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Indole-3-propionic acid, a product of intestinal flora, inhibits the HDAC6/NOX2 signalling and relieves doxorubicin-induced cardiomyocyte damage

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The heart failure-gut hypothesis indicates that damage to intestinal mucosa leads to increased microbial translocation, resulting in alterations in metabolites entering the blood circulation. This process promotes the development of heart failure. This study aimed to reveal the involvement of indole-3-propionic acid (IPA), a microbiota-derived tryptophan metabolite, in heart failure. Human cardiomyocytes AC16 was treated with doxorubicin to induce in vitro heart failure model, the influences of IPA on the cellular viability, apoptosis, inflammation and oxidative stress were evaluated. Molecular docking and western blotting were used to initially illustrate the potential relationship between IPA and HDAC6. Through HDAC6 overexpression, its mediating role in the regulatory mechanism of IPA in the above aspects was further investigated. IPA was found to reduce the apoptosis, inflammation and oxidative stress in doxorubicin-treated cells. The visualized structure displayed that IPA bound to HDAC6 and that IPA reduced HDAC6 level. Additionally, HDAC6 overexpression reversed the regulation of IPA in the above aspects, indicating the HDAC6/NOX2 signals mediated the mechanism of IPA. Together, the present study revealed that IPA reduced oxidative stress, inflammatory response and apoptosis in cardiomyocytes via inhibiting the HDAC6/NOX2 signalling. The findings suggest that gut microbiota metabolites have potential in the treatment of heart failure.
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Keywords: gut microbiome metabolites, heart failure, doxorubicin, IPA, oxidative stress

INTRODUCTION

Heart failure refers to a group of syndromes in which functional or structural diseases of the heart lead to impaired ejection ability or ventricular filling, and is the terminal stage of various heart diseases [17, 19]. Cerebrovascular, pulmonary artery, metabolic and other diseases are common factors leading to heart failure. Pathways such as gut dysbiosis, immune and systemic inflammatory responses, and catecholamines build a complex mesh between other systems and the heart [4, 10, 22]. Although great progress has been made in the prevention and treatment of

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heart failure, the morbidity and mortality remain high, and it has become one of the leading causes of death worldwide [26], reflecting the urgent requirement for novel and effective treatment options. Limited research suggests a direct or indirect link between gut microbiota and heart failure. The heart failure-gut hypothesis [28, 34] indicates that patients with chronic heart failure develop intestinal ischaemia and oedema due to reduced cardiac output and systemic congestion. The intestinal mucosa is damaged, and the translocation of microorganisms increases, resulting in corresponding changes in the metabolites entering the blood circulation [27]. This process may cause or exacerbate systemic inflammatory responses, causing malnutrition and cachexia in patients with chronic heart failure, and promoting the development of heart failure. It has been shown that plasma concentrations of endotoxin and inflammatory cytokines are higher in heart failure patients with peripheral oedema compared with heart failure patients without oedema. Diuretic therapy reduces serum endotoxin concentrations but not inflammatory cytokines [18].

The impact of the microbiota on human health is extensive. In fact, the gut microbiome acts like an endocrine organ, influencing the physiology of the host by producing bioactive metabolites [20]. Gut microbes interact with the host through multiple pathways [28], including trimethylamine/trimethylamine N-oxide, short-chain fatty acid, primary and secondary bile acid pathways, etc. Although bacterial protein degradation products are generally considered harmful to the host, emerging evidence suggests that the gut tryptophan metabolic pathway is fundamental to gut homeostasis and metabolic health. Tryptophan is an essential amino acid from the diet for protein synthesis, and its metabolism in the body is influenced by enzyme activity and gut microbes [21]. Indole-3-propionic acid (IPA), a microbiota-derived tryptophan metabolite, accumulates in host serum and exhibits high inter-individual variability. Under physiological conditions, human serum IPA concentrations range from 1 to 10 μM. Liquid chromatography-mass spectrometry analysis of plasma samples from germ-free and conventional mice revealed that IPA production was highly dependent on gut microbes [30], and colonization with *Clostridium sporogenes* and *botulinum* could promote plasma IPA concentrations [24]. In previous studies, IPA was found to suppress inflammation and ER stress in liver fibrosis [14] and enhance mitochondrial function in experimental diabetic neuropathy [8]. In addition, IPA inhibits atherosclerosis, a risk factor for cardiovascular disease, by promoting reverse cholesterol transport [31]. Therefore, the hypothesis that IPA can alleviate heart failure was proposed.

This study used doxorubicin to induce *in vitro* heart failure model, and the purpose was to explore whether IPA could alleviate myocardial injury. In addition, this study further investigated the regulatory mechanism of IPA acting on cardiomyocytes, providing a theoretical basis for gut microbiota metabolites for the treatment of heart failure.

MATERIALS AND METHODS

Cell culture

Human cardiomyocytes AC16 (Sigma-Aldrich, Saint Louis, MI, USA) were cultured in DMEM/F-12 supplemented with 10% fetal serum and 1% penicillin-streptomycin solution. AC16 cells (10^5/mL) were stimulated with doxorubicin (5 μM, cell signalling technology) to mimic heart failure *in vitro*. IPA (Sigma-Aldrich) with concentrations of 12.5, 25, 50 μM was applied to treat AC16 cells for 24 h [16].

Cell transfection

To study the regulation mechanism, AC16 cells were transfected with specific pEXP plasmids (Ribobio, Guangzhou, Guangdong, China) to gain HDAC6 overexpression. The day before transfection, AC16 cells were seeded in six-well plates. The plasmids were mixed with the transfection reagent (Thermo Fisher, Waltham, MA, USA) and added to the cells the next day. 48 h after transfection, cells were harvested to assess transfection efficiency.

CCK8

The CCK8 assay was used to assess cell viability. After the cells were treated with doxorubicin or IPA for 24 h, CCK8 reagent (Meilunbio, Shanghai, China) was added to the wells and the system was incubated for another 2 h. The cell viability was calculated based on the absorbance and the standard curve.

Oxidative stress parameters

MDA, SOD and reactive oxygen species (ROS) were applied as parameters to assess oxidative stress. AC16 cells were lysed and centrifuged at 10,000 g for 10 min to obtain the supernatant. The protein concentra-

tion was determined with a BCA protein concentration assay kit. The resulting supernatant was used to measure MDA and SOD levels using commercial kits (Beyotime, Shanghai, China). The values were calculated based on the absorbance and the standard curve. DCFH-DA probe (Elabscience, Wuhan, Hubei, China) was used to assess ROS. Diluted DCFH-DA was supplemented to the wells and incubated in the dark for 30 min. Afterward, cells were washed twice with PBS. The results were photographed under a fluorescence microscope.

Western blotting

AC16 cells were lysed in RIPA buffer, lysates were then separated on SDS-polyacrylamide gels for electrophoresis. The separated proteins were transferred to PVDF membranes, followed by the blocking with 5% skim milk. The membranes were incubated with primary antibodies overnight at 4°C and an HRP-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. Blots were visualized using an ECL chemiluminescence reagent (Hanbio Biotechnology, Seoul, South Korea) and semi-quantified using ImageJ software.

ELISA

The levels of inflammation factors TNF-α, IL-1β and IL-6 were assessed using ELISA kits (Beyotime). Cell supernatant, as the sample, was obtained by centrifugation at 300 g for 5 min. The values were calculated based on the absorbance and the standard curve.

Flow cytometry

AC16 cells were washed twice with PBS, suspended in 1x binding buffer, and incubated with Annexin V-FITC for 10 min in the dark. Thereafter, propidium iodide in 1x binding buffer was added to the cells. Cell apoptosis was then analysed using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

RT-qPCR

AC16 cells were lysed with TRIzol® Reagent (Invitrogen, Waltham, MA, USA) and the RNA was precipitated using isopropanol. Reverse transcription and the qPCR reaction were conducted using a cDNA Synthesis Kit (Bio-Rad, Tokyo, Japan) and a SYBR Green RT-qPCR kit (Yeasen, Shanghai, China). Relative mRNA levels were measured using the $\Delta\Delta$Ct method after normalization to actin.

Molecular docking

The structure of HDAC6 (PDB: 6CE6) was obtained from the RCSB PDB. The water molecules and redundant ligands around the protein were deleted, and the structure was optimized. The structure of IPA was hydrogenated and converted into a mol2 format file. The docking ran in AutoDock version 4.2 and the composite structures were displayed.

Statistics analysis

All data are presented as means ± SD and comparisons were analysed with One-way ANOVA followed by Tukey's test in the SPSS 26 software. $P < 0.05$ was considered to be statistically significant.

RESULTS

IPA on cell viability and oxidative stress

IPA with concentrations of 0, 12.5, 25, 50 μM had no significant effect on cell viability (Fig. 1A). After the cells were induced by doxorubicin, the cell viability decreased significantly, and additional IPA treatment rescued the cell viability in a concentration-dependent manner (Fig. 1B). The level of MDA in cells was significantly increased in response to doxorubicin treatment, and IPA could reduce the increase of MDA in a concentration-dependent manner (Fig. 1C). The alteration trend of SOD in cells was opposite to that of MDA, and IPA could increase the level of SOD (Fig. 1D). Similarly, IPA significantly reduced doxorubicin-induced ROS generation (Fig. 1E).

IPA on the inflammation and apoptosis

The p38 and p65 pathways were activated upon exposure to proinflammatory signals, and here, doxorubicin induced phosphorylation of p38 and p65 proteins in AC16 cells, whereas IPA reduced the phosphorylation of both proteins in a concentration-dependent manner (Fig. 2A). By directly detecting the level of inflammatory factors, IPA was found to reduce the secretion of TNFα, IL-1β, and IL-6, and partially alleviate the induction of inflammatory response by doxorubicin (Fig. 2B). The results of flow cytometry showed that IPA reduced the apoptosis induced by doxorubicin stepwise (Fig. 1C, D). Doxorubicin caused an increase in the protein content of Bax and cleaved caspase 3, and a decrease in Bcl2, whereas IPA increased the content of Bcl2 and decreased the protein content of Bax and cleaved caspase 3 in cells (Fig. 2E).

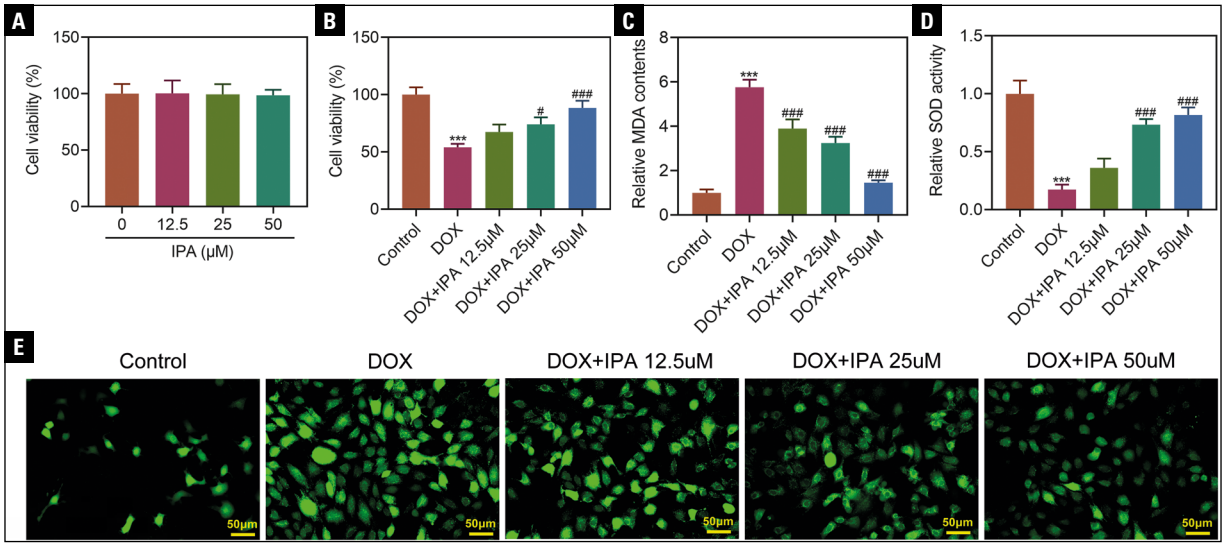

Figure 1. IPA on cell viability and oxidative stress. **A.** The effects of different concentrations of IPA without or **B.** with doxorubicin on the viability of AC16 cells were determined using the CCK8 assays. **C.** MDA; **D.** SOD and **E.** ROS were applied as parameters to assess oxidative stress. $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. DOX.

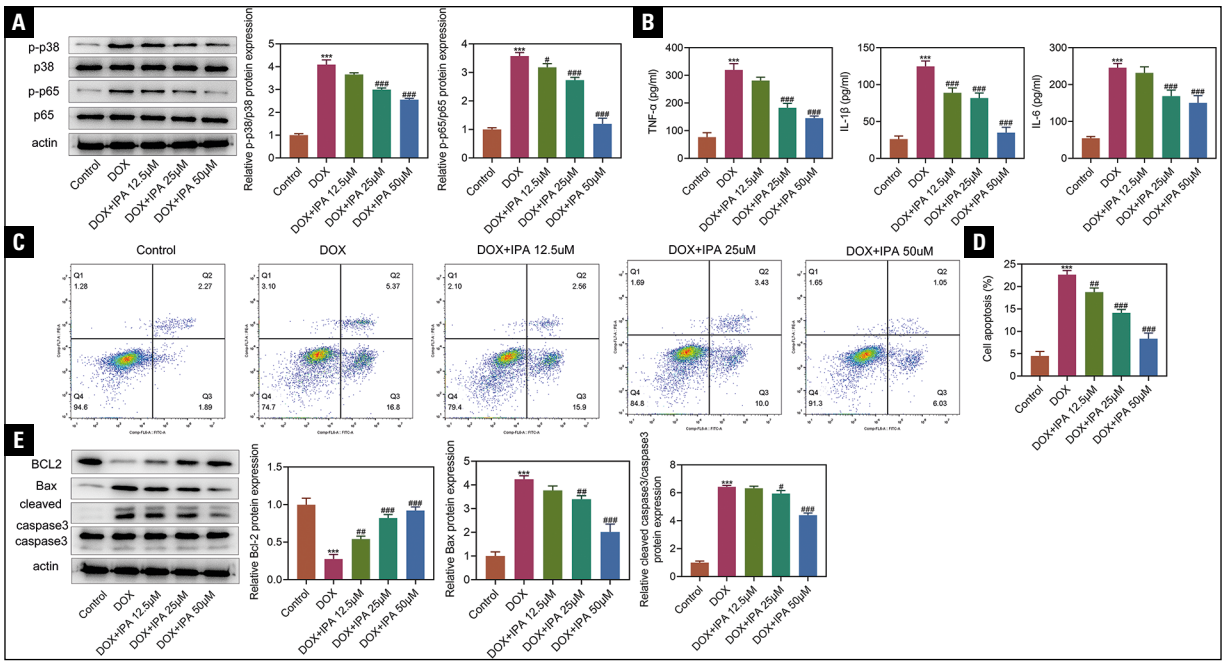

Figure 2. IPA on the inflammation and apoptosis. **A.** The effects of IPA on the phosphorylation of p38 and p65 were assessed using western blotting; **B.** The levels of inflammatory factors were determined using ELISA; **C, D.** The effect of IPA on the apoptosis induced by doxorubicin was determined using flow cytometry; **E.** The contents of apoptosis-related proteins were assessed using western blotting. $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. DOX.

IPA suppresses the HDAC6/NOX2 signalling to regulate cell apoptosis

Molecular docking results indicated that IPA could bind HDAC6 in terms of three-dimensional structure (Fig. 3A, B). Thus, the levels of HDAC6 and downstream NOX2 were detected. Doxorubicin could increase their levels, while IPA caused the decrease of HDAC6 and NOX2 levels (Fig. 3C). To further study the mechanism, HDAC6 expression was promoted in cells (Fig. 3D), and the effect of HDAC6 overexpression on IPA regulation was explored. The results suggested that the overexpression of HDAC6 destroyed the protection of IPA on cell viability and promoted cell apoptosis. Specifically, CCK8 assay revealed that

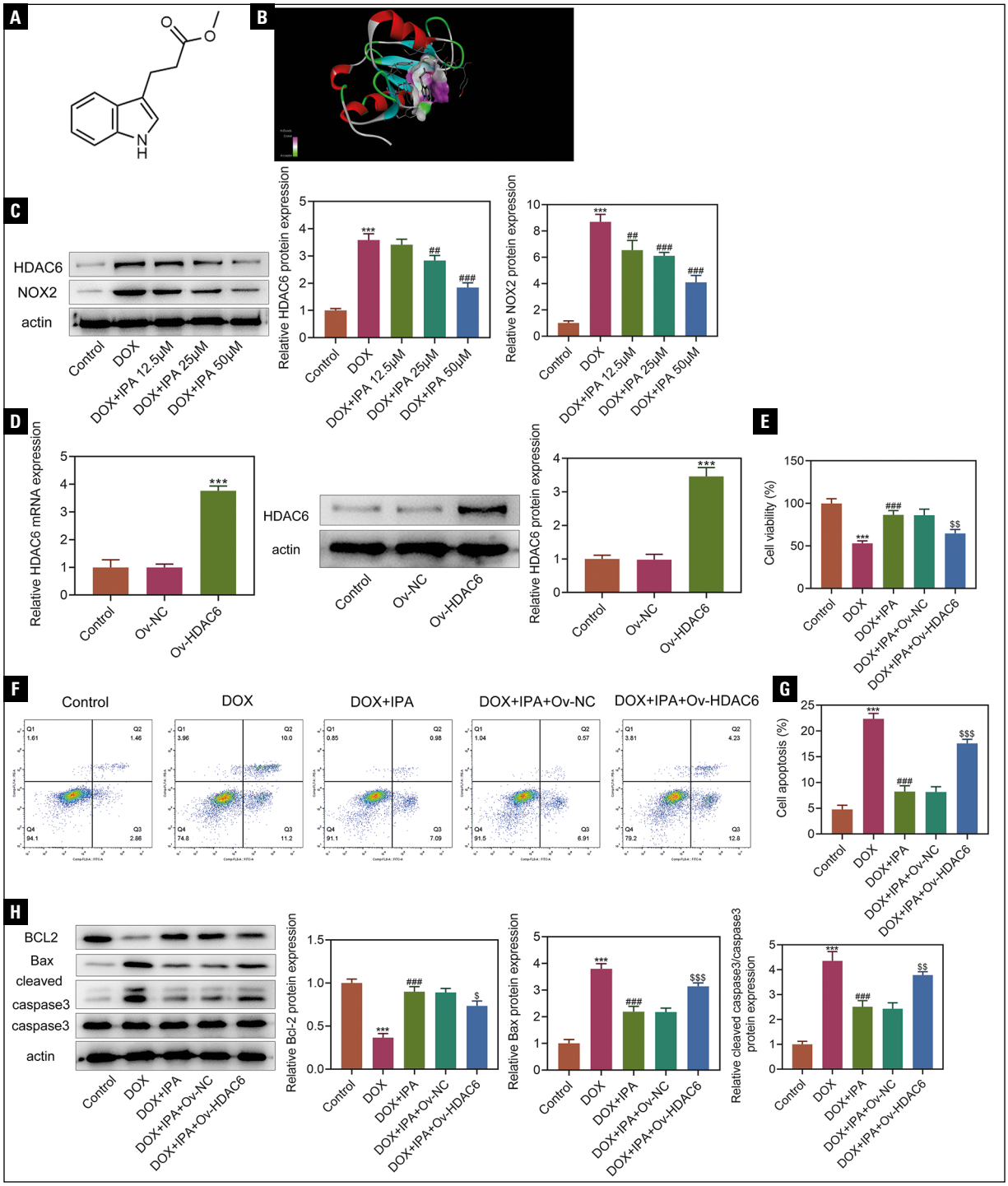

Figure 3. IPA suppresses the HDAC6/NOX2 signalling to regulate cell apoptosis. **A.** The structure of IPA; **B.** Molecular docking results indicated that IPA could bind HDAC6 in terms of three-dimensional structure; **C.** The levels of HDAC6 and downstream NOX2 were detected using western blotting; **D.** HDAC6 expression was promoted in cells via transfection; **E.** The effect of HDAC6 overexpression on the viability of AC16 cells were determined using the CCK8 assay; **F, G.** The effect of HDAC6 overexpression on the apoptosis was determined using flow cytometry and **H.** western blotting. $^{***}p < 0.001$ vs. control or Ov-NC; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. DOX; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOX + IPA + Ov-NC.

HDAC6 overexpression greatly reduced cell viability, which was closer to doxorubicin treatment alone (Fig. 3E). Compared with the Ov-NC group, the proportion of apoptotic cells increased significantly (Fig. 3F, G), accompanied by the increase of Bax and cleaved caspase 3 content and the decrease of Bcl2 (Fig. 3H).

HDAC6/NOX2 signalling influences oxidative stress and inflammation

HDAC6 overexpression triggered the increase of intracellular MDA and the decrease of SOD, reversing the effect of IPA on them (Fig. 4A, B). Intracellular ROS generation was also elevated (Fig. 4C), indicating enhanced oxidative stress. Compared with the Ov--NC group, the phosphorylation of p38 and p65 was increased upon HDAC6 overexpression (Fig. 4D), and the levels of inflammatory factors TNFα, IL-1β, and IL-6 secreted by cells were all raised (Fig. 4E).

DISCUSSION

Whether experimental or clinical studies, they have identified oxidative stress as an important pathophysiological pathway in the development of heart failure. Oxidative stress is defined as an imbalance between the production of ROS and endogenous antioxidant defence systems, the excess of ROS can impair cardiomyocyte electrobiology, myofilament calcium sensitivity, induce energy deficits and extracellular remodelling [19]. It happens that the key role of doxorubicin in inducing dose-dependent cardiotoxicity is attributed to the formation of ROS, which is characterized by irreversible cardiac damage and congestive heart failure [20]. In the past, several clinical studies have targeted producers of oxidative stress (xanthine oxidase and unconjugated NOS) or the endogenous antioxidant N-acetylcysteine (NAC), exogenous antioxidants (vitamin C/E or folic acid) to treat heart failure [11]. The results showed that only NAC could increase the level of endogenous GSH to enhance the antioxidant capacity and improve the prognosis of patients [29]. This suggests that future oxidative stress therapies for heart failure should focus on enhancing endogenous antioxidant capacity. In this study, IPA can increase the level of SOD in cardiomyocytes, and SOD is an endogenous oxidant, which highlights the feasibility of using gut microbes or their metabolites to alleviate the disease process of heart failure.

Intestinal flora creates numerous metabolites, some of which are absorbed into the systemic blood circulation and are biologically active, and the other portion of which is metabolized by host enzymes, which serve as an intermediary for the intestinal flora to affect the host [3]. In this way, the intestinal flora functions as a remote endocrine organ that connects with nearby organs via metabolic pathways. The potential role of the gut microbiome in altering the health of the host has received considerable attention, with considerable evidence showing a link between the gut microbiota and various diseases, including colorectal cancer, diabetes, liver cirrhosis, atherosclerosis and arthritis [9]. Many microbial markers specific to these diseases have been discovered, and therapeutic regimens targeting fecal microbes have been adopted as powerful tools for early diagnosis and treatment of various diseases [32, 33]. More importantly, through the fecal transplantation test and the reconstruction of intestinal flora, some specific mechanisms of intestinal flora pathogenicity have been revealed [5, 25], and the metabolic potential of intestinal flora has been clearly identified as a factor that promotes the occurrence of disease. This study also continues to explore the mechanism by which IPA regulates cardiomyocytes. Through molecular docking and induction of HDAC6 overexpression, we confirmed that IPA alleviated cardiomyocyte injury by inhibiting HDAC6/NOX2 signalling. Therein, HDAC6 is a key enzyme in the process of histone modification, responsible for catalyzing and regulating histone deacetylation, which was also found to govern mouse cardiac myofibril hardness [12]. NOX2 can generate ROS and participate in apoptosis as a second messenger [7].

Direct administration of bacterial metabolites as a therapeutic approach has several advantages over colonization. For example, bacterial colonization involves the introduction of live bacteria into the body, which can lead to infection [13], especially in individuals with compromised immune systems [23]. In contrast, direct administration of bacterial metabolites eliminates the risk of bacterial overgrowth and infection. Bacterial metabolites are usually stable compounds that can be synthesized or produced in large quantities under controlled conditions, both for precise dosage and targeted delivery, and for better regulation. In addition, the risk of adverse effects or unintended outcomes is generally lower with metabolite administration compared with live bacteria [2, 15], making it a safer option. Nevertheless, this study is limited to *in vitro* experiments and further *in vivo* studies are worthwhile.

CONCLUSIONS

The present study reveals that IPA reduces oxidative stress, inflammatory response and apoptosis in cardiomyocytes via inhibiting the HDAC6/NOX2

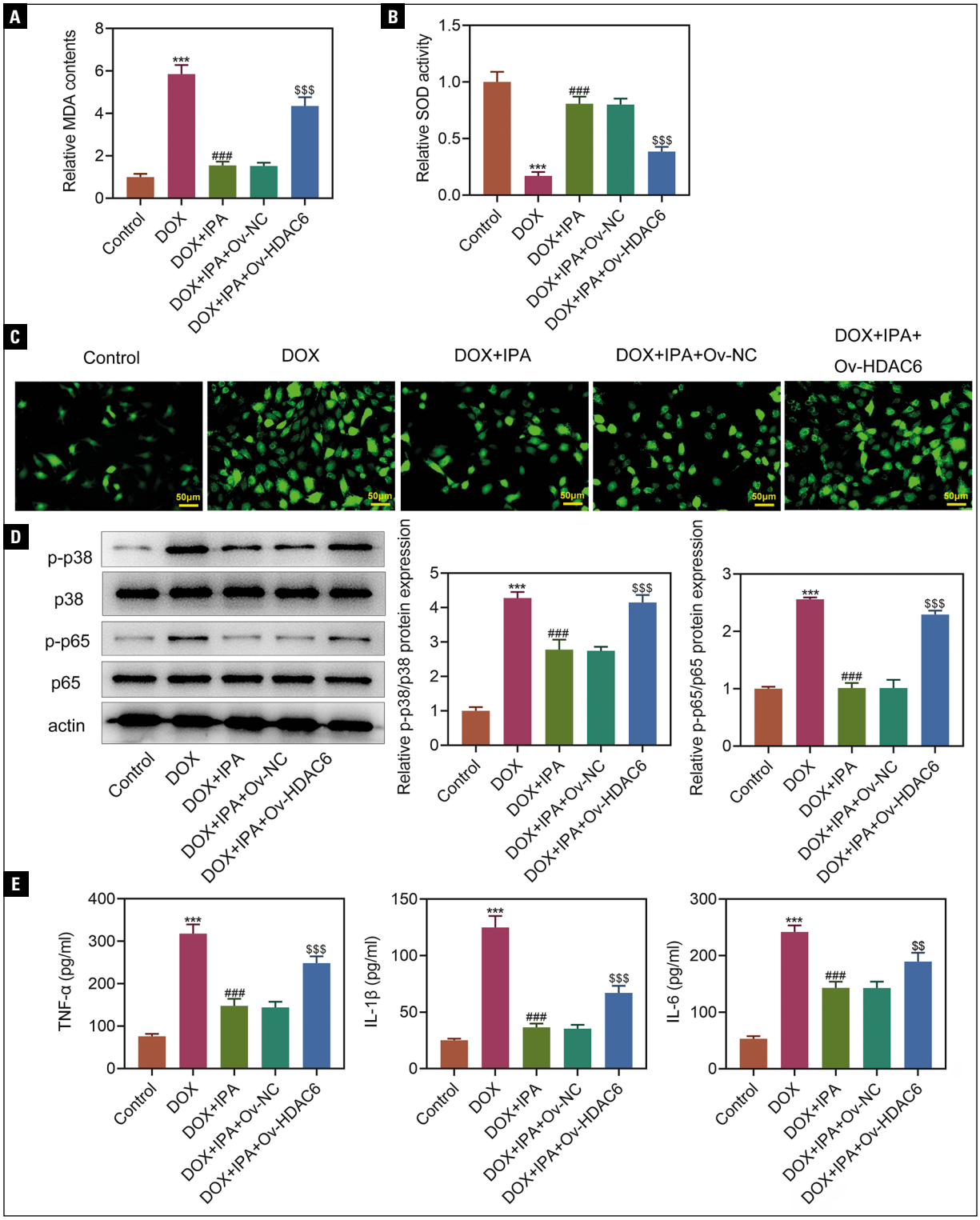

Figure 4. HDAC6/NOX2 signalling influences oxidative stress and inflammation. **A.** MDA; **B.** SOD and **C.** ROS were applied as parameters to assess oxidative stress. The effect of HDAC6 overexpression on the oxidative stress was evaluated; **D.** The effects of HDAC6 overexpression on the phosphorylation of p38 and p65 were assessed using western blotting; **E.** The levels of inflammatory factors were determined using ELISA. $^{***}p < 0.001$ vs. control; $^{\#\#\#}p < 0.001$ vs. DOX; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. DOX + IPA + Ov-NC.

signalling. IPA reduces oxidative stress in cardiomyocytes through endogenous antioxidant pathways, suggesting that gut microbiota metabolites have potential in the treatment of heart failure.

ARTICLE INFORMATION AND DECLARATIONS

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Conflict of interest: The authors declare that they have no competing interests.

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