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ORIGINAL RESEARCH

Artesunate inhibits ROS production and ameliorates mitochondrial damage through the SIRT1/FOXO3a/MnSOD pathway to alleviate heart failure

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Abstract

Heart failure (HF) is a complex and multifactorial disease responsible for over 17.3 million deaths annually, primarily mediated by oxidative stress-induced mitochondrial dysfunction. Artesunate (AS), an artemisinin derivative, possesses anti-inflammatory and antioxidative properties and is used to enhance mitochondrial function and reduce reactive oxygen species (ROS) generation. However, its specific effects and mechanisms in HF remain poorly understood. In this study, we investigated the impact of AS on Doxorubicin hydrochloride (Dox)-induced injury in H9C2 cardiomyocytes. The results showed that AS promoted cell viability, decreased ROS production and mitigated mitochondrial damage in Dox-exposed H9C2 cells. Importantly, AS also modulated the Silent information regulator 1 (SIRT1)/Forkhead box O3a (FOXO3a)/Manganese superoxide dismutase (MnSOD) pathway, indicating its potential therapeutic utility in HF by inhibiting ROS production and preserving mitochondrial function in doxorubicin-treated H9C2 cardiomyocytes.

Keywords

Heart failure (HF); Artesunate (AS); Dox; Mitochondrial damage; SIRT1/FOXO3a/MnSOD pathway

1. Introduction

Heart failure (HF) is a complex disease responsible for over 17.3 million deaths annually, making it the leading cause of cardiovascular mortality [1, 2]. Oxidative stress-induced mitochondrial dysfunction and resultant cellular damage are important pathophysiological mechanisms in the initiation and progression of HF [3]. Elevated reactive oxygen species (ROS) levels due to oxidative stress within the heart impair DNA, proteins, lipids, and other vital components of cardiomyocytes, thereby diminishing mitochondrial energy production and cardiac output [4]. Thus, safeguarding mitochondrial function and controlling ROS levels are pivotal for managing HF.

Artesunate (AS) is a water-soluble derivative of artemisinin recognized for its anti-inflammatory, antitumor, anti-apoptotic, and immunomodulatory properties [5]. It has been shown to enhance SIRT1 expression in lung slices, thereby mitigating sepsis-induced acute lung injury [6]. Moreover, AS exerts protective effects against cerebral ischemia-reperfusion injury by activating Nrf2 and suppressing ROS-mediated p38 Mitogen-activated protein kinase (MAPK) signaling, thereby mitigating oxidative stress and inflammation [7]. Additionally, AS has been implicated in alleviating myocardial injury associated with ischemia-reperfusion [7]. Nevertheless, the precise effects and underlying mechanisms of AS in HF remain to be elucidated.

Recent studies have also emphasized the role of AS in modulating mitochondrial function [8], such as enhancing mitochondrial biogenesis and functionality by upregulating essential regulatory proteins involved in mitochondrial dynamics and energy metabolism [8]. This action promotes the activity of mitochondrial enzymes and preserves mitochondrial membrane potential, thereby supporting efficient Adenosine triphosphate (ATP) production and reducing mitochondrial ROS generation [8]. The dual capacity of AS to enhance mitochondrial function while mitigating oxidative damage highlights its potential as a promising therapeutic agent for HF.

Silent information regulator 1 (SIRT1) is a metabolic regulator that belongs to the sirtuin family as a class III histone deacetylase [9]. It plays an essential role in maintaining tissue homeostasis and has been implicated in various metabolic disorders [10]. It is also closely associated with oxidative stress management, particularly through the SIRT1/FOXO3a/MnSOD signaling pathway, which is essential for reducing mitochondrial ROS levels [11]. MnSOD is predominantly found in mitochondria and is responsible for scavenging mitochondrial ROS, thereby mitigating oxidative

stress [11]. Activation of this antioxidant pathway has been shown to effectively suppress ROS production.

This study investigates the role of AS in reducing ROS production and mitigating mitochondrial damage in doxorubicin-treated H9C2 cardiomyocytes, which represent a model of oxidative stress and mitochondrial dysfunction. Understanding these mechanisms could lead to novel therapeutic strategies for HF by targeting these critical pathways.

2. Materials and methods

2.1 Cell culture and treatment

H9C2 cardiomyocytes (ATCC, CRL-1446) were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA, 11995065) supplemented with 10% FBS (Fetal Bovine Serum, Gibco, Grand Island, NY, USA, 16000044) and 1% PS (Penicillin-Streptomycin, Invitrogen, Carlsbad, CA, USA, 15140122) at 37 °C in a humidified atmosphere with 5% carbon dioxide (CO_2).

For the experimental treatments, H9C2 cells were categorized into the following groups: control, Dox (1 μM), and Dox combined with varying concentrations of AS (2.5, 5, 10, 20 μM). Doxorubicin hydrochloride (Dox, Sigma-Aldrich, D1515, St. Louis, MO, USA) was purchased from Sigma-Aldrich (D1515, A3731, St. Louis, MO, USA), and AS was obtained from Sigma-Aldrich (A3731, St. Louis, MO, USA).

2.2 Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (C0038, Beyotime, Beijing, China). Briefly, H9C2 cells were seeded in 96-well plates and treated according to the experimental groups. After 24 hours, 10 μL of CCK-8 solution was added to each well and incubated for 2 hours. Then, their optical density was measured at 450 nm (OD450) to evaluate cell viability.

2.3 Apoptosis assay

Apoptosis was evaluated using flow cytometry with an Annexin Fluorescein isothiocyanate (FITC)/Propidium iodide (PI) apoptosis detection kit (556547, BD Biosciences San Jose, CA, USA). H9C2 cells were stained as per the manufacturer's instructions and analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

2.4 ROS measurement

Intracellular ROS levels were quantified using the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Sigma-Aldrich, St. Louis, MO, USA). H9C2 cells were treated with 10 μM DCFH-DA at 37 °C for 30 minutes in the dark, and fluorescence intensity was assessed using a fluorescence microscope (LSM710, ZEISS, Oberkochen, Germany).

2.5 Mitochondrial membrane potential assay

Mitochondrial membrane potential was assessed using JC-1 staining (Beyotime, Beijing, China). Briefly, treated H9C2 cells were incubated with 5 μM JC-1 at 37 °C for 30 minutes. Fluorescence images were captured using a fluorescence microscope (Olympus IX73, Japan). In this experiment, green fluorescence indicated JC-1 monomers (indicative of damaged mitochondria), and red fluorescence indicated JC-1 aggregates (indicative of healthy mitochondria).

2.6 ATP measurement

ATP levels were quantified using an ATP assay kit (S0026, Beyotime, Beijing, China). H9C2 cells were lysed, and ATP content was determined according to the manufacturer's instructions.

2.7 Immunoblot

H9C2 cells were lysed, and protein concentrations were assessed using the Bicinchoninic acid (BCA) protein assay kit (23225, Thermo Fissher, Rockford, IL, USA). Equal amounts of protein were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk and then incubated overnight at 4 °C with primary antibodies against SIRT1 (Abcam, ab110304, 1:1000, Cambridge, UK), FOXO3a (Cell Signaling Technology, #2497, 1:1000, Danvers, MA, USA), MnSOD (Abcam, ab13533, 1:1000, Cambridge, UK), and β-actin (Abcam, ab8226, 1:3000, Cambridge, UK). After washing, the membranes were incubated with Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) and visualized using an Enhanced chemiluminescence (ECL) detection reagent (32016, Thermo Fisher Scientific, Rockford, IL, USA).

2.8 Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 AS promotes the viability of Dox-stimulated H9C2 cells and reduces apoptosis

Cell viability and apoptosis assays were conducted to investigate the protective effects of AS on Dox-stimulated H9C2 cells, and the viability of H9C2 cells treated with various concentrations of AS (0, 2.5, 5, 10, 20 μM) was assessed using the CCK-8 assay (Fig. 1A, $p < 0.001$). The results showed that AS exhibited no significant cytotoxicity up to 10 μM but significantly reduced cell viability at 20 μM (Fig. 1A, $p < 0.001$). Subsequently, cells were treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). In addition, Dox significantly decreased cell

viability, whereas AS co-treatment markedly improved cell viability (Fig. 1B, $p < 0.05$). Apoptosis in H9C2 cells treated with Dox alone and in combination with AS was analyzed using flow cytometry. Dox treatment significantly increased apoptosis, whereas AS co-treatment reduced the percentage of apoptotic cells (Fig. 1C, $p < 0.001$). Together, these findings indicate that AS effectively enhances the viability of Dox-stimulated H9C2 cells and mitigates apoptosis.

3.2 AS reduces ROS production in Dox-stimulated H9C2 cells

Here, DCFH-DA staining was performed to evaluate the impact of AS on ROS production in Dox-stimulated H9C2 cells. The results showed that Dox treatment significantly increased ROS production, which is evident based on the strong green fluorescence, while co-treatment with AS significantly de-

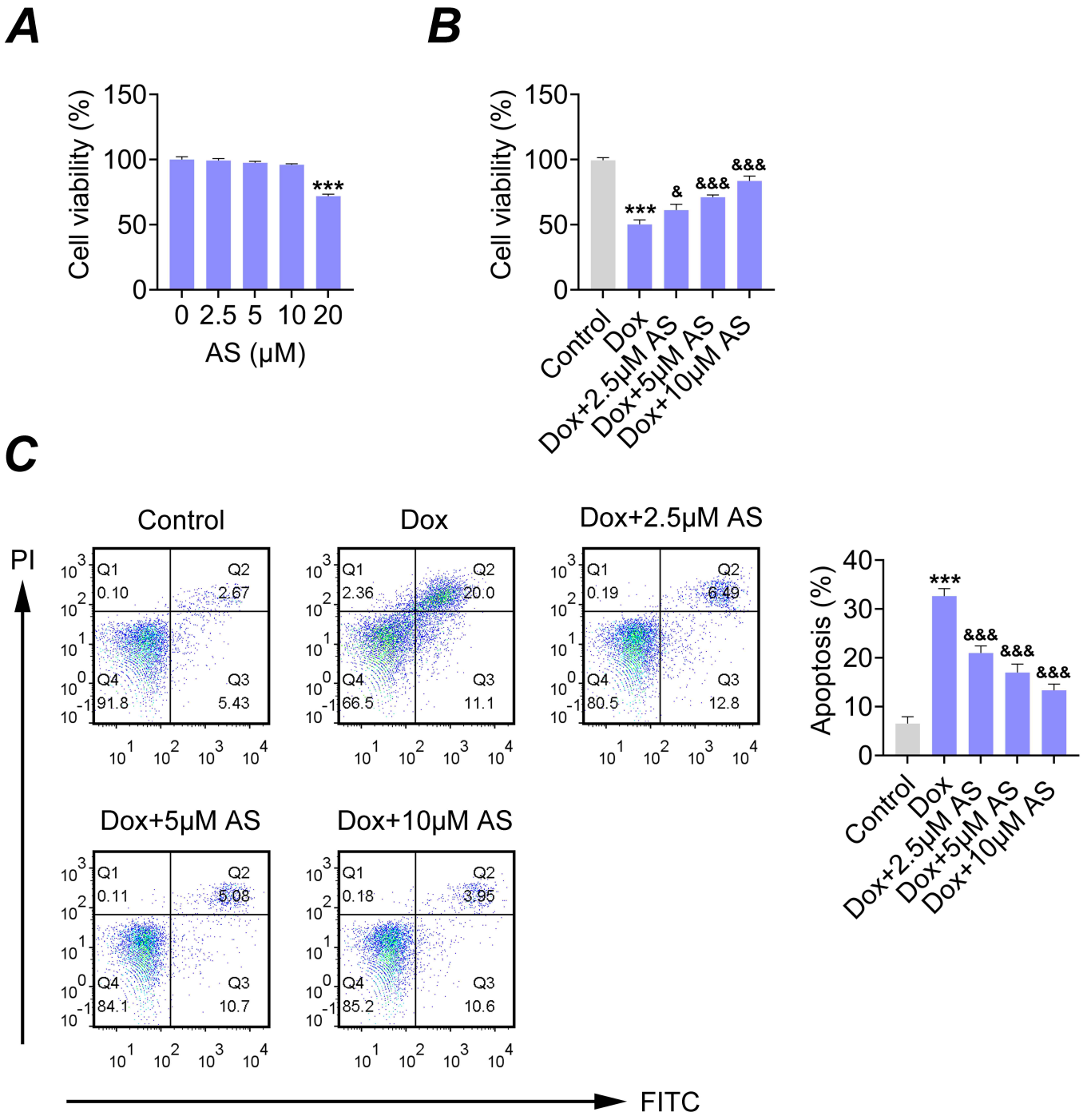

FIGURE 1. Artesunate (AS) promotes viability and reduces apoptosis of Dox-stimulated H9C2 cells. (A) Cell viability assay using CCK-8 method in H9C2 cells treated with varying concentrations of AS (0, 2.5, 5, 10, 20 μM). (B) Cell viability assay in H9C2 cells treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). (C) Flow cytometry analysis of apoptosis in H9C2 cells treated with Dox (1 μM) alone and with AS (2.5, 5, 10 μM). Quantification of apoptosis. ***$p < 0.001$ compared to the control group; &$p < 0.05$, &&&$p < 0.001$ compared to Dox group. Dox: Doxorubicin hydrochloride; PI: Propidium iodide; FITC: Fluorescein isothiocyanate.

creased ROS levels (reduced green fluorescence intensity) (Fig. 2, $p < 0.05$). These findings demonstrate AS's effective reduction of ROS production in Dox-stimulated H9C2 cells, highlighting its antioxidative potential in mitigating Dox-induced oxidative stress.

3.3 AS alleviates mitochondrial damage in Dox-stimulated H9C2 cells

Next, JC-1 staining was performed to evaluate mitochondrial damage in H9C2 cells treated with Dox alone and in combination with AS. In this experiment, green fluorescence denotes JC-1 monomers (indicative of damaged mitochondria), and red fluorescence signifies JC-1 aggregates (indicative of healthy mitochondria). The results showed that Dox treatment increased green fluorescence and decreased red fluorescence, indicating substantial mitochondrial damage (Fig. 3A, $p < 0.05$). In contrast, AS co-treatment reduced green fluorescence and increased red fluorescence, suggesting alleviation of mitochondrial damage (Fig. 3A). Additionally, ATP production was measured under the same conditions. Dox significantly reduced ATP levels, whereas AS co-treatment restored ATP production (Fig. 3B, $p < 0.05$). Thus, these results indicate that AS can effectively mitigate mitochondrial damage in Dox-stimulated H9C2 cells.

3.4 AS regulates the SIRT1/FOXO3a/MnSOD pathway in H9C2 cells

Lastly, immunoblot assays were conducted to explore the underlying mechanism. Dox treatment was found to suppress the expression of SIRT1 and FOXO3a in H9C2 cells (Fig. 4A). Conversely, AS treatment reversed this suppression and significantly upregulated the expression of these regulators in Dox-stimulated H9C2 cells (Fig. 4A, $p < 0.05$). Similarly, MnSOD expression was downregulated following Dox treatment, whereas AS treatment enhanced MnSOD expression in Dox-stimulated H9C2 cells (Fig. 4B, $p < 0.05$). These results indicate that AS effectively activates the SIRT1/FOXO3a/MnSOD pathway, potentially contributing to its protective effects against Dox-induced damage in H9C2 cells.

4. Discussion

HF affects millions of individuals annually [2]. Its pathogenesis involves multiple mechanisms, with oxidative stress and mitochondrial dysfunction playing pivotal roles [12]. Excessive accumulation of ROS in the myocardium leads to cellular damage, reduces mitochondrial energy production, and impairs cardiac output [13]. Therefore, strategies targeting oxidative stress mitigation and preservation of mitochondrial function are essential for improving the management of HF.

Mitochondrial dysfunction is a hallmark of HF, characterized by decreased mitochondrial biogenesis, altered dynamics, and impaired respiratory function [14–16]. This dysfunction results in excessive ROS production, which damages mitochondrial DNA, proteins and lipids, disrupts the mitochondrial membrane potential, and exacerbates energy deficits and cardiac dysfunction [14]. Therapeutic strategies aimed at reducing ROS levels and enhancing mitochondrial function hold potential for significantly improving outcomes in HF patients.

AS is a water-soluble derivative of artemisinin, originally developed as an antimalarial drug [17]. Recent studies have expanded its pharmacological activities to also include anti-inflammatory, antitumor and antioxidative properties [6, 7]. AS has been reported to be effective in alleviating oxidative stress and inflammatory responses in various disease models, suggesting its potential as a therapeutic candidate for HF [6, 7, 18]. However, its specific effects and underlying mechanisms in HF remain largely unexplored.

In this study, we demonstrated that AS promotes the viability of Dox-stimulated H9C2 cardiomyocytes, reduces ROS production, and alleviates mitochondrial damage. These findings are consistent with previous reports on AS's protective effects against oxidative stress and mitochondrial dysfunction. AS enhances mitochondrial biogenesis and function by increasing the expression of key regulatory proteins involved in mitochondrial dynamics and energy metabolism, and this preservation of ATP production helps to reduce mitochondrial ROS generation.

The SIRT1/FOXO3a/MnSOD pathway plays a crucial role in mitigating oxidative stress and maintaining mitochondrial function [19, 20]. SIRT1, a class III histone deacetylase, is central to cellular metabolism, stress resistance, and longevity [20]. Activation of SIRT1 has been associated with various beneficial effects in metabolic diseases, including HF [19]. SIRT1 regulates FOXO3a, a transcription factor that promotes MnSOD expression, a primary mitochondrial antioxidant enzyme [20]. By enhancing MnSOD levels, the SIRT1/FOXO3a axis effectively reduces mitochondrial ROS and protects against oxidative damage [20].

In this study, it was found that 20 μM AS could significantly reduce the viability of H9C2 cardiomyocytes, suggesting a potential cytotoxic effect at this concentration and the importance of carefully considering the therapeutic dosage of AS to ensure both safety and efficacy. Previous studies have established the safety and efficacy of AS in treating malaria and cancer using well-defined dosing protocols. However, higher doses warrant cautious consideration due to potential cytotoxicity. Future investigations could incorporate comprehensive dose-response assessments and explore the pharmacokinetic and pharmacodynamic profiles of AS *in vivo* to delineate its therapeutic range and ensure its safe application across different cell types and disease models.

Our findings indicate that AS significantly activates the SIRT1/FOXO3a/MnSOD pathway in Dox-stimulated H9C2 cells, suggesting a novel mechanism by which AS exerts protective effects in HF. This aligns with previous studies demonstrating the beneficial effects of SIRT1 activation in reducing oxidative stress and improving mitochondrial function in HF models.

Despite these promising results, our study has several limitations. Firstly, experiments were conducted *in vitro* using H9C2 cardiomyocytes, which may not fully replicate the complexity of HF observed *in vivo*. Secondly, the precise molecular mechanisms through which AS activates the SIRT1/FOXO3a/MnSOD pathway require further elucidation.

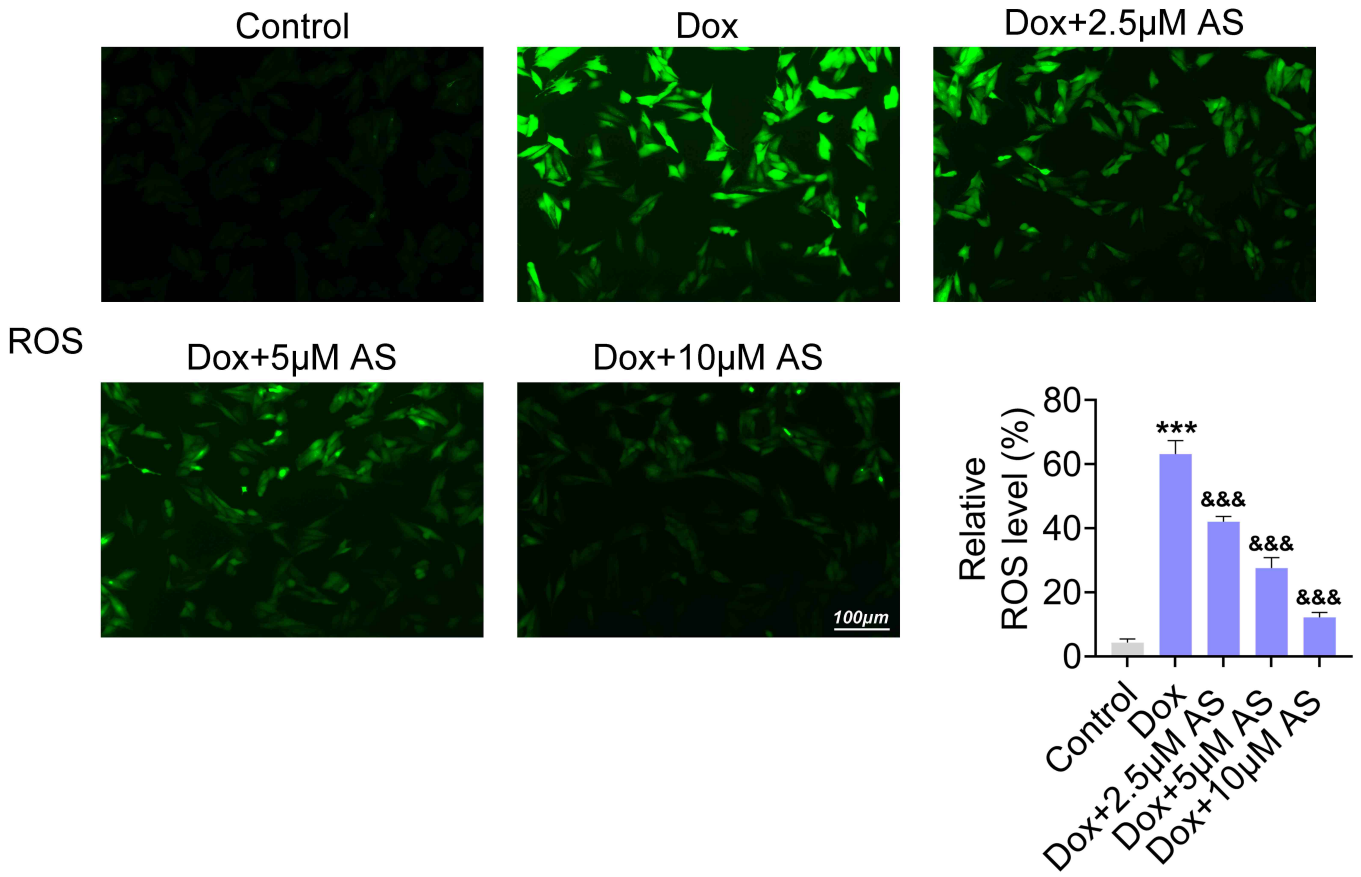

FIGURE 2. AS reduces ROS production in Dox-stimulated H9C2 cells. DCFH-DA fluorescence indicating ROS levels in H9C2 cells treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). The green panel represents DCFH-DA fluorescence. Scale bar, 100 μm. ROS: reactive oxygen species; Dox: Doxorubicin hydrochloride; AS: Artesunate. ***$p < 0.001$ compared to the control group; &&&$p < 0.001$ compared to Dox group.

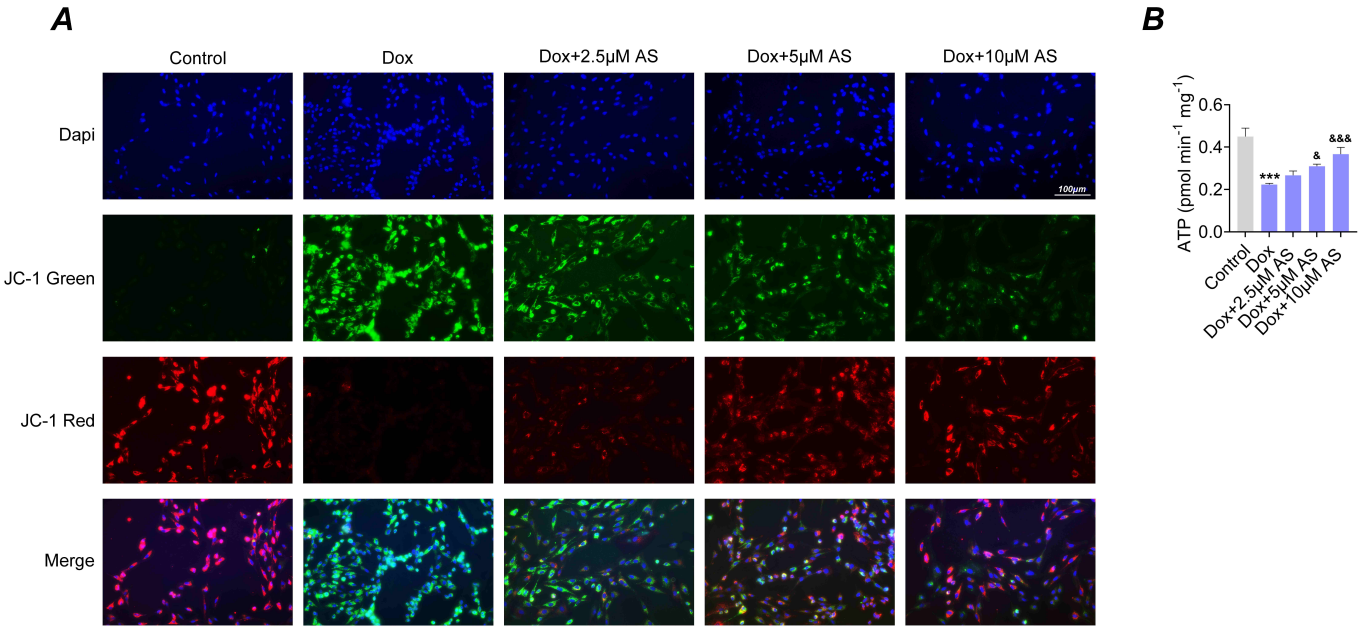

FIGURE 3. AS alleviates mitochondrial damage in Dox-stimulated H9C2 cells. (A) JC-1 staining showing mitochondrial damage in H9C2 cells treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). The green panel indicates JC-1 monomers and the red panel indicates aggregates. Scale bar, 100 μm. (B) ATP production in H9C2 cells treated with Dox (1 μM) alone and with AS (2.5, 5, 10 μM). ***$p < 0.001$ compared to the control group; &$p < 0.05$, &&&$p < 0.001$ compared to Dox group. Dox: Doxorubicin hydrochloride; AS: Artesunate; ATP: Adenosine triphosphate.

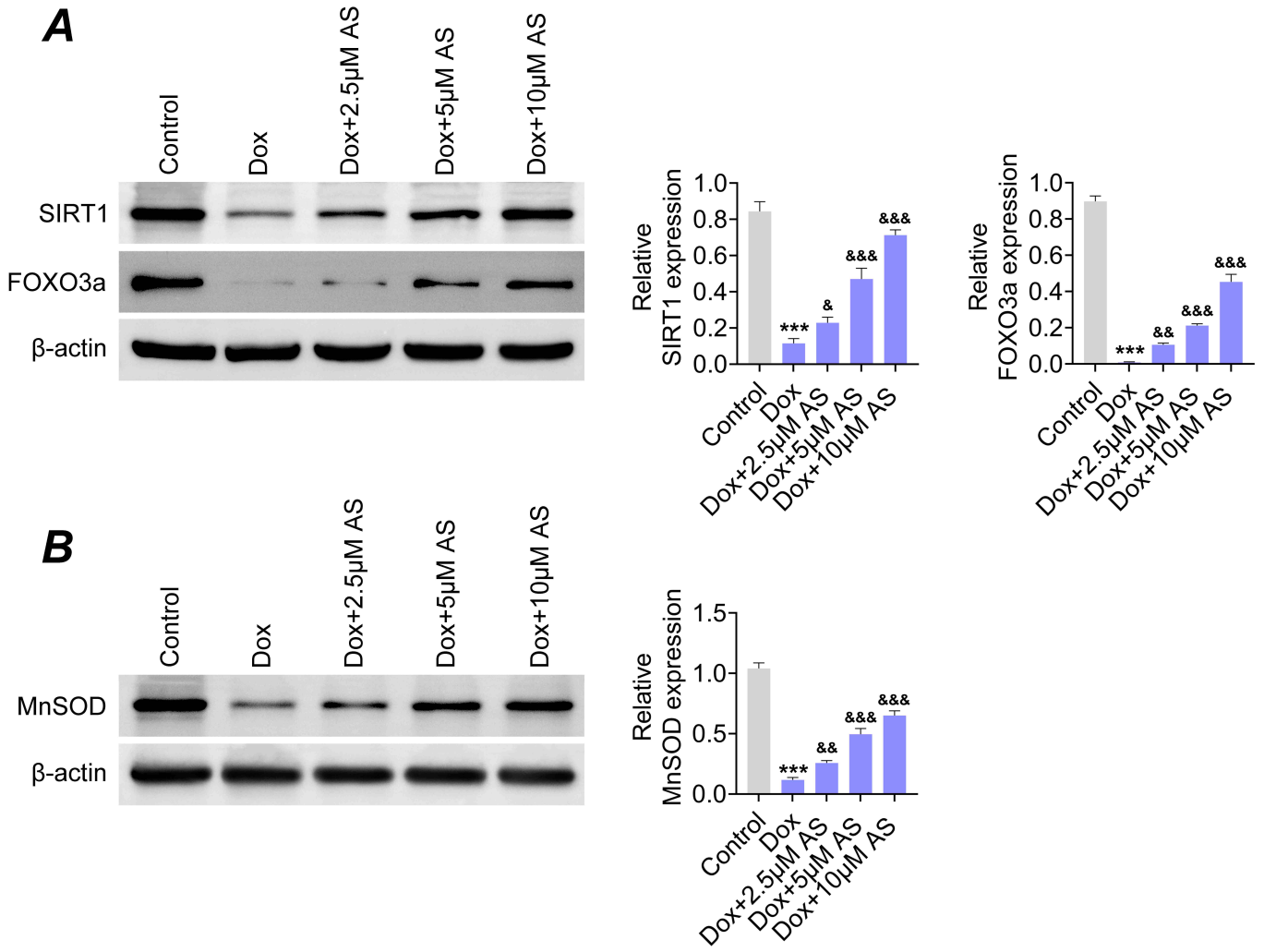

FIGURE 4. AS regulates the SIRT1/FOXO3a/MnSOD pathway in H9C2 cells. (A) Immunoblot assays showing SIRT1 and FOXO3a expression in H9C2 cells treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). (B) Immunoblot assays showing MnSOD expression in H9C2 cells treated with Dox (1 μM) alone and in combination with different concentrations of AS (2.5, 5, 10 μM). ***$p < 0.001$ compared to the control group; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ compared to Dox group. SIRT1: Silent information regulator 1; Dox: Doxorubicin hydrochloride; AS: Artesunate; FOXO3a: Forkhead box O3a; MnSOD: Manganese superoxide dismutase.

Additionally, long-term studies are necessary to assess the therapeutic potential and safety of AS in animal models and clinical trials.

While our study primarily focuses on the acute effects of AS on H9C2 cardiomyocytes, it is important to consider the implications for chronic conditions such as HF. The observed reductions in ROS production and mitigation of mitochondrial damage suggest that AS may protect cardiomyocytes from oxidative stress and mitochondrial dysfunction, which are both essential factors associated with HF progression. To comprehensively understand AS's therapeutic potential, future research could explore its long-term effects in chronic HF models, including sustained administration and continuous evaluation of cardiac function over extended periods.

This study provides new insights into how AS reduces ROS production and alleviates mitochondrial damage in cardiomyocytes under oxidative stress. Previous research has highlighted AS's antioxidative properties in various cellular models, such as its ability to mitigate oxidative stress and inflammation in myocardial ischemia-reperfusion injury [19] and protect against doxorubicin-induced cardiotoxicity *in vivo* [19]. However, the specific mechanisms through which AS achieves these protective effects in cardiomyocytes remained unclear. In this regard, our findings contribute to this understanding by revealing the involvement of the SIRT1/FOXO3a/MnSOD pathway in mediating AS's antioxidative effects. These results underscore AS's potential as a protective agent against oxidative stress-induced damage in cardiomyocytes, warranting further investigation in more complex and clinically relevant models.

While our study demonstrates AS's potential to reduce ROS production and enhance mitochondrial integrity in doxorubicin-treated H9C2 cells, we acknowledge the need to isolate these effects. Future studies could employ Analysis of Covariance (ANCOVA) or multivariate linear regression analysis to assess how AS influences mitochondrial integrity independently of its effects on ROS levels.

5. Conclusions

In conclusion, AS promotes cardiomyocyte viability, reduces ROS production and alleviates mitochondrial damage by activating the SIRT1/FOXO3a/MnSOD pathway, suggesting its potential as a promising therapeutic agent for HF, targeting oxidative stress and mitochondrial dysfunction. Further research is necessary to validate these effects *in vivo* and explore the clinical potential of AS in HF treatment.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YPL, PH—designed the study and carried them out, supervised the data collection, analyzed the data; prepare the manuscript for publication and reviewed the draft of the manuscript. YPL—interpreted the data. Both authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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