiomyocyte marker α -actinin was used to stain the cells to measure changes in cardiomyocyte size (in Fig. 5A in green, with intact nuclei in blue) and then using laser scanning confocal microscope to observe them, revealing that Ang II treatment led to an increase in cardiomyocyte size more than the control group, while such elevation was significantly suppressed by the NOT treatment (30 and 100 μ M, not 10 μ M) (Figs. 5A–B). The anti-hypertrophy effect of NOT is exhibited in a dose-dependent manner. Consequently, hypertrophic gene expression was examined in the cardiomyocytes. NOT significantly suppressed Ang II-induced upregulation of ANF, BNP, and β -MHC mRNA levels (Fig. 5C). The results revealed that NOT alleviated Ang II-induced myocardial hypertrophy response.

Based on the CCK-8 experiment, 100 μ M NOT decreased Ang II-stimulated HL1 cell cellular viability ($P < 0.001$), and other doses did not affect cellular viability (Fig. 5D). Therefore, we used NOT 30 μ M in the subsequent experiments.

NOT-evoked cardioprotection is mediated by GSK3 β

GSK3 β is recognized as a negative regulator of heart disease [6–8]. Furthermore, NOT is demonstrated as a natural inhibitor of GSK3 β [20, 21]. We first confirmed NOT regulation on GSK3 β activity. In HL1 cells, Ang II down-regulated the ser9-phosphorylation levels of GSK3 β , compared to the control group, suggested GSK3 β overactive in myocardial injury, consistent with a previous study[6–8]. NOT treatment significantly inactivated GSK-3 β , evidenced by increased ser9-phosphorylation levels (Fig. 6G). The expression of GSK3 β was also investigated in HF mice. To support the results in vitro, phosphorylation of GSK3 β as an inactivated state was down-regulated in MI heart, while NOT treatment partly reversed the downregulation (Fig. S1). In summary, the activation of GSK3 β is regulated by NOT, and NOT promotes inhibitory phosphorylation of GSK3 β .

To further define the GSK3 β function in NOT-evoked cardiomyocyte protection, intervention studies were conducted in vitro by GSK3 β inhibitor 1-AKP. In HL1 cells, NOT (30 μ M) inhibited cardiomyopathy hypertrophy, which was supported by a decrease in cell area detected from α -actinin immunofluorescence (Figs. 6A and E) and a reduced hypertrophy marker mRNA expressions (ANP, BNP, and β -MHC) (Figs. 6B–D), as well as the downregulation of β -actinin expression (Fig. 6F). The action of NOT on cardiomyocyte was partially reversed by an antagonist of GSK3 β in 1-AKP treated mice, suggesting that NOT alleviated the hypertrophy reaction of cardiomyocyte by regulation GSK3 β .

Discussion

Herein, for the first time, we show that NOT directly inhibits cardiomyocyte hypertrophy by reducing the activity of the GSK3 signal pathway. The MI sudden onset and its exceedingly limited therapeutic window make accurate drug administration challenging. Time-insensitive strategy is urgently needed to develop against ischemic heart injury[1]. We tried multiple dosing times to explore the action of NOT. Interestingly, NOT has a cardioprotective effect on HF development and progression as well as targets numerous HF pathogeneses, such as cardiac remodeling, hypertrophy, fibrosis, as well as inflammatory mechanisms, when administered either early (0.5 h p-MI) or delayed (12 h p-MI) or therapeutically (post-HF). Our data supported NOT with a broad therapeutic window after acute MI, and the longest effective time for the first dose was 12 h after MI in mice. The duration of administration was suggested at 28 days, although it works at 14 days with a slightly weaker effect. In vitro, NOT presented an anti-hypertrophy property in cultured HL1 cells subjected to Ang -stimulation. It can regulate the activity of GSK3 β (inhibitory phosphorylation), which is a known combining target of NOT and a negative regulator of HF. Pharmacological inhibition of GSK3 β abolished the anti-hypertrophy action NOT. The findings confirm that the beneficial action of NOT against myocardial hypertrophy is mediated by GSK3 β . Herein, regarding toxicity, no significant changes were found in the NOT-treated mice body or organ weight, except for the injured heart, indicating that there was no significant toxicity resulting from NOT treatment.

NOT, a furanocoumarin-type is an active compound extracted from *N. incisum*; the latter had an extensive spectrum impact on biomedical pharmacology[21]. NOT exhibited anti-arthritis, anti-cancer, and anti-dementia activities, respectively, by inhibiting the JAK-STAT pathway and reducing inflammatory reaction; by suppressing proliferation and promoting apoptosis in acute human leukemia HL-60 and HEPG-2 cell lines; and by suppressing BACE1 and GSK3 β activity, then decreasing A β and phosphorylated tau in APP/PS1 AD mice[18–23]. Focus on heart disease; there is only one paper about PAH[23]. In MCT-induced PAH rats, NOT improved mortality rate and right ventricular function, paralleled with decreased right ventricular hypertrophy and fibrosis, eased pulmonary vascular remodeling and attenuated inflammation of the lung. In vitro, the human pulmonary arterial smooth muscle cells (HPASMCs) proliferation and migration were inhibited by NOT. To our knowledge, pulmonary hypertension leads to right ventricle overload and eventually progresses to right ventricular failure. In that study, MCT induced PAH model did not affect left ventricular ejection fraction (LVEF). Comparably, LVEF showed no significant effect after NOT administration. HF can be triggered by several risk factors, and HF developed from MI still accounts for the largest proportion[27]. Therefore, our study first focuses on AMI-induced HF. Using LAD-ligation for four weeks, chronic HF was induced, and it has similar features to human disorders in hemodynamic changes, vascular remodeling, and HF [3]. Similarly to an earlier study, we found the beneficial NOT effect on HF development and progression by alleviating heart dysfunction and inhibiting cardiac remodeling, hypertrophy, fibrosis, and inflammation. Our study enriched efficacy data of pharmacology. NOT (100 mg/kg, not 10 and 30 mg/kg) exhibited cardioprotection, not only by early (0.5 h-pMI) and delayed pretreatment (12 h-pMI) but also by treatment after HF. The dose-effect and time-effect relationship were exhibited. In addition, GSK3 β is recognized as a key target of NOT and is involved in the anti-hypertrophy action, supported by intervention studies. It should be noted that, in the same as in intragastric administration, our effective dose of NOT is higher than that used in previous studies. In

murine RA mice, the therapeutic schedule of NOT is 20 mg/kg for 22 days. In APP/PS1 mice, NOT works at 40 mg/kg, not 20 mg/kg, for two months. In PAH rats, a dosage of 20 mg/kg for 21 days was administered. Our cardioprotective dose of NOT is 100 mg/kg daily for 28 days in HF mice, with 30 mg/kg tending to affect heart function. Moreover, the beneficial effect is already observed after NOT-treated 14 days, in a time-dependent manner, which is slightly weaker than that after 28 days. Our broad dose setting to cover the range of effective doses may partially explain the difference, which weakens the ability to explore the minimum effective dose.

To explicit the targets of NOT is very important to evaluate the feasibility of patent medicine and also pave the way for developing more specific agents. Previous studies have shown that NOT, as a small natural molecule, can inhibit BACE1, GSK3 β , and JAK2[19–22]. After recognizing NOT as BACE1 and GSK3 β dual inhibitor, researchers designate a series of triple inhibitors to lead compounds on the basis of NOT furocoumarin scaffold, aimed to suppress the three AD classical targets (AChE/BACE1/GSK3 β), which is expected to obtain ideal anti-cognitive impairment activity[27]. In the RA model and in zebrafish, direct NOT binding to JAK2 and JAK3 is necessary for its anti-inflammatory effects, with 931/arginine, 980/asparagine, and 981/leucine of JAK2 are essential binding sites[22]. In our study, NOT regulates the activation of GSK3 β , in agreement with previous studies. Intervention with GSK3 β inhibitors abolished NOT hypertrophy in the myocardium, suggesting that it is a critical target of NOT in cardioprotection. According to the literature, NOT showed a strong inhibitory effect against GSK3 β (IC₅₀: 1 μ M)[21]. Their binding mode was predicted by virtual screening in silico, and NOT bound in kinase GSK3 β hinge region and establishes two firmly hydrogen-bonding interactions with Asp133 and Tyr134 backbones. BACE1 and JAK2 target functions were not studied in our study, which cannot be ruled out and require further studies.

GSK-3 β is consistently active in resting cells, and it is crucial to maintain normal cardiac homeostasis that governs cardiac development, proliferation, hypertrophy, and fibrosis [28, 29]; it is found to be suppressed in hypertrophy and HF and serves as a negative regulator. Activated GSK-3 β caused heart injury. GSK-3 β pharmacological inhibition or knockdown is beneficial in several cardiovascular pathologies[7–16]. GSK-3 β intervention results in increased cardiomyocyte proliferation in cultured cardiomyocytes through overexpression growth regulators (GATA4, cyclin D1, and c-Myc) and canonical Wnt signaling[30, 31]. It also promotes angiogenesis, prevents adverse cardiac remodeling by inhibition of Akt-GSK-3 β -mTOR pathways, and triggers GSK-3 β -catenin[14, 32, 33], downregulates mPTP opening, and induces antiapoptotic (decreased Bax/Bcl-2 ratio and the apoptotic cascade)[9–13]. GSK-3 β phosphorylates a wide range of substrates, voltage-dependent anionic channel 1 (VDAC1) and E2-related factor (Nrf2) are also reported as downstream substrates [15, 16]. Nevertheless, other studies show contradicting results. GSK-3 β inhibition conferred physiological hypertrophy induction[7]. In GSK-3 β KO mice subjected to transverse aortic constriction surgery, no significant differences in hypertrophy were observed compared to WT mice[17]. We cannot explain this inconsistency in data, yet, we supposed that it might be because of protein phosphorylation at a different site that defines the protein activity. GSK-3 β residues have the ability to modulate their substrate binding potential[28, 29]. GSK-3 β phosphorylation at tyrosine 216 elevates GSK-3 β enzymatic activity. In contrast, GSK-3 β phosphorylation at serine 9

inactivates GSK-3 β by blocking its active site [29]. These phosphorylation and dephosphorylation switches of GSK-3 β can affect several molecular pathways. The GSK-3 β phosphorylation inhibition at serine 9 was well demonstrated.

Divergent GSK-3 β inhibitor cardioprotective effect, including SB216763, copper nanoparticles (NPs), and resveratrol, was reported[7, 8]. However, in human heart clinical trials, there were no GSK3 β inhibitors; only limited inhibitors were utilized for conducting distinct phases of clinical trials for the diseased conditions, including neurodegenerative disorders, myotonic dystrophy, and several cancers. In the interest of fighting myocardial injury with cutting-edge therapeutic approaches, researchers should consider developing GSK3 β inhibitors. Now we recognize NOT as a potential agent. A diverse-acting substrate of GSK3 β may confer NOT to extensively participate in different pathogenesis of HF. Notably, NOT is a natural compound, which means it is safer than synthetic chemicals and more affordable to manufacture. Moreover, It can be easily extracted from *N. incisum*.

Our research attained crucial novel insights, but it also had some drawbacks. First, JAK2 and JAK3 are possible target pathways of NOT, but we did not identify the specific target molecule. Additional research is required to explore their role. Second, our effective dosage in vivo is greater than that in previous research. The dose setting of NOT in mice is broad, which weakens the capability to explore minimum effective dose. Although the action of NOT has been confirmed, the minimum effective dose is still obscure. A more refined dose setting is needed to clear NOT pharmacological activity.

Conclusions

In conclusion, NOT treatment protects against HF development and progression. NOT early and delayed pre-administration alleviated cardiac dysfunction following MI and postponed the onset of HF. Following HF, intragastrically admistred NOT prevented adverse cardiac remodeling and attenuated hypertrophy and fibrosis against cardiac injury, as well as inflammation. In vitro, NOT presented an anti-hypertrophy property in cultured HL1 cells subjected to Ang -stimulation. NOT served as an inhibitor of GSK3 β , and its beneficial action against heart hypertrophy is partially mediated by GSK3 β in vitro. These results could give support for using NOT in preventing and treating HF.

Declarations

Ethics approval and consent to participate

All the authors confirmed that all experimental protocols were approved by the Ethics Committee of the 960th Hospital of the Joint Logistics Support Force of the People's Liberation Army. We confirmed that all methods were carried out in accordance with the guidelines of animal care carried out by the Institute for Laboratory Animal Research of the 960th Hospital. (No. 47). We confirmed that all methods were reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent for publication

Data Availability

All data supporting the conclusions are presented in the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' Contributions

CY, QS and LW: participated in the design of the study, performed the experiments, analyzed the data, and drafted the manuscript. JG and YT: designed and guided the work; contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

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Figures

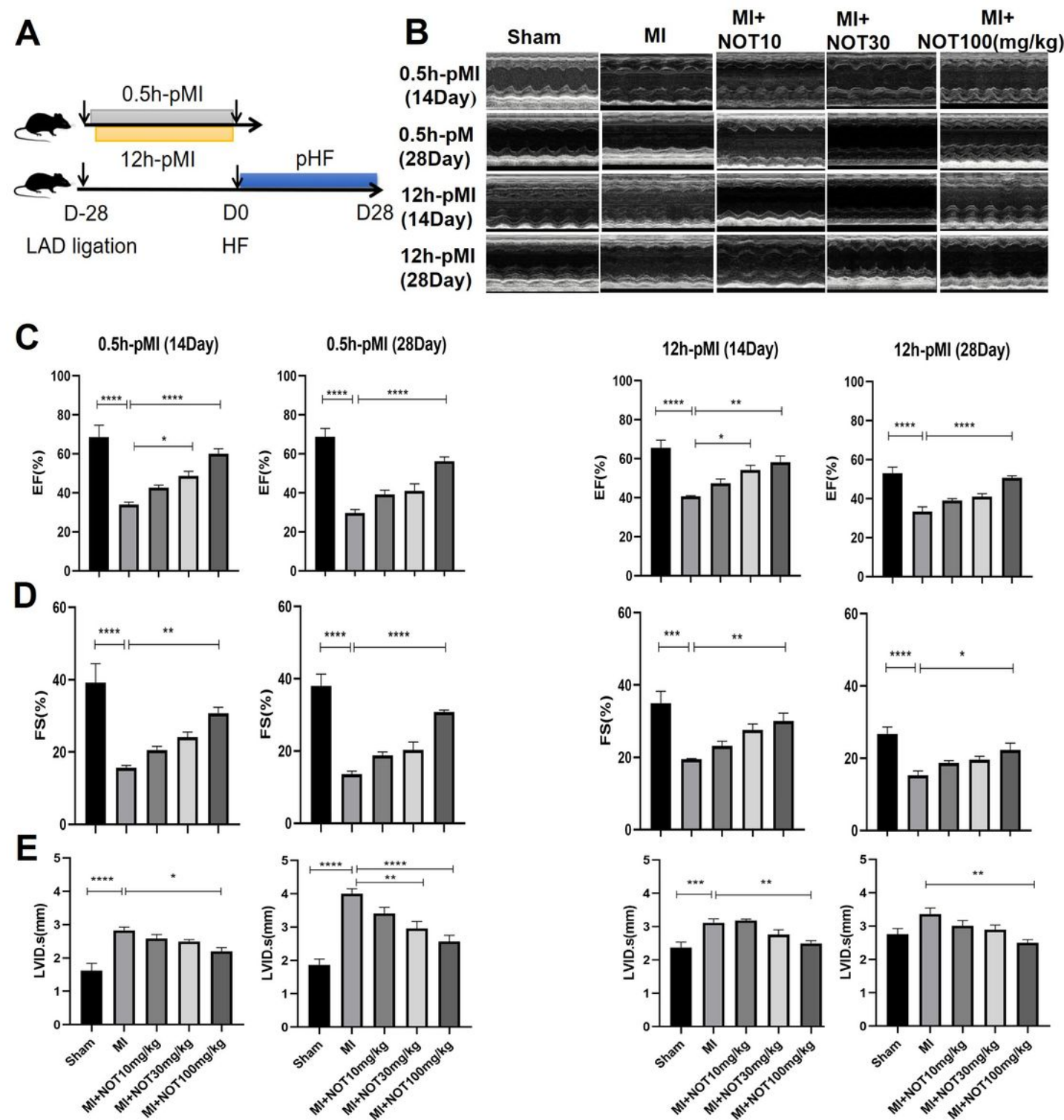


Figure 1

Notopterol (NOT) pretreatment attenuated myocardial infarction (MI)-induced cardiac dysfunction. (A) Scheme showing the experiment design for notopterol (NOT) administration, intragastric once a day for 28 days. According to the administration time from heart failure (HF), three ways were included. **0.5h-pMI**, early pretreatment of NOT with the first dose at 0.5h post MI. **12h-pMI**, delayed pretreatment with the first dose at 12h post MI. **pHF**, treatment after HF, with the first dose at 28 days after MI. **(B)** The cardiac function was monitored by echocardiograms after NOT administration 14 and 28 days. Representative image of an M-model photograph from vehicle and NOT treated groups. **(C-E)** Quantified values of ejection fraction (EF), fractional shortening (FS), left ventricular internal diameter at end systole (LVID.s) are presented as mean±SEM. n=6. * $P<0.05$, ** $P<0.01$.

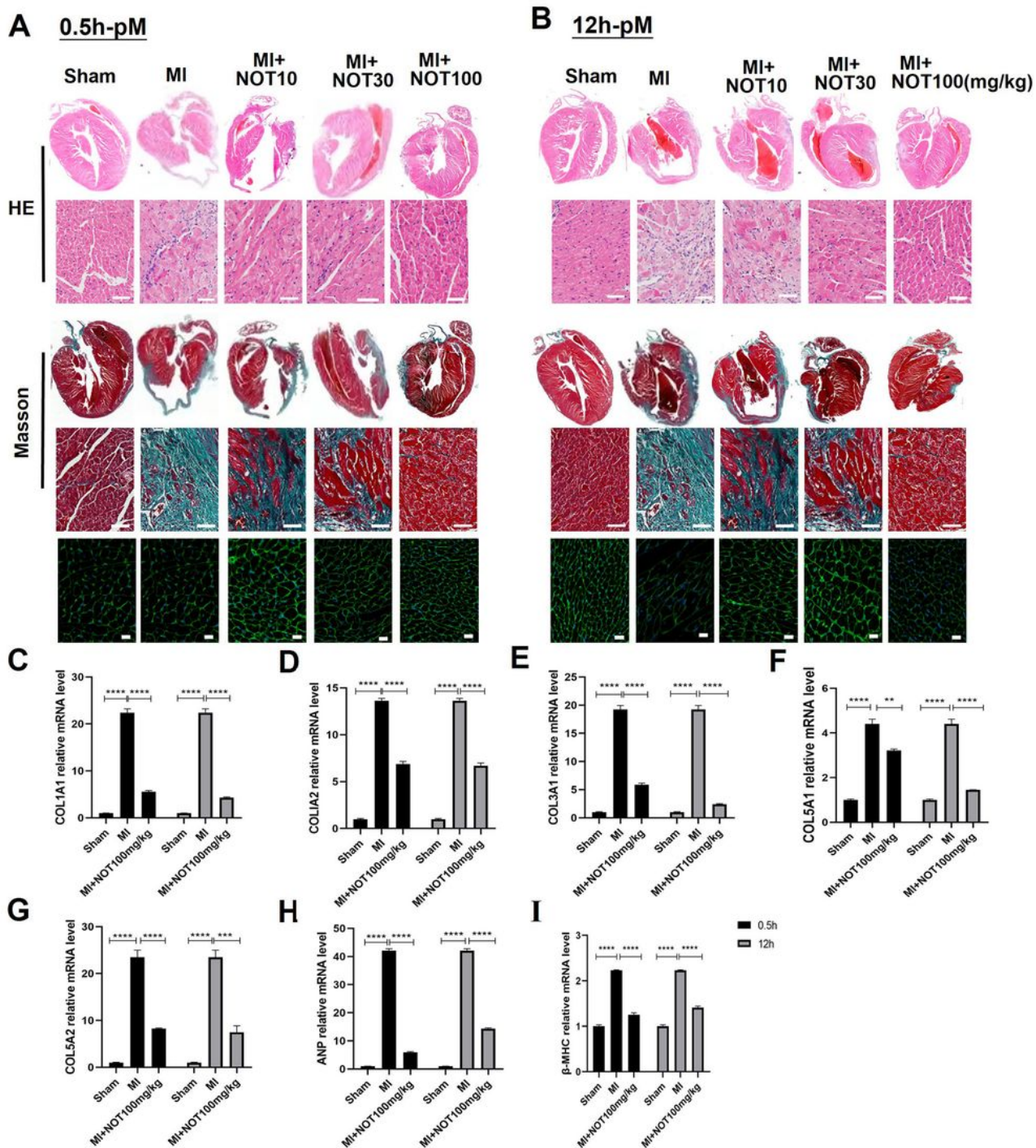


Figure 2

NOT pretreatment alleviated MI-induced myocardia remodeling, cardiac hypertrophy and fibrosis. (A-B) Representative histopathological images of heart sections with haematoxylin-eosin (H&E, scale bars: 1.5 mm and 50 μ m) staining for myocardia remodeling, Masson (scale bars: 1.5 mm and 50 μ m) staining for collagen deposition and wheat germ agglutinin (WGA, scale bars: 50 μ m) staining for cardiac hypertrophy. A exhibited the images of 0.5h-pMI groups, while B showed figures of 12h-pMI groups. **(C-H)**

Real-time PCR for myocardial Collagen IA1 (COLIA1), Collagen IA2 (COLIA2), CollagenIIIA1(COL3A1),Collagen VA1 (COL5A1), Collagen VA2 (COL5A2) , atrial natriuretic peptide (ANP) andβ-major histocompatibility complex β-MHC expression. Data represent the mean±SEM.n=3 **P*<0.05, ***P*<0.01

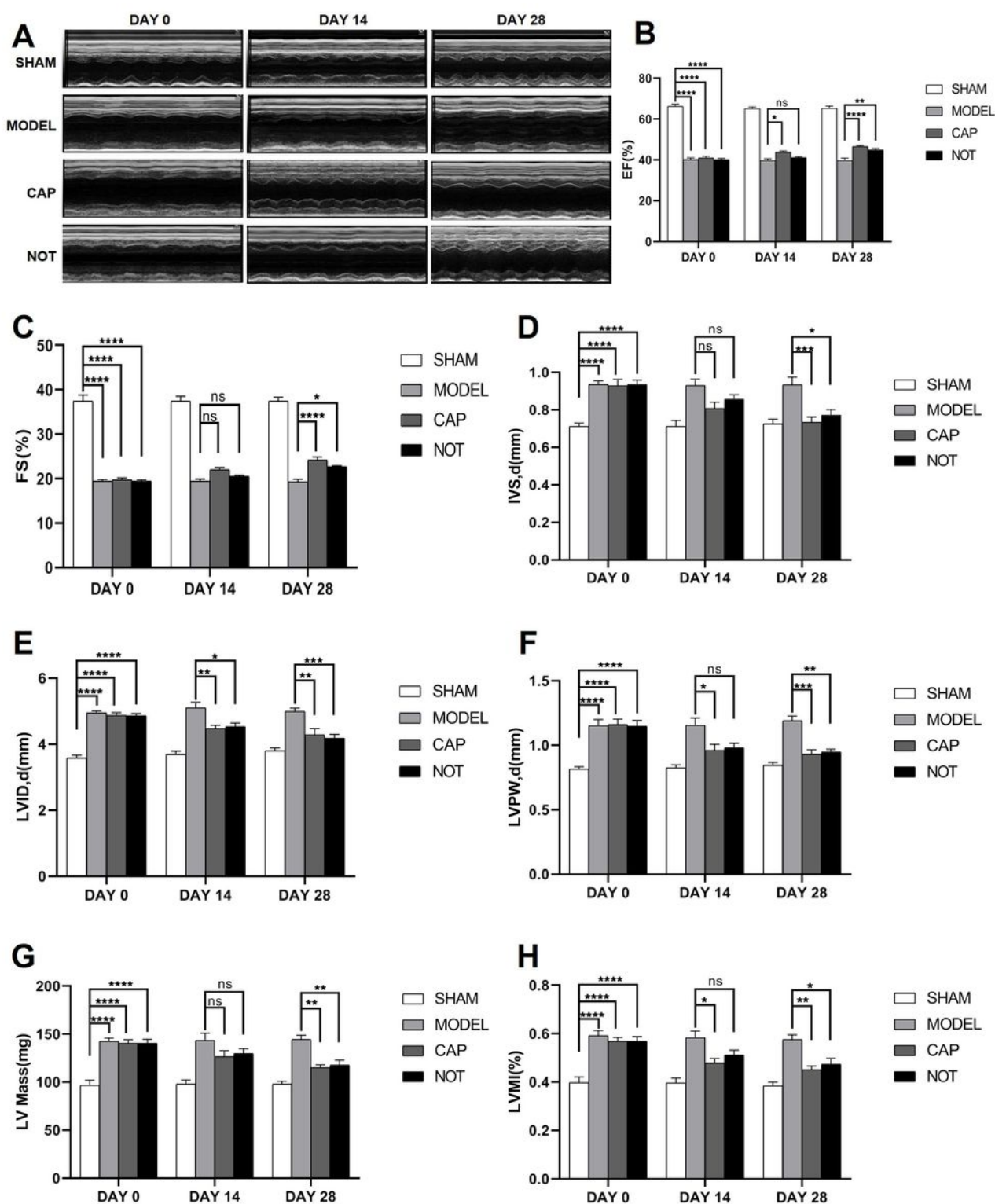


Figure 3

NOT treatment attenuated cardiac injury in HF mice. (A) In heart failure (HF) mice induced by left anterior descending (LAD) 4 weeks, the cardiac function was monitored by echocardiograms after vehicle, captopril (CAP) and notopterol (NOT) administration 14 and 28 days. Representative image of an M-mode photograph from vehicle and NOT treated groups. **(B-H)** Quantified values of ejection fraction (EF), fractional shortening (FS), inter ventricular septum diameter (IVS.d), left ventricular end-diastolic diameter (LVID.d), left ventricular diastolic posterior wall thickness (LVPWd), Left ventricular mass (LV Mass) and left ventricular mass index (LVMI) are presented as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, ns, no significance. n=12, 13, 13, 15 in sham, LAD, LAD+CAP and LAD+NOT, respectively.

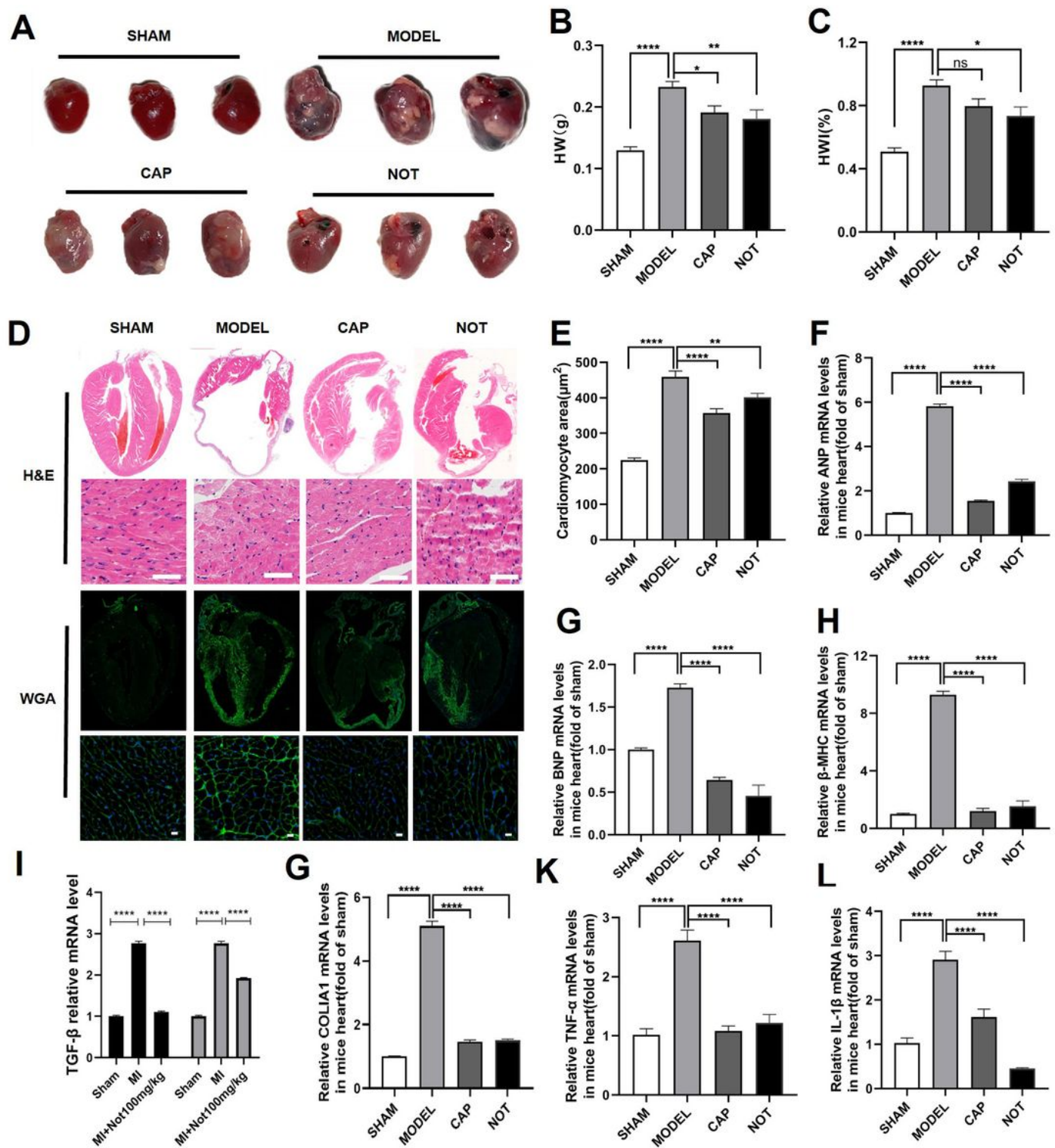


Figure 4

NOT treatment attenuated myocardia remodeling, cardiac hypertrophy and fibrosis in HF mice. (A) Representative macroscopic appearance of the heart in each group. Scale bar = 2 mm. **(B-C)** Heart weight (HW) and heart weight/body weight ratio. **(D)** Histological sections from hearts taken 4 weeks after sham surgery or left anterior descending (LAD) were stained with haematoxylin-eosin (H&E, scale bars, 1.5 mm and 50 μ m) for myocardia remodeling and wheat germ agglutinin (WGA, scale bars, 1mm and 50 μ m) for myocardia fibrosis.

cardiac hypertrophy. (E) Quantitative analysis of relative cardiomyocyte cell size from WGA staining. (F-L) Real-time PCR for myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β -major histocompatibility complex (β -MHC) expression, Transforming growth factor- β (TGF- β), Collagen IA1 (COLIA1), (Tumor necrosis factor- α) TNF- α and (Human IL-1 beta protein) IL-1 β expression. Data represent the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, ns, no significance. n = 4.

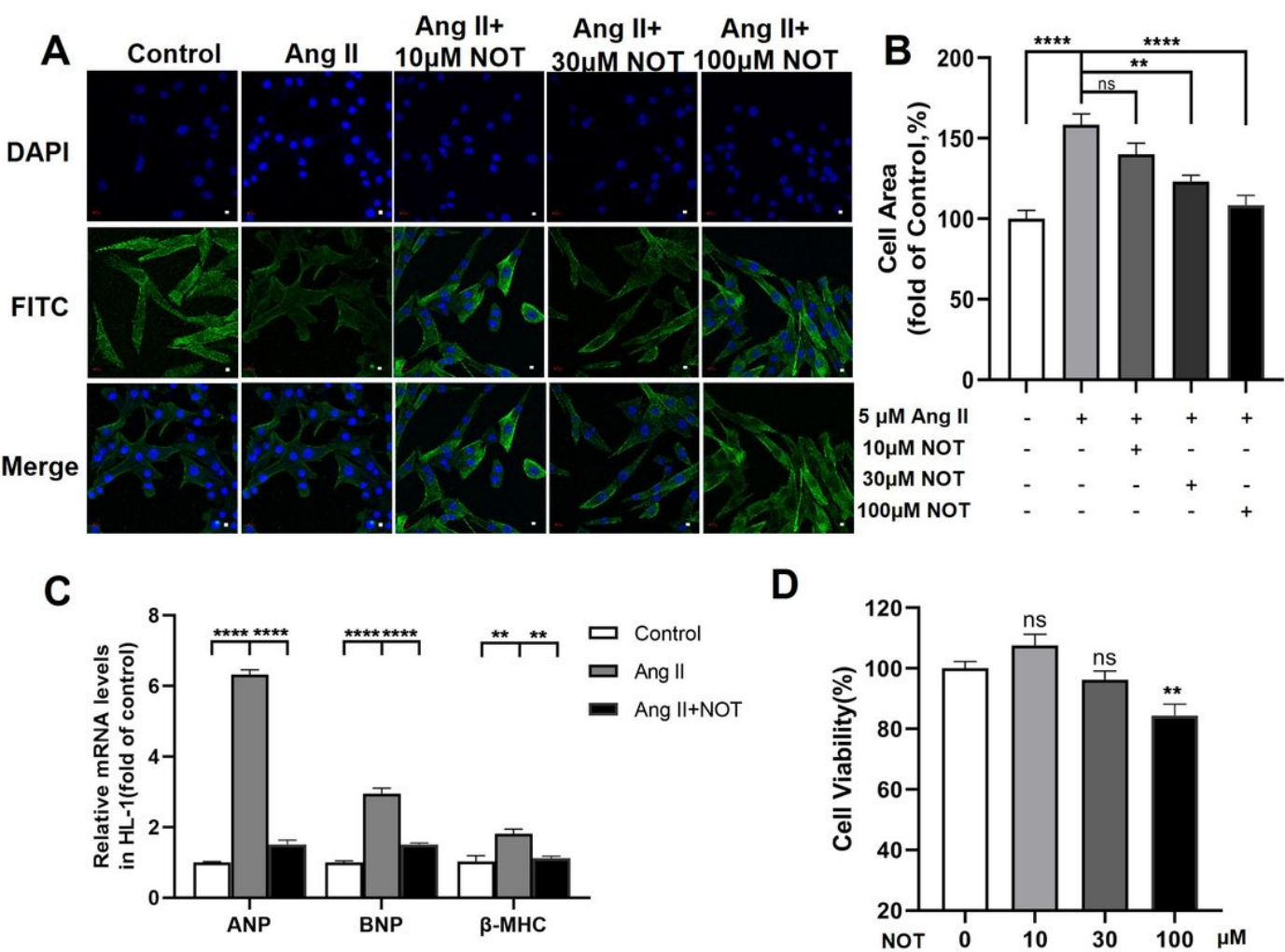


Figure 5

NOT alleviated AngII-induced myocardial hypertrophy of HL1. Effects of different concentrations of notopterol (NOT) in HL-1 hypertrophy induced by Ang II. (A) Cardiomyocyte size of HL-1 was determined using the α -actinin immunofluorescence. Representative images observed by aser scanning confocal microscope. Scale bar = 25 mm. (B) Quantitative analysis of relative cardiomyocyte cell size from α -actinin immunofluorescence. (C) Real-time PCR for myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and β -major histocompatibility complex (β -MHC) expression. HL-1 were incubating with NOT 10 μ M, 30 μ M, 100 μ M with or without Ang II (5 μ M) for further 24 h.(D) Effect of NOT on cellular viability in normal HL-1. Data represent the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, ns, no significance. n = 4.

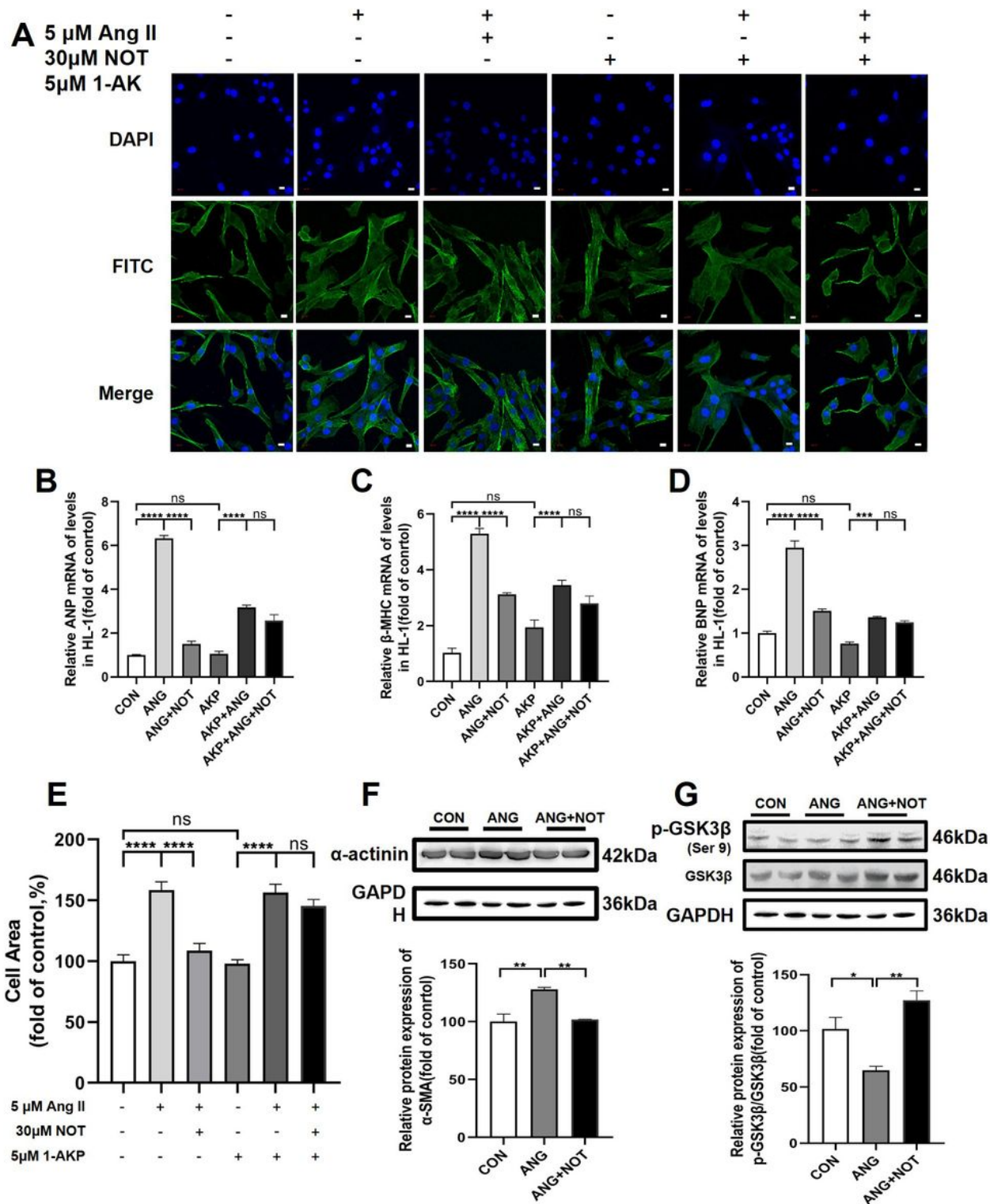


Figure 6

GSK3 β is involved in NOT-evoked cardioprotection. (A) Effects of GSK3 β inhibitor 1-AKP on the anti-hypertrophy effect of notopterol (NOT) in HL-1. Cell shape of HL-1 was determined by α -actinin immunofluorescence. Representative images of HL-1 observed by auser scanning confocal microscope. Scale bar = 25 mm. (B-D) Real-time PCR for myocardial atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and β -major histocompatibility complex (β -MHC) expression. (E) Quantitative analysis of

relative cardiomyocyte cell size from α -actinin immunofluorescence. **(F)** Representative images and quantitative analysis of α -actinin expression detected by western blotting. **(G)** Representative images and quantitative analysis of p-GSK3 β , GSK3 β and GAPDH in HL-1. Data represent the mean $\pm$ SEM. * P < 0.05, ** P < 0.01, ns. no significance. n = 4.

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