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AP39 ameliorates doxorubicin-induced cardiotoxicity by regulating the AMPK/UCP2 pathway

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Order of Authors:	Bin Zhang
	Yangxue Li
	Ning Liu
	Bin Liu, M.D.
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AP39 ameliorates doxorubicin-induced cardiotoxicity by regulating the AMPK/UCP2 pathway

First author:Bin Zhang

Corresponding author:Bin Liu

Authors:Bin Zhang[a],Yangxue Li[a],Ning Liu[a],Bin Liu[a,*]

a The Second Hospital of Jilin University,Changchun, Jilin,130000, China.

*Corresponding author at:No. 4026 Yatai Street, Nanguan District, Changchun City, Jilin Province, China.The Second Hospital of Jilin University.E-mail address:liubin3333@vip.sina.com

Abstract

Doxorubicin (DOX) is a broad-spectrum, highly effective antitumor agent, however, its cardiotoxicity has greatly limited its use.Hydrogen sulfide (H_2S) is an endogenous gaseous transmitter that exerts cardioprotective effects via the regulation of oxidative stress and apoptosis and maintenance of mitochondrial function, among other mechanisms.AP39 is a novel mitochondria-targeted H_2S donor that, at appropriate concentrations, attenuates intracellular oxidative stress damage, maintains mitochondrial function, and ameliorates cardiomyocyte injury.In this study, DOX-induced cardiotoxicity models were established with H9c2 cells and Sprague–Dawley rats to evaluate the protective effect of AP39 and its mechanisms of action.Both in vivo and in vitro experiments showed that DOX induces oxidative stress injury, apoptosis, and mitochondrial damage in cardiomyocytes and decreases the expression of p-AMPK/AMPK and UCP2.All DOX-induced changes were attenuated by AP39 treatment. Furthermore,the protective effect of AP39 was significantly

attenuated by the inhibition of AMPK and UCP2. The results suggest that AP39 ameliorates DOX-induced cardiotoxicity by regulating the expression of AMPK/UCP2.

Key words:AP39,Doxorubicin,Cardiotoxicity,AMPK,UCP2

1 Introduction

Doxorubicin (DOX), a broad-spectrum anthracycline antineoplastic drug, is widely used for the treatment of leukemia, breast cancer, ovarian cancer, lymphoma, and osteosarcoma (1).However, its severe dose-dependent cardiotoxicity (2)affects the quality of life of patients with cancer and can even shorten life expectancy.There is evidence that DOX exerts cardiotoxicity via oxidative stress, apoptosis, inflammation, and fibrosis.Furthermore,due to its cationic nature,DOX readily binds to mitochondrial intramembranous membranes and forms an irreversible complex with cardiac phospholipid proteins, leading to cardiotoxicity by inducing mitochondrial damage in cardiomyocytes (3, 4).The only drug currently approved by the FDA for the treatment of DOX cardiotoxicity is dexrazoxane, which still has various side effects, including myelotoxicity in patients with soft-tissue sarcoma (5). Therefore, there is an urgent need to identify safe and effective drugs to improve DOX cardiotoxicity.

DOX-induced cardiotoxicity is related to adenosine

monophosphate-activated protein kinase (AMPK) (6). AMPK is an important regulator of cellular energy homeostasis and mitochondrial homeostasis. The activation of AMPK modulates cellular metabolism, autophagy, apoptosis, and fibrosis (7). Uncoupling protein 2 (UCP2) is located within the inner mitochondrial membrane and affects mitochondrial function and metabolism through oxidative phosphorylation uncoupling. AMPK attenuates oxidative stress damage, reduces apoptosis (8), attenuates mitochondrial damage (9), and attenuates inflammatory responses (10) by upregulating UCP2.

Hydrogen sulfide (H_2S), a gaseous signaling molecule, exerts a potent protective effect on the cardiovascular system by regulating oxidative stress, apoptosis, autophagy, inflammation, mitochondrial function, neovascularization, and fibrosis at reasonable concentrations and has been shown to function via AMPK(11, 12).AP39 is a novel mitochondria-targeted H_2S donor that attenuates intracellular oxidative stress at appropriate concentrations while maintaining cell viability, mitochondrial respiration, and mitochondrial DNA integrity (13, 14).AP39 prevents myocardial ischemia-reperfusion injury independently of the cytoplasmic RISK pathway (15),it also inhibits mitochondrial autophagy, antagonizes cardiomyocyte iron death, and ameliorates myocardial fibrosis in rats with myocardial infarction via the PINK1/Parkin pathway (16).

Based on its effects on oxidative stress, apoptosis, and mitochondrial processes, we hypothesized that the exogenous H_2S donor AP39 may

attenuate DOX-induced cardiotoxicity. The aim of this study was to assess whether AP39 exerts a protective effect against DOX-induced cardiotoxicity and to investigate its mechanism of action,including its effects on the mitochondrial pathway and AMPK/UCP2.

2. Materials and methods

2.1 Reagents and antibodies

DOX (S1208) was purchased from Selleck (Houston, TX, USA), AP39(HY-126124) was purchased from MCE , Compound C(CC; 171260) and genipin (G4796)were purchased from Sigma–Aldrich (St. Louis, MO, USA).Primary antibodies for the following proteins were purchased from Cell Signaling Technology (Danvers, MA, USA):Caspase-3(9662, 1:1000),Cleaved Caspase-3 (9664, 1:1000),AMPKα (5831, 1:1000), p-AMPKα (Thr172) (50081, 1:1000), UCP2 (89326, 1:1000).Primary antibodies for Bax(A0207, 1:1000) and Bcl-2 (A19693, 1:1000) were purchased from ABclonal.Small interfering RNA against UCP2 (siUCP2) and its negative control (NC) were synthesized by IBSBIO.Lipofectamine 2000 was purchased from Invitrogen(Waltham, MA, USA).Annexin V-FITC and SYTOX Red were purchased from Thermo Fisher Scientific(Waltham, MA, USA).The Cell Counting Kit-8(CCK-8) and BCA Protein Assay Kit were purchased from Bioss.

Additionally,2′,7′-dichlorofluorescein diacetate (DCFH-DA) was purchased from Bestbio.The Mitochondrial Membrane Potential Assay Kit with JC-1 and

H2S Content Assay Kit were purchased from Solarbio (Beijing, China).The ATP assay kit was purchased from Nanjing Jiancheng Bioengineering Institute.The Superoxide Dismutase (SOD) Activity Assay Kit,Glutathione Peroxidase (GPX) Activity Assay Kit,Malondialdehyde (MDA) Content Assay Kit,Coenzyme II NADP (H) Content Assay Kit, and Lactate Dehydrogenase (LDH) Activity Assay Kit were all purchased from Beijing Boxbio Science & Technology Co., Ltd. (Beijing, China). The Rat Troponin T Type 2, Cardiac (TNNT2) ELISA Kit, Rat Creatine Kinase MB Isoenzyme (CKMB) ELISA Kit, and Rat Brain Natriuretic Peptide (BNP) ELISA Kit were all purchased from Jianglai Biology (Shanghai, China).

2.2 Animals and treatment

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Committee for the Protection and Utilization of Animals of Jilin University(2023 No. 463).

Male 8 to 10-week-old SPF Sprague–Dawley rats, weighing 300–320 g, were purchased from Yeast Laboratory Animal Technology.The rats were housed at the Animal Center of Jilin University, with the room temperature controlled at 21 ± 1°C, humidity controlled at 50-60%, 12h day/night cycle, free access to water and food, and acclimatization for 1 week. They were then randomly assigned to groups (10 rats per group) and administered treatments according to different protocols.The specific dosing regimens were as follows: (1) Con (equal amounts of 0.9% NaCl + DMSO); (2) DOX(5mg/kg once a

week,ip, cumulative dose 15mg/kg); (3) AP39(50nmol/kg every other day,ip); (4) DOX + AP39; (5) DOX + AP39 + CC(20mg/kg/d for 1 week,ip); (6) DOX + AP39 + Genipin(20mg/kg/d for 3 days,ip).The above doses were based on previous study reports (17,18,19,20) and experimental data.

Weighing was performed every 3 days during the experiment, and the rats' mental status, activity status, and any pain or discomfort were also paid attention to and recorded. The duration of this experiment was 21 days, and no rats died before euthanasia. 21 days later, cardiac ultrasound was performed after isoflurane anesthesia was given, and then euthanasia was given by CO_2 inhalation method (a total of 60 rats). The above experiments were supervised and directed by the Institutional Committee for the Protection and Utilization of Animals of Jilin University.

2.3 Cell culture and treatments

The rat H9c2 cell line was purchased from Beijing Zhongke QC Biotechnology Co. (Beijing, China). DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin was used for cell culture in an incubator at 37°C and a CO_2 concentration of 5%.Different drugs were given to stimulate the cells for 24h according to the experimental protocol including DOX (1 μmol/L), AP39 (100 nmol/L), and CC (10 μmol/L) (21). To reduce UCP2 expression in vitro, cells were transfected with siUCP2 (50 nmol/L) using the transfection reagent Lipofectamine2000 for 48h, and the effectiveness of transfection was evaluated by qPCR and western blotting.

2.4 Cell activity assay

H9c2 cells were inoculated in 96-well plates (4×10^3/well) and incubated with different concentrations of DOX (0, 0.5, 1, and 2 μmol/L) and AP39 (0, 30, 50, 100, 300, and 500 nmol/L), with a final volume of 100μL in each well.After 24 h, 10 μL of CCK-8 reagent was added to each well, the cells were incubated in the cell incubator for 60 min, and absorbance was measured at 450 nm.

2.5 Detection of ROS

H9c2 cells were inoculated in 6-well plates (5×10^4/well), and different stimuli were applied when cells reached approximately 70% confluence. Cells were incubated for 24h in a cell culture incubator. The DCFH-DA probe was diluted with serum-free DMEM at a ratio of 1:1000 and added to the 6-well plates at 1 mL/well, followed by incubation 37°C in the dark for 20 min. Cells were washed gently with phosphate-buffered saline and images were obtained under a fluorescence microscope. The average fluorescence intensity was evaluated using ImageJ.

2.6 Flow cytometry

H9c2 cells were resuspended under different conditions and diluted with 1× Binding Buffer to a concentration of 1×10^6 cells/mL. Then, 100 μL of the cell suspension was used for flow cytometry; briefly, 5 μL of Annexin V-FITC and 5 μL of SYTOX Red were added, samples were incubated at room temperature (25°C) in the dark for 15 min, 400 μL of 1× Binding Buffer was added, and samples were assayed immediately using the flow

cytometer(Cytoflex,Beckman).

2.7 Western blotting

Total protein was extracted from cell samples and cardiac tissues using RIPA buffer, and the protein concentration was determined using a BCA Kit. Equal concentrations of protein samples were separated by 10% SDS-PAGE and then transferred to PVDF membranes, which were blocked with 5% skim milk powder at room temperature for 60 min. The primary antibody was incubated overnight at 4°C, followed by incubation with the secondary antibody at room temperature for 1 h. Chemiluminescent color development was performed by adding the developing solution.

2.8 Mitochondrial membrane potential assay

Mitochondrial membrane potential was assayed using the JC-1 probe according to the manufacturer's instructions. When the mitochondrial membrane potential was high, JC-1 aggregated in the mitochondrial matrix and formed a polymer, producing red fluorescence; when the mitochondrial membrane potential was low, JC-1 did not aggregate in the mitochondrial matrix, and the monomers produced green fluorescence. Images were obtained using a fluorescence microscope, and the fluorescence intensity was analyzed using ImageJ. The ratio of red to green fluorescence was used to measure the change in mitochondrial membrane potential.

2.9 Quantitative real-time PCR

Total RNA was extracted with TransZol, and reverse transcription and

qRT-PCR were performed according to the instructions provided with the relevant kits. *GAPDH* was selected as the internal reference gene, and the relative expression was calculated by the $2^{-\Delta\Delta CT}$ method.

2.10 Oxidative stress and ATP assays

According to the manufacturer's instructions, oxidative stress levels were measured using SOD, GSH-Px, MDA and NADPH kits, and cellular ATP levels were measured using ATP kits.Absorbance values were measured at different wavelengths using an enzyme meter and analyzed according to the standard curves and corresponding formulas.

2.11 ELISA

Cardiomyocyte injury was assessed using ELISA kits for TNNT2, CK-MB, and BNP in rat serum according to the manufacturer's instructions.

2.12 Transmission electron microscopy

Different groups of rat myocardial specimens and different drug-stimulated H9c2 cells were fixed with 2.5% glutaraldehyde phosphate and stained with1% phosphotungstic acid. The mitochondrial ultrastructure was observed and analyzed by using a JEM-1400 microscope at a magnification of 5000×, 8000×, and 25000×.

2.13 HE and Masson staining

Rat myocardial tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 3-μm-thick wax slices. The sections were stained with hematoxylin and eosin (HE), Masson Lichtenstein acidic reagent, and toluidine

blue and observed under a light microscope.

2.14 Statistical analyses

All statistical analyses were performed using GraphPad Prism 9.0. Datas are expressed as the mean ± standard deviation (SD). Comparisons between two groups were performed using Student's *t*-test, comparisons among multiple groups were performed using one-way ANOVA and post hoc Tukey's tests. Values of $p < 0.05$ were statistically significant . All data used in statistical analyses were obtained from three or more independent repeated experiments.

3 Results

3.1 DOX induces H9c2 cell damage

H9c2 cells were stimulated with various concentrations of DOX (0, 0.5, 1, or 2 μmol/L) for 24 h for CCK-8 detection.Exposure to 1 μmol/L DOX for 24 h decreased H9c2 cell viability by approximately 50% (compared with that in the control group), and the DOX-induced decrease in cell viability was dose-dependent. We stimulated H9c2 cells with 1 μmol/L DOX for different durations (0, 6, 12, 24, and 48 h). A CCK-8 assay showed that cell viability decreased by about 50% at 24 h. Therefore, we stimulated H9c2 cells with 1 μmol/L DOX for 24 h for subsequent experiments (Fig. 1 A, B).

Free radical production is the main cause of cardiomyocyte damage by DOX, and cardiotoxicity occurs progressively with ROS production and lipid

peroxidation (22). As determined using the DCFH-DA probe, DOX increased ROS levels in cardiomyocytes (Fig. 1 C), resulting in decreased SOD and GSH-Px activity and increased MDA and NADPH levels (Fig. 1 D), suggesting that DOX causes oxidative stress injury in cardiomyocytes. Flow cytometry revealed that the apoptosis rate was significantly higher($p < 0.01$) in the DOX group than in the Con group (Fig. 1 E), suggesting that DOX caused apoptosis in H9c2 cells.

Figure1.DOX induces H9c2 cell damage.

3.2 DOX induces mitochondrial damage in H9c2 cells

Previous studies have shown that DOX can lead to cardiomyocyte apoptosis via endogenous pathways (23), particularly the mitochondrial pathway. Furthermore, DOX can lead to mitochondrial damage (4). In this study, DOX increased the expression levels of the apoptosis-related protein Bax, decreased expression levels of Bcl-2, and increased expression levels of Cleaved Caspase-3/Caspase-3 (Fig. 2 A), indicating that DOX promotes apoptosis in cardiomyocytes and its mechanism of action involves mitochondria. We further evaluated mitochondrial membrane potential and ATP levels, revealing that DOX could lead to a decrease in mitochondrial membrane potential and ATP levels in cardiomyocytes (Fig. 2 B, C), while mitochondrial damage (mitochondrial structural disorganization, fragmentation, and cristae rupture) was observed by transmission electron microscopy (Fig. 2

D).

We performed AMPK and UCP2 assays. Western blotting showed that DOX resulted in decreased levels of p-AMPK/AMPK and UCP2 in cardiomyocytes (Fig. 2 E), suggesting that the damage to cardiomyocytes caused by DOX may be related to AMPK/UCP2.

Figure2.DOX induces mitochondrial damage in H9c2 cells.

3.3 AP39 ameliorates DOX-induced myocardial injury

AP39 has a concentration-dependent effect on mitochondrial activity. At low concentrations (30–100 nmol/L), AP39 stimulates mitochondrial electron transport and cellular bioenergetic functions, and at high concentrations (300 nmol/L), it has an inhibitory effect on mitochondrial activity (14). Therefore, we first stimulated H9c2 cells with different concentrations of AP39 (0, 30, 50, 100, 300, and 500 nmol/L) for 24 h and performed CCK-8 assays. The results were in accordance with those of previous reports indicating that AP39 at lower concentrations (30–100 nmol/L) does not significantly reduce cell viability. A decrease in cell viability was detected at 300 nmol/L, and a significant decrease in cell viability was detected at 500 nmol/L. Subsequently, we co-stimulated H9c2 cells with 1 μmol/L DOX and different concentrations of AP39 for 24 h. The CCK-8 results showed that the improvement of cell viability was statistically significant at AP39 concentrations of 50 nmol/L and 100 nmol/L, and the improvement was particularly obvious at an AP39

concentration of 100 nmol/L. In summary, we chose 100 nmol/L AP39 for subsequent experiments (Fig. 3 A–C).

We measured the intracellular H_2S content under different conditions, demonstrating that DOX stimulation decreases the H_2S content in H9c2 cells, and this decrease was attenuated by the exogenous administration of AP39 (Fig. 3 D). In addition, AP39 significantly ameliorated DOX-induced oxidative stress injury in H9c2 cells, with a significant decrease in intracellular ROS levels (Fig. 3 E), improvements in SOD and GSH-Px activity, and decreases in MDA and NADPH levels after co-treatment with AP39 compared with corresponding levels in the DOX group (Fig. 3 F). AP39 ameliorated DOX-induced cardiomyocyte apoptosis, which was significantly lower in the DOX+AP39 group than in the DOX group (Fig. 3 G).

Figure3.AP39 ameliorates DOX-induced myocardial injury.

3.4 AP39 ameliorates DOX-induced mitochondrial damage

We further investigated the mechanisms by which AP39 exerted protective effects against DOX-induced myocardial injury. Western blotting showed that AP39 decreased the expression of the apoptosis-related proteins Bax and Cleaved Caspase-3/Caspase-3 and increased the expression of Bcl-2 (Fig. 4 A). Additionally, AP39 attenuated the DOX-induced decrease in mitochondrial membrane potential and ATP levels in cardiomyocytes (Fig. 4 B, C). Transmission electron microscopy revealed that mitochondrial damage (i.e.,

289 the disorganization of mitochondrial structure, fragmentation, and cristae
290 breakage) was attenuated by AP39 (Fig. 4 D).
291 As determined by western blotting, cardiomyocyte p-AMPK/AMPK and
292 UCP2 levels were elevated after co-treatment with AP39 and DOX than after
293 DOX stimulation alone (Fig.4 E), suggesting that the beneficial effect of AP39
294 on DOX cardiotoxicity may be related to AMPK/UCP2.
295 *Figure4.AP39 ameliorates DOX-induced mitochondrial damage.*
296
297 3.5 Inhibition of AMPK expression limits the beneficial effect of AP39 on DOX
298 cardiotoxicity
299 To verify whether the beneficial effect of AP39 on DOX cardiotoxicity was
300 related to AMPK, we inhibited AMPK using the AMPK inhibitor Compound C
301 (CC) and demonstrated the effectiveness of CC by western blotting (Fig. 5 A).
302 As determined by a CCK-8 assay, CC did not influence cell viability (Fig. 5 B).
303 ROS levels were significantly higher in the DOX+AP39+CC group than in the
304 DOX+AP39 group (Fig. 5 C). SOD and GSH-Px activities were lower and MDA
305 and NADPH levels were higher in the DOX+AP39+CC group than in the
306 DOX+AP39 group (Fig. 5 D), suggesting that the inhibition of AMPK
307 expression limited the beneficial effect of AP39 on DOX-induced oxidative
308 stress injury in cardiomyocytes. The apoptosis rate was higher in the
309 DOX+AP39+CC group than in the DOX+AP39 group (Fig. 5 E). Western
310 blotting showed that the expression levels of Bax and Cleaved

Caspase-3/Caspase-3 were higher and expression levels of Bcl-2 were lower in DOX+AP39+CC group than in the DOX+AP39 group (Fig. 5 F), suggesting that the inhibition of AMPK limited the effect of AP39 on DOX-induced apoptosis in cardiomyocytes. Furthermore, the beneficial effects of AP39 on both mitochondrial membrane potential and ATP levels in cardiomyocytes were weakened by the inhibition of AMPK expression (Fig. 5 G, H). These results suggest that AP39 ameliorates DOX-induced cardiotoxicity by regulating AMPK expression.

We further evaluated the regulatory relationship between AMPK and UCP2. Although the down-regulation of UCP2 by DOX was improved by co-treatment with AP39, the expression level of UCP2 in the DOX+AP39+CC group was still significantly lower than that in the DOX+AP39 group (Fig. 5 I), indicating that the inhibition of AMPK expression suppressed the up-regulation of UCP2 by AP39. These findings suggest that UCP2 may function downstream of AMPK in the regulation of DOX cardiotoxicity by AP39.

Figure5.Inhibition of AMPK expression limits the beneficial effect of AP39 on DOX cardiotoxicity.

3.6 AP39 improves DOX-induced cardiotoxicity by preventing the down-regulation of UCP2

To clarify whether the beneficial effect of AP39 on DOX cardiotoxicity was achieved by modulating the expression of UCP2, we inhibited the expression

of UCP2 using small interfering RNA and confirmed the effectiveness of transfection by qPCR and western blotting (Fig. 6 A). CCK-8 results showed that cell viability did not differ significantly in the NC and siUCP2 groups compared with Con group(Fig. 6 B). Oxidative stress damage, apoptosis, and mitochondrial damage were not significantly improved in the DOX+AP39+siUCP2 group compared with those in the DOX+AP39 group. In particular, ROS levels were high (Fig. 6 C), SOD and GSH-Px levels were low, and MDA and NADPH levels were high (Fig. 6 D). The apoptosis rate remained high, and western blotting showed that Bax and Cleaved Caspase-3/Caspase-3 levels were high and Bcl-2 levels were low (Fig. 6 E, F). Mitochondrial membrane potential and ATP levels remained low (Fig. 6 G, H). The above results suggested that the inhibition of UCP2 inhibited the beneficial effect of AP39 on DOX cardiotoxicity, suggesting that UCP2 mediates the effects of AP39.

We hypothesized that UCP2 acts downstream of AMPK. To verify this, we further evaluated the levels of p-AMPK/AMPK. DOX decreased the expression of p-AMPK/AMPK. AP39 upregulated p-AMPK/AMPK, and the inhibition of UCP2 did not influence the effect of AP39 (Fig. 6 I). We have previously confirmed that the inhibition of AMPK could affect the expression of UCP2. These findings further demonstrated that UCP2 functions downstream of AMPK.

Collectively, these findings demonstrated that AP39 ameliorates

DOX-induced oxidative stress damage, apoptosis, and mitochondrial damage in H9c2 cells by regulating the expression of AMPK/UCP2.

Figure6.AP39 improves DOX-induced cardiotoxicity by preventing the down-regulation of UCP2.

3.7 AP39 attenuates DOX-induced cardiotoxicity in rats by regulating the AMPK/UCP2 pathway

To further validate our experimental results, we conducted in vivo experiments with rats. DOX administration resulted in a significant decrease in body weight and an elevated heart/body weight ratio in rats over those in the control group (Fig. 7 A, B). Cardiac ultrasound showed a significant decrease in EF%, FS%, and E/A, suggesting that there was a significant decline in cardiac function (Fig. 7 C). The levels of TNNT2, CK-MB, LDH, and BNP were significantly increased in abdominal aorta blood after DOX administration (Fig. 7 D), indicating obvious myocardial damage. HE staining of the rat myocardium was observed under an optical microscope; myocardial cells in the DOX group were deformed, broken, and dissolved, with edema, an enlarged myocardial interstitial space, unevenly colored myocardial fibers, and inflammatory cell infiltration. Masson staining showed a disrupted arrangement of cardiomyocytes, obvious increase in blue collagen fibers in the interstitium of the myocardium, and obvious myocardial fibrosis in the DOX group (Fig. 7 E). Mitochondrial swelling, structural disorder, fragmentation, ridge breakage,

and vacuole-like degeneration of cardiomyocytes in the DOX group were observed by transmission electron microscopy (Fig. 7 F). We also tested indexes of serum oxidative stress in rats. SOD and GSH-Px activities were lower and MDA and NADPH levels were higher in the DOX group than in the control group (Fig. 7 G). Western blotting showed that DOX increased the expression of the apoptosis-related protein Bax, decreased the expression of Bcl-2, increased the expression of Cleaved Caspase-3/Caspase-3, and decreased expression levels of p-AMPK/AMPK and UCP2 (Fig. 7H). In the DOX+AP39 group, the toxic effects of DOX were ameliorated to varying degrees, consistent with the results of our in vitro experiments. We also confirmed the mechanism by which AP39 improves DOX cardiotoxicity in vivo by administering the AMPK inhibitor CC and UCP2 inhibitor genipin.Cardiomyocyte injury, oxidative stress injury, mitochondrial injury, and apoptosis, as described above, did not differ significantly in the DOX+AP39+CC and DOX+AP39+Genipin groups, also consistent with results of in vitro experiments. These findings suggest that the beneficial effect of AP39 on DOX-induced cardiotoxicity in rats is achieved by modulating AMPK/UCP2 expression.

Figure7.AP39 attenuates DOX-induced cardiotoxicity in rats by regulating the AMPK/UCP2 pathway.

4 Discussion

DOX is a broad-spectrum and highly effective antitumor drug commonly used in the treatment of different types of tumors. It can significantly improve the survival rate of patients with cancer. However, its severe cardiotoxicity greatly limits its application. Therefore, there is an urgent need to find drugs that can reduce the cardiotoxicity of DOX. In this study, both in vivo and in vitro experiments demonstrated that the exogenous mitochondria-targeted H_2S donor AP39 could attenuate DOX-induced cardiotoxicity by ameliorating oxidative stress, apoptosis, and mitochondrial damage. Mechanistically, we found that AP39 exerts its protective effects by activating the expression of AMPK/UCP2, and inhibitors of AMPK and UCP2 can attenuate or even eliminate the beneficial effect of AP39.These results clearly indicate that AP39 is promising for the prevention or treatment of DOX cardiotoxicity.

Increasing focus on DOX cardiotoxicity has led to extensive research. Studies have shown that DOX decreases levels of SOD, CAT, and GSH-Px and increases levels of MDA in the rat heart, and the amelioration of oxidative stress injury can ameliorate cardiotoxicity (24, 25), consistent with our findings. In our experiments, DOX induce ROS production in H9c2 cardiomyocytes, decreased SOD and GSH-Px activity in cardiomyocytes and rat serum, and increased MDA and NADPH levels, indicating that it induces oxidative stress in cardiomyocytes. DOX can induce cardiomyocyte apoptosis through both endogenous and exogenous pathways (23). For example, DOX can induce apoptosis and pyroptosis via the Akt/mTOR signaling pathway (26), heat shock

proteins (HSP-10, HSP-20, HSP-22, HSP-27, and HSP60), and lipocalin, and it is possible to reduce the cardiotoxicity of DOX by promoting antiapoptotic activity (27, 28), as demonstrated in our experiments. In particular, we found that DOX can significantly increase the apoptosis rate of H9c2 cells, up-regulate Bax and Cleaved Caspase-3/Caspase-3, and down-regulate Bcl-2, suggesting that DOX can induce endogenous apoptosis via the mitochondrial pathway. Compared with other cell types, cardiomyocytes have more mitochondria, and DOX mainly acts on cardiomyocyte mitochondria, interfering with mitochondrial electron transport and leading to the formation of superoxide (O2-) free radicals (29). DOX induces mitochondrial DNA (mtDNA) mutations and defects along with elevated ROS in mitochondria, and these changes have been implicated in the development of cardiomyopathy (30). DOX can also induce excessive opening of mitochondrial permeability transition pore (31) and affect mitochondrial KATP channel activity (32), thus leading to myocardial injury. In our experiments, DOX decreased mitochondrial membrane potential and ATP levels in cardiomyocytes. Mitochondrial structure disorganization, fragmentation, and cristae rupture were observed. These in vivo and in vitro experiments clearly show that DOX causes the structural damage and dysfunction of mitochondria in cardiomyocytes.

Hydrogen sulfide (H_2S), initially described as a toxic gas with a rotten egg odor, is similar in nature to nitric oxide (NO) and carbon monoxide (CO), an endogenous gaseous signaling molecule in mammals. Increasing studies have

443 shown that it is involved in a variety of pathophysiological processes, such as
444 oxidative stress, inflammation, apoptosis, and angiogenesis; additionally, it
445 plays a protective role in the pathogenesis and progression of cardiovascular
446 diseases (33). H_2S reduces lipid peroxidation by hydrogen peroxide and
447 superoxide scavenging in a model of isoprenaline-induced myocardial injury
448 (34). H_2S-mediated activation of Nrf2-dependent pathways leads to the
449 upregulation of genes involved in endogenous antioxidant defense (35). It
450 protects mitochondrial function by inhibiting respiration, thereby limiting ROS
451 production and reducing mitochondrial uncoupling (36). Furthermore, H_2S
452 significantly prevents high glucose-induced apoptosis in cardiomyocytes by
453 modulating the expression of Bax and Bcl-2 (37). AP39, a novel
454 mitochondria-targeted H_2S donor, can ameliorate high-fat-diet-induced liver
455 injury in young rats by attenuating oxidative stress and mitochondrial damage
456 (38). It can support cellular bioenergetics and prevent Alzheimer's disease by
457 maintaining mitochondrial function in APP/PS1 mice and neurons (18). It can
458 prevent 6-hydroxydopamine-induced mitochondrial dysfunction (39). In this
459 study, both in vivo and in vitro experiments confirmed that exogenous
460 mitochondrial targeting of AP39 ameliorates DOX-induced oxidative stress by
461 decreasing cardiomyocyte ROS levels, elevating SOD and GSH-Px contents,
462 and decreasing MDA and NADPH levels; it improved cardiomyocyte apoptosis
463 by regulating the expression of apoptosis-related proteins, such as Bax, Bcl-2,
464 and Cleaved Caspase-3/ Caspase-3, and improved DOX-induced

mitochondrial injury by elevating mitochondrial membrane potential and ATP levels, consistent with results of previous studies on the mechanisms underlying the myocardial protective effects of H_2S or AP39.

Cardiac tissues have high metabolic energy requirements, and growing evidence suggests that AMPK plays a key role as an energy sensor and a major regulator of metabolism in regulating cell survival in vivo and in vitro (40). In 2005, Tokarska-Schlattner et al. were the first to demonstrate that AMPK inactivation plays an important role in DOX cardiotoxicity (41). Since then, additional studies have shown that AMPK is closely related to multiple molecular mechanisms underlying DOX-induced cardiomyocyte injury. DOX is able to inhibit the expression and phosphorylation of AMPK proteins in the rat heart via DNA damage-induced Akt signaling, which activates a negative feedback loop of mTOR signaling and leads to cardiac remodeling (42). DOX can lead to myocardial fibrosis and cardiomyocyte apoptosis in APN-SE mice by inhibiting AMPK expression (43). Some AMPK activators, such as metformin, statins, resveratrol, and thiazolidinediones, have the potential to prevent DOX cardiotoxicity (44). Located within the mitochondrial membrane, UCP2 acts as an anion carrier and regulates the transmembrane proton electrochemical gradient in many human tissues; it is involved in a number of processes, including mitochondrial membrane potential, ROS production within the mitochondrial membrane, and calcium homeostasis (45). UCP2 is involved in the reduction of ROS production and mitochondrial ROS

scavenging (46) and can protect cardiomyocytes from oxidative stress by inhibiting ROS production (47). UCP2 prevents neuronal apoptosis and attenuates brain dysfunction after stroke and traumatic brain injury (48). UCP2 protects the heart from I/R injury by inducing mitochondrial autophagy (49). Studies on the interaction between AMPK and UCP2 have yielded conflicting results. It has been suggested that UCP2 affects the autophagic process in septic cardiomyopathy via AMPK signaling (50) an regulates cholangiocarcinoma cell plasticity via mitochondrial-AMPK signaling (51). However, there is substantial evidence that AMPK functions upstream of UCP2. For example, in a model of nonalcoholic fatty liver disease, LB100 regulated UCP2 expression by inhibiting AMPK (21). Malvidin alleviates mitochondrial dysfunction and ROS accumulation by activating the AMPK-α/UCP2 axis, thereby preventing inflammation and apoptosis in SAE mice (10). Indole sulfate induces oxidative stress and hypertrophy in cardiomyocytes by inhibiting the AMPK/UCP2 signaling pathway (52). In our experiments, we found that the protective effect of AP39 against DOX cardiotoxicity was mediated by AMPK/UCP2, and the use of AMPK inhibitors affected the expression of UCP2, while the inhibition of UCP2 expression did not have a significant effect on the expression level of AMPK. These findings suggest that AMPK is an upstream signal of UCP2 and regulates the expression of UCP2. The differences in the regulatory relationship between AMPK and UCP2 among studies may be related to differences in disease models, stimuli, and

other factors. The present study clarifies the role of the AMPK regulation of UCP2 in the attenuation of DOX cardiotoxicity by AP39; however, the specific mechanism underlying these regulatory effects is not clear. A downstream pathway of AMPK is Sirt1/PGC-1α, and AMPK activates the NAD+-dependent type III deacetylase Sirt1 by increasing the intracellular NAD+/NADH ratio; Sirt1 activation leads to peroxisome proliferation-activated receptor-γ coactivator 1α (PGC-1α) deacetylation and activity regulation, and Sirt1/PGC-1α may be involved in the regulation of UCP2 (8). Accordingly, the roles of Sirt1/PGC-1α need to be studied further. Despite these limitations, our experimental results provide possible therapeutic strategies for DOX cardiotoxicity and support the beneficial effects of AP39.

5 Conclusions

Taken together, our findings suggest that AP39 ameliorates DOX cardiotoxicity by attenuating oxidative stress, apoptosis, and mitochondrial damage via the modulation of AMPK/UCP2 expression. These findings indicate that AP39 is a promising new therapeutic agent for preventing DOX-induced cardiotoxicity.

CRediT authorship contribution statement

Bin Zhang: Conceptualization,Methodology,Validation,Formal analysis,Writing - Original Draft;Yangxue Li:Formal analysis,Writing - Review & Editing;Ning Liu: Resources,Writing - Review & Editing,Project administration;Bin Liu*:

Resources,Supervision,Project administration,Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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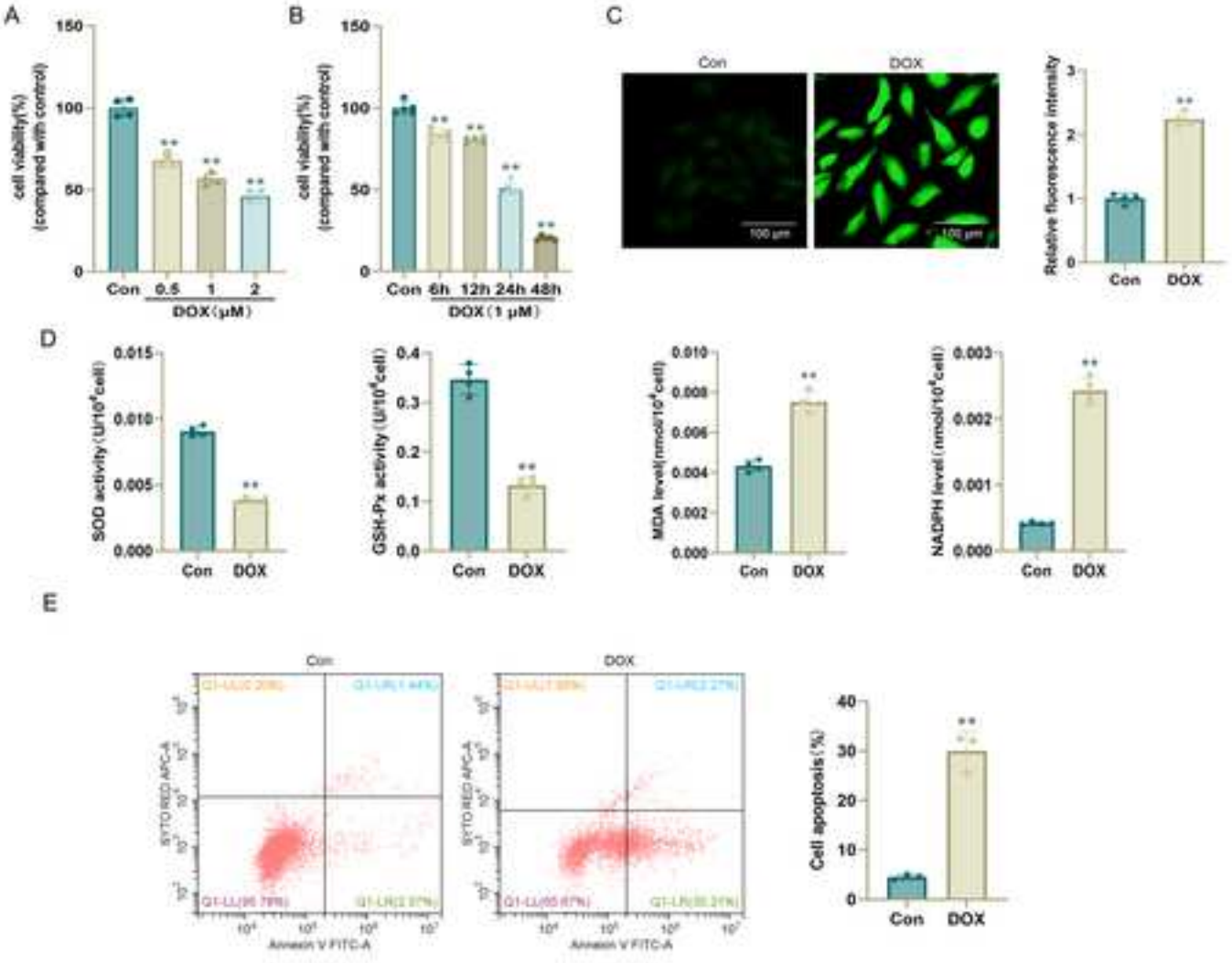

Figure 1 DOX induces H9c2 cell damage. (A) and (B) Cell viability determined by CCK-8 assays after treatment with DOX at different concentrations for 24 h and treatment with 1 μmol/L DOX for different times (n = 4 or 5); (C) Representative DCFH-DA images and statistical results (n = 4); (D) SOD, GSH-Px, MDA, and NADPH levels in H9c2 cells (n = 4); (E) Apoptosis rate measured by flow cytometry (n = 3).Values represent the mean ± SD. *p < 0.05 vs. Con group, **p < 0.01 vs. Con group.

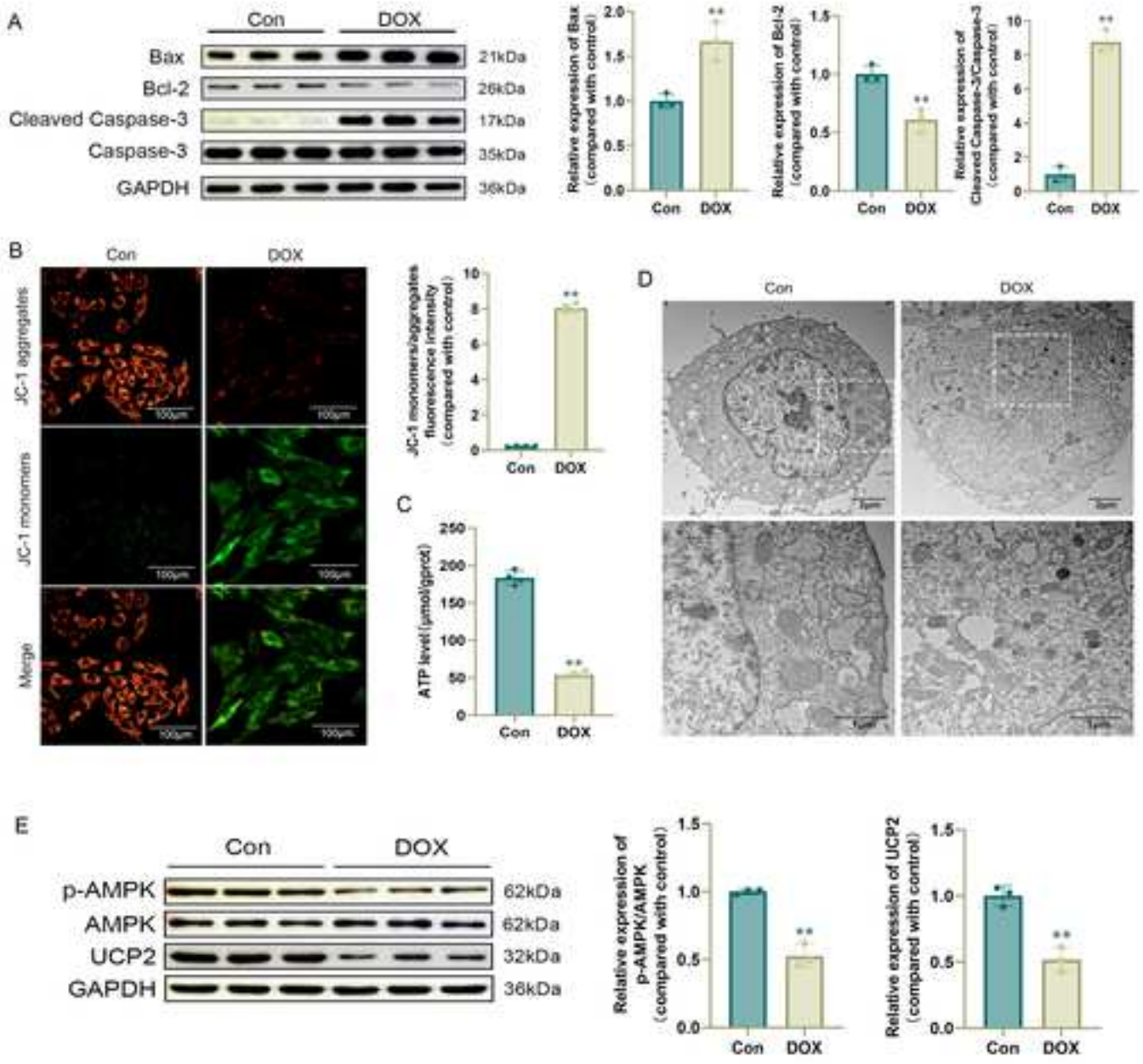

Figure 2 DOX induces mitochondrial damage in H9c2 cells. (A) Western blot detection of apoptosis-related protein levels and statistical results (n = 3); (B) Representative JC-1 images and quantification of fluorescence intensity for JC-1 monomers/aggregates (n = 4); (C) ATP level (n = 4); (D) Representative images of mitochondria in H9c2 cells observed by transmission electron microscopy; (E) Western blot detection of p-AMPK, AMPK, and UCP2 levels and statistical results (n = 3). Values are presented as the mean ± SD. *p < 0.05 vs. Con group, **p < 0.01 vs. Con group.

Figure3

Click here to access/download;Figure;FIG3.TIF.tif

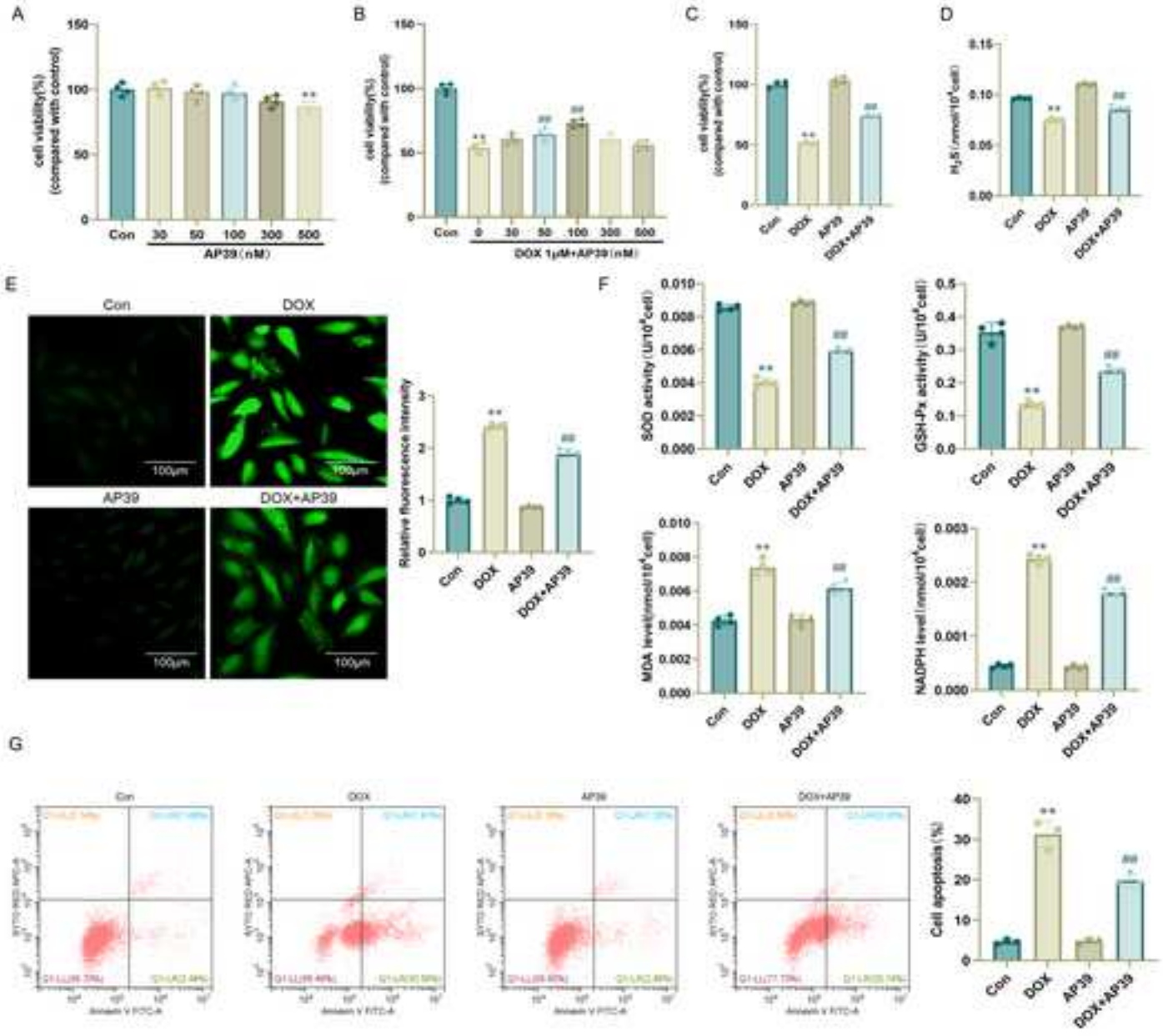

Figure 3 AP39 ameliorates DOX-induced myocardial injury. (A)-(C) Cell viability determined by CCK-8 assays after H9c2 cells were treated with different concentrations of AP39 for 24 h, 1 μmol/L DOX, and different concentrations of AP39 for 24 h (n = 4); (D) H2S contents (n = 4); (E) Representative DCFH-DA images and statistical results (n = 4); (F) SOD, GSH-Px, MDA, and NADPH levels in H9c2 cells (n = 4); (G) Apoptosis rate measured by flow cytometry (n = 3). Values are presented as the mean ± SD. *p < 0.05 vs. Con group, **p < 0.01 vs. Con group. #p < 0.05 vs. DOX group, ##p < 0.01 vs. DOX group.

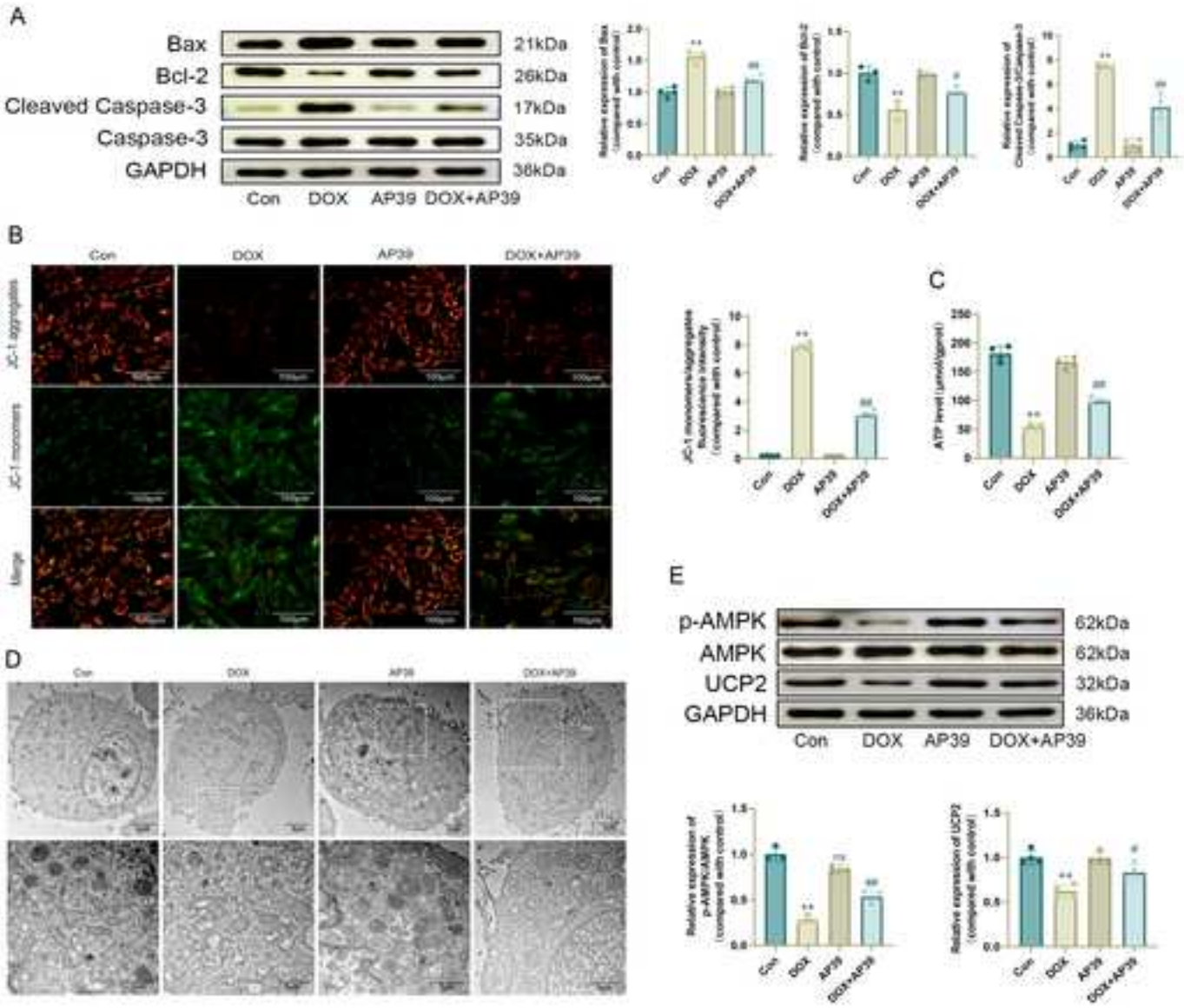

Figure 4 AP39 ameliorates DOX-induced mitochondrial damage. (A) Western blot detection of apoptosis-related protein levels and statistical results (n = 3 or 4); (B) Representative JC-1 images and quantification of fluorescence intensity for JC-1 monomers/aggregates (n = 4); (C) ATP level (n = 4); (D) Representative images of mitochondria in H9c2 cells observed by transmission electron microscopy; (E) Western blot detection of p-AMPK, AMPK, and UCP2 levels and statistical results (n = 3 or 4). Values are presented as the mean ± SD. *p < 0.05 vs. Con group, **p < 0.01 vs. Con group. #p < 0.05 vs. DOX group, ##p < 0.01 vs. DOX group. NS indicates no significant difference vs. Con group.

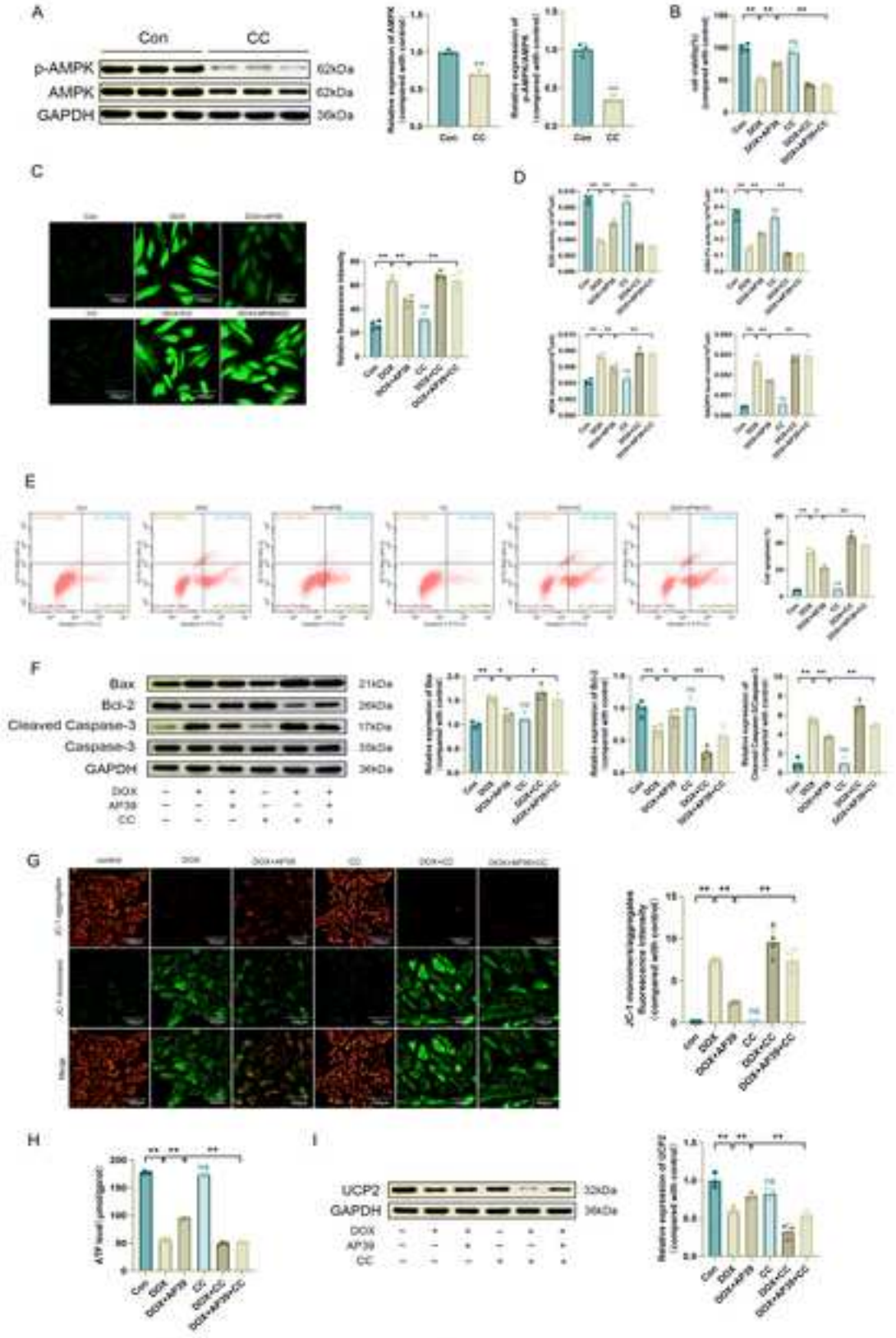

Figure 5 Inhibition of AMPK expression limits the beneficial effect of AP39 on DOX cardiotoxicity. (A) Western blot detection of p-AMPK and AMPK levels and statistical results (n = 3); (B) CCK-8 assay of cell viability (n = 4); (C) Representative DCFH-DA images and statistical results (n = 4); (D) SOD, GSH-Px, MDA, and NADPH levels in H9c2 cells (n = 4); (E) Apoptosis rate measured by flow cytometry (n = 3); (F) Western blot detection of apoptosis-related protein levels and statistical results (n = 3 or 4); (G) Representative JC-1 images and quantification of fluorescence intensity for JC-1 monomers/aggregates (n = 4); (H) ATP level (n = 4); (I) Western blot detection of UCP2 levels and statistical results (n = 3). Values are presented as the mean ± SD. *p < 0.05, **p < 0.01. NS indicates no significant difference vs. Con group.

Figure6

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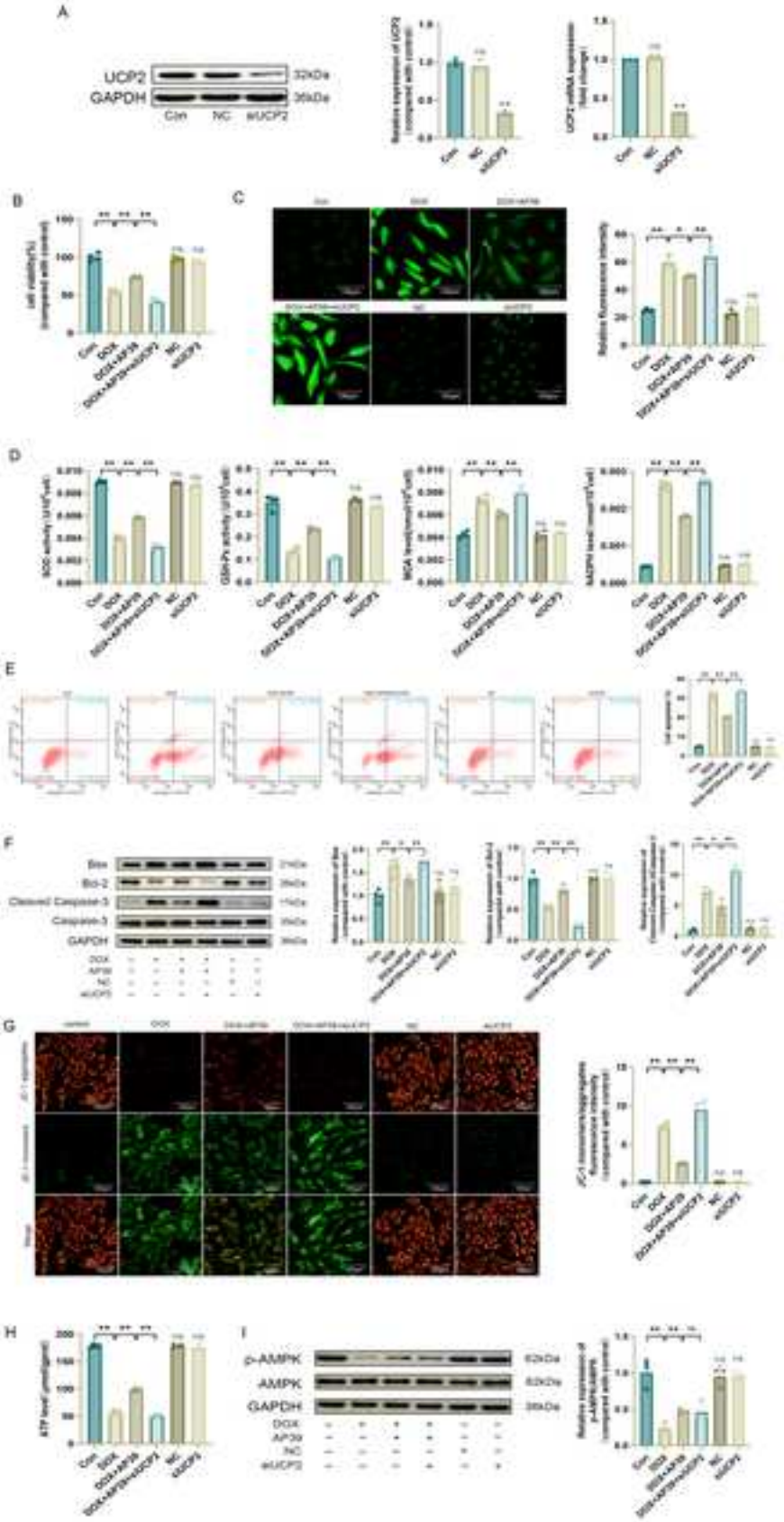

Figure 6 AP39 improves DOX-induced cardiotoxicity by preventing UCP2 down-regulation. (A) Western blot analysis of UCP2 (n = 4) and PCR for UCP2 mRNA levels (n = 3). (B) CCK-8 assay of cell viability (n = 4). (C) Representative DCFH-DA images and statistical results (n = 4). (D) SOD, GSH-Px, MDA, and NADPH levels in H9c2 cells (n = 4). (E) Apoptosis rate measured by flow cytometry (n = 3). (F) Western blot detection of apoptosis-related protein levels and statistical results (n = 3 or 4). (G) Representative JC-1 images and quantification of fluorescence intensity for JC-1 monomers/aggregates (n = 4). (H) ATP levels (n = 4). (I) Western blot detection of p-AMPK and AMPK levels and statistical results (n = 3). Values are presented as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. NS indicates no significant difference vs. Con group.

Figure7

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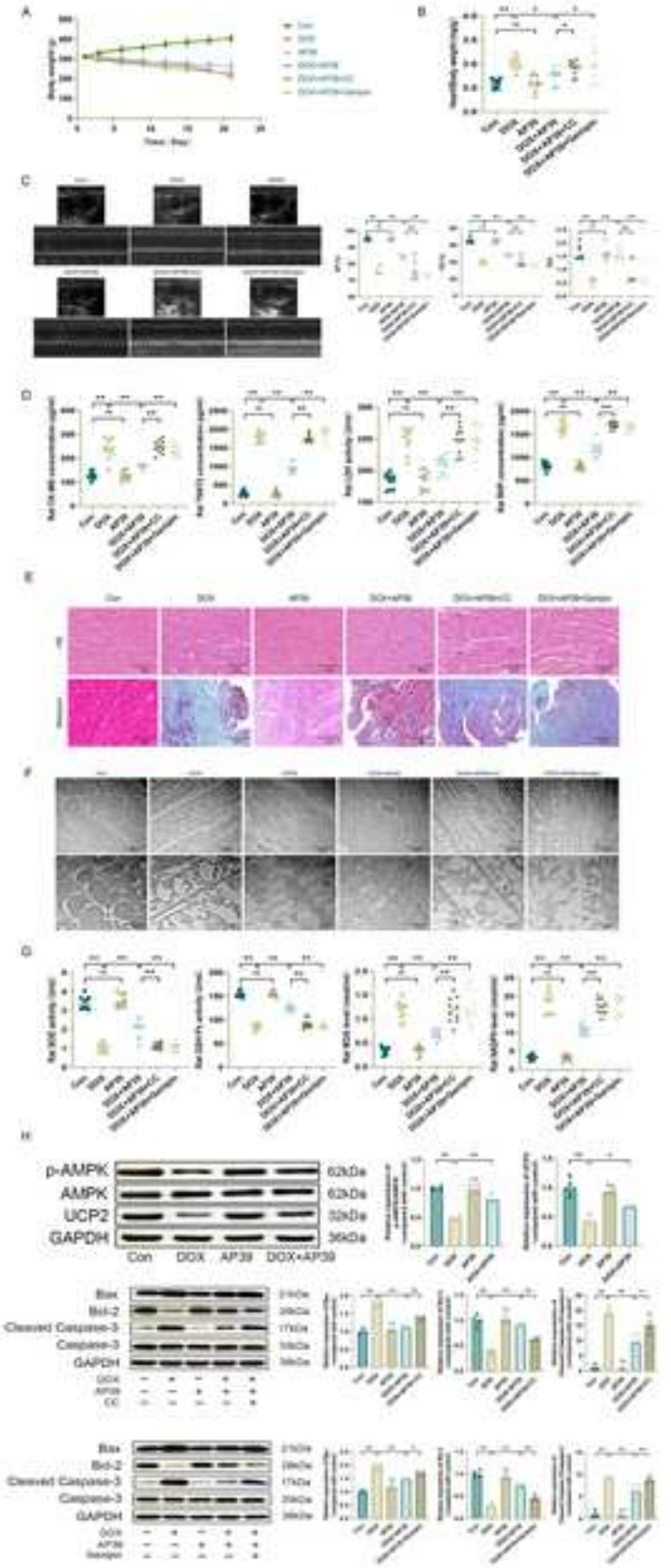

Figure 7 AP39 ameliorates DOX-induced cardiotoxicity in rats by regulating the AMPK/UCP2 pathway (A) Body weight in different groups of rats (n = 10). (B) Heart/body weight ratio in different groups of rats (n = 10). (C) Representative echocardiographic images and quantitative analysis of EF%, FS%, and E/A (n = 7). (D) Serum TNNT2, CK-MB, LDH, and BNP levels in rats (n = 10). (E) Representative HE and Masson staining images of the rat myocardium. (F) Representative images of mitochondria in rat cardiomyocytes observed by transmission electron microscopy. (G) SOD, GSH-Px, MDA, and NADPH levels in rat serum (n = 10). (H) Western blot detection of apoptosis-related protein, p-AMPK, AMPK and UCP2 levels and statistical results (n = 3 or 4). Values are presented as the mean ± SD. *p < 0.05, **p < 0.01. NS indicates no significant difference vs. Con group.