

The apelin-13 peptide protects the heart against apoptosis through the ERK/MAPK and PI3K/AKT signaling pathways.

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Research article

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Abstract

Background: It has been acknowledged that endocrine activity is associated with the function of multiple systems in vivo. The apelin-13 peptide has been demonstrated to play a crucial role in various physiological and pathological processes. However, whether apelin-13 peptide function in DOX induced cardiotoxicity is unknown.

Methods: We explored the function and mechanism of the apelin-13 peptide in apoptosis and oxidative stress by CCK-8, trypan blue staining, TUNEL, LDH, JC-1 and western blot in vitro. Then we verified the effect of apelin-13 in vivo by serum CKMB and LDH, echocardiography, sirius red staining and HE staining assay.

Results: Treatment with the apelin-13 peptide significantly enhanced cell viability, mitochondrial membrane potential, and reduced LDH release, rate of apoptosis and activation of caspase-3 in vitro. In mice, the apelin-13 peptide alleviated the heart failure induced by DOX treatment. ML-221 inhibited the activation of ERK, PI3K and AKT proteins phosphorylation by apelin-13.

Conclusion: The apelin-13 and APJ interaction on the cell membrane inhibits apoptosis through the ERK/MAPK and PI3K/AKT signaling pathways. The application of apelin-13 may be a novel therapeutic strategy in oxidative stress-induced heart failure therapy.

Introduction

Doxorubicin (DOX) has been identified as one of the most common treatment for tumor[1]. Chemotherapeutics could lead to severe side effect, most common of which is cardiotoxicity[2]. Mounting evidences manifested that long-term use of DOX could lead to arrhythmias and even heart failure[3]. Because of its cardiac toxicity, the clinical application is severely limited. For decades, cardiotoxicity caused by anthracyclines through oxidative stress injury is one of the most acceptable theories at present[4]. However, the biomolecular mechanism of DOX in cardiomyocytes needs further to be elucidated.

Oxidative stress is a pathological process that causes oxidative injury to organs and tissues. When cells are stimulated, the accumulation of oxidizing substances, such as free radicals, causes damage to organelles, leading to cell death[5]. Recent studies have demonstrated that apoptosis and ROS are associated with cellular damage incurred during oxidative stress[6]. Oxygen free radicals[7] resulting from oxidative stress can be prevented, and targeting regulated cell death pathways before oxidative stress manifestation can alleviate oxidative impairment and potentially pave the way for new therapeutic plans for use in oxidative stress injury[8]

It is well known that peptides produced by proteasome degradation play important protective roles in various diseases and act as endogenous ligands or receptors to mediate varieties of signaling pathways[9]. For example, endorphins are endogenous peptides produced by the body that have similar

effects as morphine and analgesics[10]. Cecropins play important roles in the antibacterial activity of C-terminal amides[11]. Angiotensin II binds to angiotensin receptors in the heart and kidney to regulate water and salt balance and blood pressure[12]. Furthermore, the levels of natriuretic peptide family members are measured in the diagnosis of heart failure[13]. These peptides play indispensable roles in regulating physiological function and repairing human diseases. Previous studies have found that the human gene AK092578 encodes a 54-amino acid hormone with a signal peptide[14]. The 54 aa hormone is cleaved into the mature 32 aa form, which is named ELABELA (ELA, also known as toddler or apela[15]. A small molecular peptide encoded by a gene and cut by a proteasome, preproapelin is composed of 77 amino acid residues and is decomposed to produce endogenous fragments such as apelin-36, apelin-13 and apelin-17[16]. Different subtypes of apelin are involved in different activities in different metabolic pathways. The receptor for the apelin receptor early endogenous ligand (APELA) and apelin (APLN) hormones is named APLNR or APJ and is coupled to G proteins that inhibit adenylate cyclase activity[17]. Studies have reported that the receptor for the APELA hormone, APJ [18], plays a pivotal role in early development.

Researches have demonstrated that ELA and its active fragment, apelin-13, has a potency in lower blood pressure and modulate heart development[19]. Given the protective effect of apelin-13 in cardiovascular system, we investigated its role in cardiotoxicity. Meanwhile, the biomolecular mechanism of DOX induced cardiotoxicity still has not been clarified. Our study suggested that apelin-13 bestowed cardioprotective effects against DOX-induced cardiotoxicity by inhibiting apoptosis and oxidative stress through ERK/MAPK and PI3K/AKT signaling pathway. CoCl₂ can also elicited cardiac complication by inducing oxidative stress and apoptosis. We also provide evidence that apelin-13 binds to APJ to attenuate apoptosis and oxidative stress. In summary, our results shed light on the role of apelin-13 during the molecular regulation of apoptosis and oxidative stress and provide a novel interventional treatment for cardiovascular disease.

Materials And Methods

2.1 Reagents and antibodies

Doxorubicin and cobalt chloride were purchased from MedChemExpress (New Jersey, America) and Sigma, respectively. Primary antibodies for the following proteins were purchased from cell signaling technology (Danvers, MA): actin (1:5000), total-PARP (1:1000), cleaved-PARP (1:1000), total caspase-3 (1:1000), cleaved-caspase-3 (1:1000), BCL-2-associated X protein (BAX, 1:1000), anti-B-cell lymphoma 2 (BCL-2, 1:1000), total ERK1/2 (1:1000), phosphorylated ERK1/2 (1:1000), total PI3K (1:1000), phosphorylated PI3K (1:1000), total AKT (1:1000), phosphorylated AKT (1:1000). In addition, anti-rabbit IgG and HRP-linked antibody were purchased from Biosharp Life Science (Beijing, China). Mitochondrial membrane potential assay kits with JC-1 and a LDH cytotoxicity assay kit, relative oxygen species assay kit, Cell Counting Kit-8, Trypan blue staining cell viability assay kit and one-step TdT-mediated dUTP nick

end-labeling apoptosis assay kit were obtained from Beyotime Biotechnology (Shanghai, China). ML-221 was purchased from MedChemExpress (New Jersey, United States).

2.2 Peptide Synthesis And Administration

Apelin-13 CMPLHSRVFPF peptide was synthesized with purity > 95% by Shanghai Science Peptide Biological Technology Co. LTD. (Shanghai, China). The peptide powder was dissolved in sterile water to generate a 10 μ M stock solution and was diluted to the experimental concentration.

2.3 Cell Culture

Rat primary cardiomyocytes were extracted from rats one-half day after birth. The blood, fat and connective tissues and heart tissue sections were separated and digested with trypsin at 37 °C at 60 rpm for 15 min. The solution was removed, and the digestion was repeated three times. The cell-containing digestive fluids were placed in a centrifuge tube and centrifuged together after passing through 180 mesh. Cells were suspended in 10 ml DMEM containing 10% horse serum (HS, GIBCO, USA) and incubated in a 10 cm² dish for 1.5 h. The cell suspension was removed, and the cells ($5 \sim 6 \times 10^5$ cells per dish) were inoculated into a new dish, as previously described.

2.4 Animals

Male C57BL/6J mice (6–10 weeks of age, 20 ~ 22 g) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China), and all procedures were followed in accordance with the ethical committee of Nanjing Medical University. All animals were raised at 20 ~ 25 °C and in 50 ~ 70% relative humidity. The experimental mice were randomly divided into four groups. The mice were treated with 5 mg/kg DOX or (and) 1.5 μ mol/kg DOX administered into the abdominal cavity for five consecutive weeks; electrocardiograms were obtained and the mice were sacrificed. The mice were treated according to the experimental requirements. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 85 – 23, revised 1996).

2.5 Cell Death Rates

Trypan blue staining was used to calculate the mortality of primary cells. The cells were collected at different times (0, 6, 12, 18, 24 or 36 h) and stained with the dye from a Trypan blue staining cell viability assay kit to determine the cell death rate. At different concentrations of DOX (0.1, 0.5, 1, 2 and 5 μ M) and CoCl₂ (200, 400, 600, 800 and 1000 μ M) and measurements were taken according to the manufacturer's instructions.

2.6 Lactate Dehydrogenase (Ldh) Level Detection

Levels of lactate dehydrogenase (LDH) released were detected in serum using an LDH release assay kit according to the manufacturer's protocol.

2.7 Jc-1 Assay

The mitochondrial membrane potential was measured by a mitochondrial membrane potential assay kit with JC-1 according to the manufacturer's instructions. The cells were cultured in serum-free DMEM containing (1×) JC-1 staining working fluid at 37 °C for 20 min. Then, washed twice with JC-1 buffer, added 2 ml DMEM, and the cells were photographed by a fluorescence microscope (BX61; Olympus Corporation, Tokyo, Japan). The JC-1 density was assessed by ImageJ software and calculated upon normalization to the control.

2.8 Reactive Oxygen Species (ros) Measurement

The levels of intracellular ROS were determined using a relative oxygen species assay kit following the instructions. Cells were incubated in serum-free DMEM containing 0.1% DCFH-DA at 37 °C for 20 min, washed with serum-free DMEM three times and photographed with a fluorescence microscope.

2.9 Tunel Staining Assay

The rate of apoptosis can be detected by TUNEL staining kit. Cells were seeded (1×10^5 cells per well) in 6-well dishes. After the treatments described above were performed, the cells were washed once with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Apoptotic cells were visualized with TUNEL staining according to the manufacturer's protocol. TUNEL fluorescence intensity/DAPI fluorescence density was used to calculate the percentage of positive cells, and the density was evaluated using ImageJ software 1.26 (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.10 Echocardiography

Male C57BL/6J mice were treated with echocardiography after administrated with DOX and apelin-13. These mice were detected their FS, EF and LVEDs respectively. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 85 - 23, revised 1996).

2.11 Western Blot Analysis

Proteins were isolated from cells using lysis buffer (containing RIPA and 1% PMSF). Protein quantification was performed using a BCA protein detection kit (23229; Thermo Fisher Scientific). Protein samples of the same mass were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA), blocked with 5% skim milk and then incubated with specific primary antibodies. A FluorChem M system was used to quantify the positive bands representing proteins involved in the orchestrated immune responses (ProteinSimple, San Jose, CA, USA).

2.12 Statistical Analysis

All results are expressed as the mean \pm SD. Student's t-tests were used to determine significant differences. Comparisons between groups were performed by one-way analysis of variance (ANOVA), and p values < 0.05 (*) and 0.01 (**) were considered significant. All experiments were repeated at least three times, unless otherwise specified. All data were analyzed by GraphPad Prism software.

Results

3.1 The bioinformation related to apelin.

Elabela is considered as a peptide hormone that is derived from the placenta of mammalian animals. The human Elabela gene is located on chromosome 4 GRCh 38.p12, which contains three exons. The precursor protein encoded by this gene composed of 54 amino acids (aa) peptide, which is cut by the Golgi apparatus to a mature form with 32 aa. Elabela protein contains two bi-arginine sequences that are recognized and cleaved into different sizes. Apelin, the first endogenous ligand of ELA, to be discovered expressed widely in human body. We found that apelin and its family are named according to the length of their sequence. (Fig. 1). Hence, the biological function and mechanism need to be clarified.

3.2 The effect of doxorubicin and cobalt chloride on cell viability and death.

To investigate the effect of doxorubicin (DOX) on rat primary cell viability, the proportion of viable cells was determined by trypan blue assay. The results indicated that DOX (0.1, 0.5, 1, 2, 5 μ M/L) affected cell death in dose-dependent manner and 1 μ M/L DOX significantly increased cell death rate (Fig. 2A). CCK-8 assay was performed to evaluate the cell viability of doxorubicin, and the 1 μ M DOX administration reduced cell survival index dramatically (Fig. 2B). To determine the optimal interaction time, cells were incubated for different times and evaluated using Trypan blue and CCK-8 assays. The exposure of doxorubicin for 12 h significantly increased the rate of cell death and decreased cell viability in rat primary cardiomyocytes (Fig. 2C and 2D). The oxidative stress induced by cobalt chloride (CoCl₂) was served as a complementary model. As shown in Fig. 2E, the cell viability was significantly decreased

upon 800uM CoCl₂ treatment. To determine the optimal working time points, the primary cells were incubated for 0, 6, 12, 18, 24, 30, and 36 h. Our results demonstrated that treatment with CoCl₂ for 24 h significantly decreased cell survival rate (Fig. 2F).

3.3 Apelin-13 inhibits apoptosis by attenuating doxorubicin-induced cardiotoxicity.

To figure out the biological function of apelin-13 in DOX induced cardiotoxicity in rat cardiomyocytes, pretreatment with apelin-13 at different concentrations (0.1, 0.5, 1, 2 and 5uM) for 1 h and 1uM apelin-13 dramatically reduced the mortality of DOX-treated primary cardiomyocytes (Fig. 3A). To verify the cardioprotective effect of apelin-13, we assessed cell viability and LDH release. Compared with that of the scramble group, apelin-13 significantly enhanced cell viability and reduced LDH release (Fig. 3B and 3C), which means apelin-13 could resist DOX induced cell damage. The western results showed that apelin-13 reduced DOX-induced cell death by inhibiting apoptosis (Fig. 3D). The cleaved caspase3 and PARP was alleviated in the apelin-13 group. Next, we found that the number of apoptotic cells increased in DOX induced group and apelin-13 reduced apoptotic cell rate significantly by TUNEL assay (Fig. 3E). Changes in mitochondrial membrane potential can also reflect the state of early cell apoptosis. The result indicated that apelin-13 reduced the number of JC-1 monomers formation compared with DOX treatment (Fig. 3F). Therefore, our results indicated that apelin-13 possess a protective role in DOX induced cardiotoxicity.

3.4 The effect of apelin-13 in CoCl₂ induced oxidative stress.

To test whether apelin-13 possessed function in oxidative stress, we performed comprehensive functional analysis. The results demonstrated that treatment of apelin-13 decreased cell death rates (Fig. 4A). Then, CCK-8 result suggested that apelin-13 increased cell viability compared with scramble peptide (Fig. 4B). The result of LDH release showed that apelin-13 significantly lowered LDH release (Fig. 4C). To investigate whether apelin-13 could inhibit CoCl₂ induced cell death, our result revealed that CoCl₂ increased apoptotic cell rates and apelin-13 alleviated cell apoptosis (Fig. 4D and 4E). The expression of cleaved-PARP and caspase-3 proteins was decreased in apelin-13 treated group (Fig. 4F). These findings suggested that apelin-13 reduced the apoptosis and cell damage induced by CoCl₂.

3.5 Apelin-13 significantly resists DOX-induced cardiotoxicity in vivo.

We next tested whether apelin-13 has a protective effect on rodent hearts in vivo. Male C57BL/6J mice were intraperitoneally injected with doxorubicin (5 mg/kg) for five weeks consecutively and apelin-13 was

injected daily *i.p.* Then they were subjected to echocardiography and sacrificed the following week (Fig. 5A). The release of CKMB and LDH in peripheral blood were decreased significantly in apelin-13 administrated group (Fig. 5B). The EF and FS rates of were increased, whereas the LVED values were decreased significantly by echocardiography when treated with apelin-13 (Fig. 5C). The evidence from Sirius red staining suggested that fibrosis was significantly reduced by apelin-13 (Fig. 5D). Accordingly, HE staining showed that apelin-13 treatment alleviated the collagenous fiber induced by DOX in heart tissues (Fig. 5E). Therefore, these results suggested that apelin-13 ameliorated the cardiotoxicity induced by DOX in mice.

3.6 Apelin-13 inhibits cardiotoxicity through activating ERK/MAPK and PI3K/AKT signaling pathways.

ERK/MAPK and PI3K/AKT are important pathways that protect the physiological function of the heart by inhibiting apoptosis. To verify the molecular mechanism by which apelin-13 protects primary cardiomyocytes against apoptosis, a western blot analysis was performed. The results showed the expression of the phosphorylated ERK protein was activated upon apelin-13 treatment compared with DOX treatment interference (Fig. 6A). To validate the role of apelin-13 in PI3K/AKT signaling pathways, we found that apelin-13 activated the expression of the phosphorylated AKT and PI3K protein (Fig. 6B). When the action of CoCl₂ was blunted, the expression of the phosphorylated ERK protein was upregulated (Fig. 6C). Meanwhile, apelin-13 activated the expression of the both phosphorylated proteins after CoCl₂ treatment in rat primary cells (Fig. 6D). These results revealed that apelin-13 protects cardiomyocytes from apoptosis through ERK/MAPK and PI3K/AKT signaling pathways.

3.7 Apelin-13 protects cardiac function by binding APJ.

To determine the role of ML-221, treatment with ML-221 to detect its interaction with apelin-13 by western blot. We found ML-221 inhibited the effect of apelin-13 on apoptosis of cardiomyocytes with DOX administration (Fig. 7A). To determine whether ML-221 was involved in ERK/MAPK and PI3K/AKT signaling pathways when treated with DOX, we found ML-221 significantly increased the expression of the phosphorylated AKT, PI3K and ERK protein compared with apelin-13 (Fig. 7B and 7C). Furthermore, we found ML-221 can also inhibit apoptosis in CoCl₂ induced apoptosis. The phosphorylated AKT, PI3K and ERK protein expressions were significantly upregulated after cell treatment with ML-221 (Fig. 7E and 7F). These results suggested that apelin-13 inhibits apoptosis by binding to APJ. In brief, we plotted the mechanism of apelin-13 according our research (Figure.S1) .

Discussion

Our current study revealed that apelin-13 has a protective effect on apoptosis and attenuates cardiotoxicity induced by DOX in vitro and in vivo. Furthermore, we demonstrated that apelin-13

attenuated apoptosis by targeting cardiomyocyte membranes via APJ. The apelin-13 peptide is regarded as a promising therapeutic strategy in its action toward ERK/MAPK and PI3K/AKT signaling pathway. Our findings provide evidence of the molecular mechanism by which the apelin-13 peptide inhibits apoptosis and oxidative stress, indicating its potential use for DOX-induced cardiotoxicity.

Because of cardiac toxicity caused by DOX, its clinical application is severely limited. However, the mechanism has not been illuminated. CoCl_2 is a classic and effective compound used to simulate hypoxic and ischemic processes[20]. Apelin-13 maintains activity comparable to the full-length Elabela peptide because of its integrated C-terminus[21]. Previous studies have revealed that the apelin system is an emerging pathway involved in various physiological functions[22]. The endogenous ligand of its orphan G protein-coupled receptor is named apelin. The N-terminus of apelin-13 is involved in regulating its binding to the receptor APJ, while the apelin-13 C-terminus is mainly involved in regulating its biological activity[23]. Further studies showed that the apelin-13 is more active than that of the longer apelin peptide, however the ability of the smaller one to bind to the receptor is limited. Apelin-13 is related to the formation of blood vessels, the expression of endothelial cells and the regulation of cardiac contractility and is found in peripheral tissues and the central system. In our study, we found that apelin-13 can bind with APJ to exert its protective effect on primary cells, which was rescued by the application of ML-221, a specific antagonist of APJ[24].

Studies have demonstrated that the endocrine system is indispensable during cardiovascular physiological and pathological processes. Many known bioactive substances secreted by the heart, including ANP, have been indicated to be involved in the regulation of cardiovascular disease and sensitive as indicators of disease monitoring[25]. In vivo, the increased heart rate and reduced collagen fiber formation to resist cardiotoxicity. In addition, endocrine-derived peptides play a role in different pathological processes and engage in organ cross talk. For instance, a vasoactive intestinal polypeptide of 28 amino acids is released by intestinal neurons[26]. Its level of variation is related to a variety of clinical diseases and plays different functions in different organs. It is worth observing that some long peptides can be cleaved into smaller peptides that have more consequential functions than those of longer peptides.

PI3K-AKT signaling pathway is a classical signaling pathway that regulates apoptosis[27]. PI (3, 4, 5) P3, acts as an intracellular second messenger in the cell that needs to transfer protein kinase B (AKT) to the membrane for activation[28]. Phosphorylated AKT mediates insulin and various growth factors to induce cell growth and promotes cell survival through numerous channels[29]. Apelin-13 ERK/MAPK is a classical signaling pathway of anti-apoptosis. When the downstream phosphorylated-ERK was activated, it could inhibit the process of apoptosis. In our study, the results supported that apelin-13 resist cardiotoxicity by inhibiting apoptosis and oxidative stress by activating PI3K, Akt and ERK phosphorylation.

Our study also has some limitations. First, more clinical samples are needed to determine the exact time window of its interactions for clinical application. Second, whether the modification of apelin-13

influences its function in cardiovascular diseases needs further evaluation.

Overall, we performed a full-scale functional analysis of apelin-13 in DOX-induced cardiotoxicity and found that it can inhibit cell apoptosis in vitro and in vivo. We explored the target of apelin-13 and found that it activates the ERK/MAPK and PI3K/AKT signaling pathways to inhibit apoptosis. Therefore, our study indicate apelin-13 as a novel treatment for DOX-induced cardiotoxicity.

Abbreviations

LDH: Lactate dehydrogenase

ROS: Relative oxygen species

DOX: Doxorubicin

CoCl₂: Cobalt chloride

ERK: Extracellular signal-related kinases

JNK: Jun amino-terminal kinases

PI3K: phosphatidylinositol 3 kinases

AKT: Protein kinase B

JC-1: Mitochondrial membrane potential assay kit with JC-1

Scr: Scramble

AA: amino acid

Declarations

Conflict of Interest

All authors read and approved the final manuscript. The authors declare no competing financial interests.

Data statement

This work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Lingmei Qian designed the research. XueJun Wang, Li Zhang and MengWen Feng performed the experiments. Hao Zhang and Jia Xu analysed the data. XueJun Wang and ZiJie Cheng wrote this manuscript. Lingmei Qian and ZiJie Cheng supervised this work.

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Figures



Figure 1

The bioinformation of apelin. Apelin-13 is a cleaved fragment of ELA (19–32) that contains 11 amino acids.

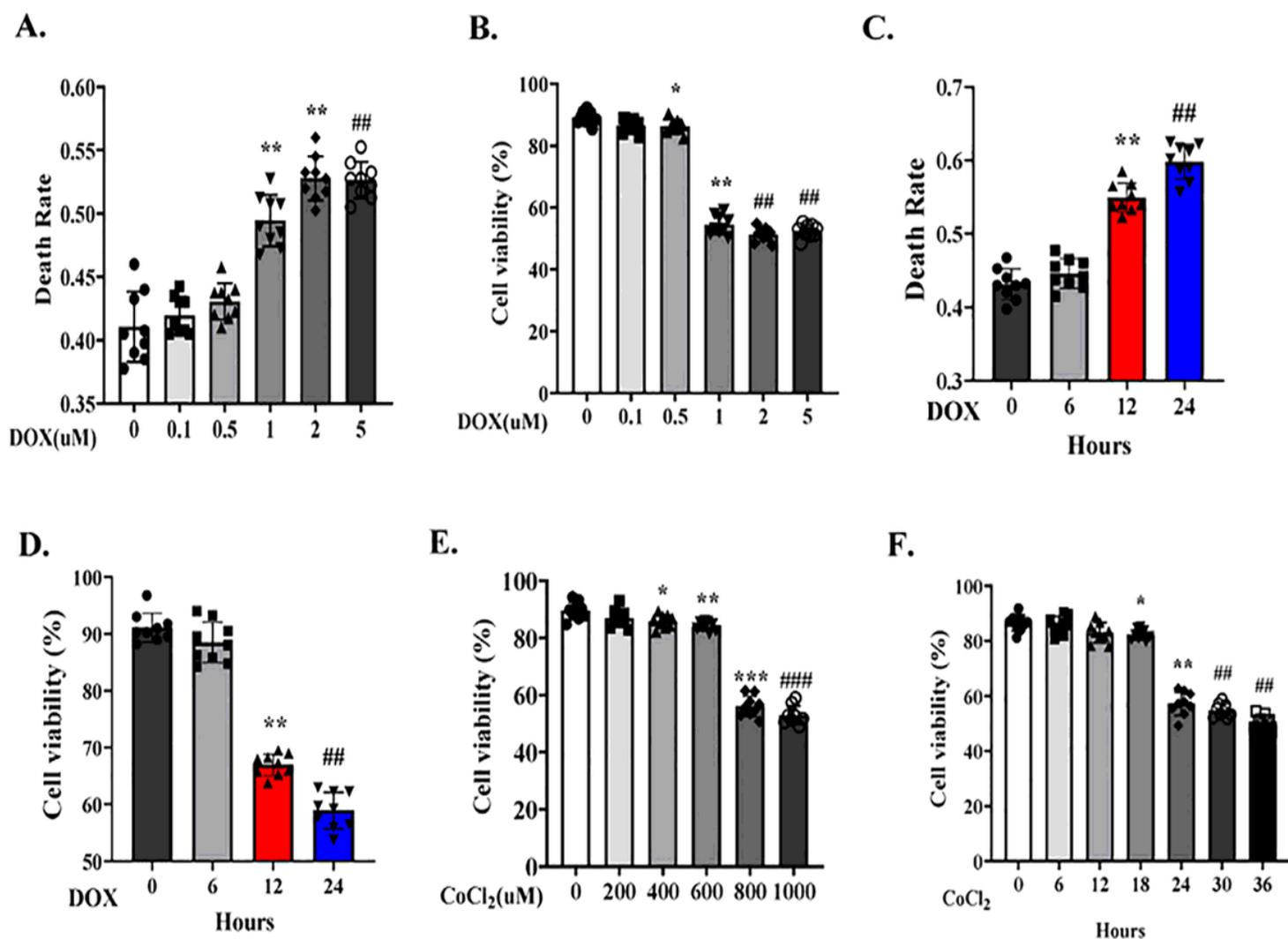


Figure 2

The effect of doxorubicin and cobalt chloride on cell viability and death. A The death rate of rat primary cells treated with doxorubicin in a dose-dependent manner. $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 6$. B The cell survival rate was significantly reduced by 1 μM DOX. $*P < 0.05$, $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 6$. C The effect of doxorubicin varies over time. $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$. D The cell viability decreased most obviously after treatment with 1 μM doxorubicin for 12 h. $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$. E The application of 800 μM CoCl₂ was the most effective in reducing rat primary cell activity. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $###P < 0.001$, one-way ANOVA, $n = 6$. F The survival rate was the most significant when cells were treated with 800 μM CoCl₂ for 24 h. $*P < 0.05$, $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 7$.

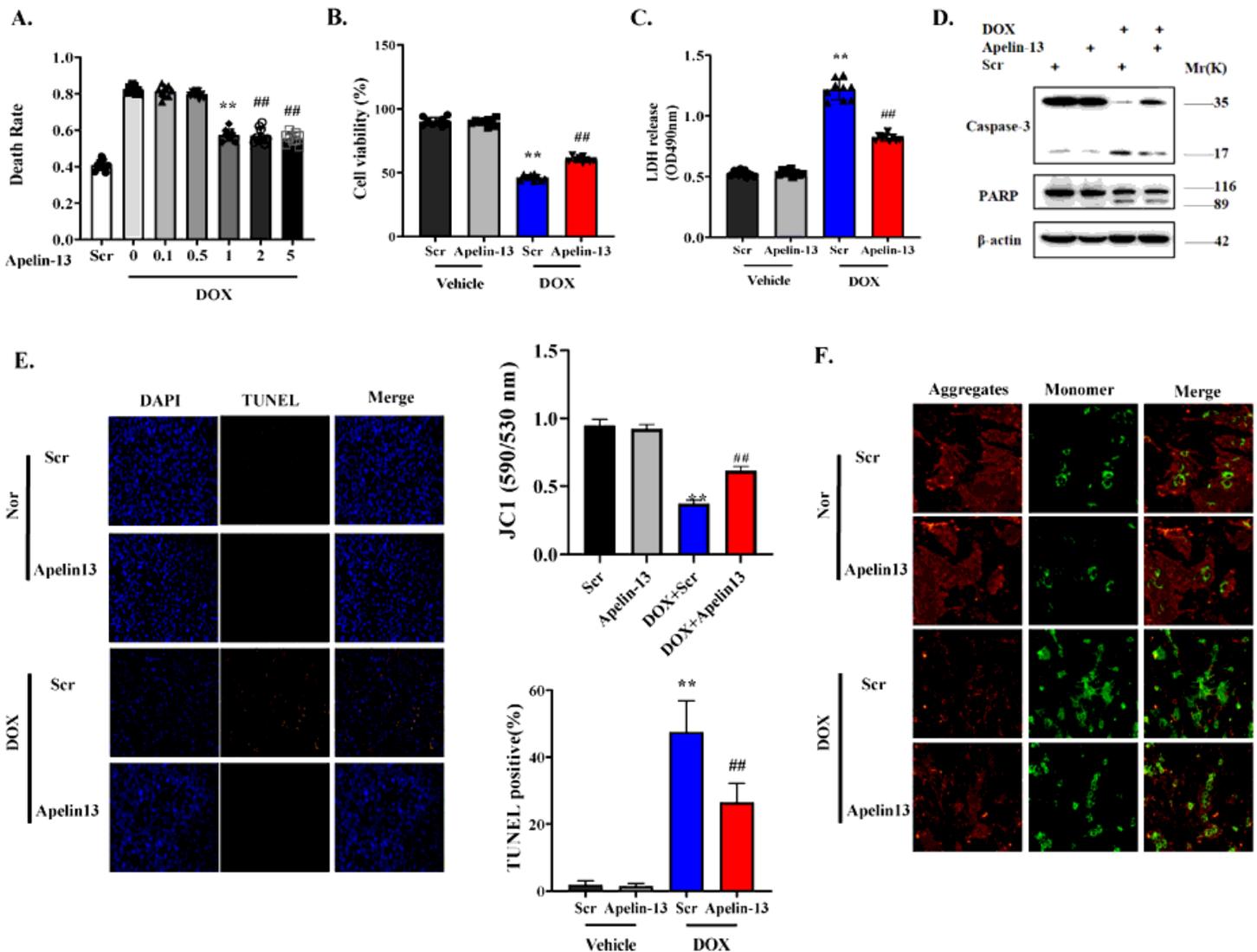


Figure 3

Apelin-13 inhibits apoptosis by attenuating doxorubicin-induced oxidative stress.. Apelin-13 significantly reduced the primary cell death rate in rat cells in a dose-dependent manner. $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 7$. B Treatment with 1 μM apelin-13 was most effectively in increasing the activity of

the primary cells. ****P < 0.01** and **##P < 0.01**, one-way ANOVA, n = 4. C LDH release decreased significantly in the presence of apelin-13. ****P < 0.01** and **##P < 0.01**, one-way ANOVA, n = 4. D Western blotting was used to detect apelin-13 efficiency. ****P < 0.01**, one-way ANOVA, n = 4. E Apelin-13 reduced the formation of apoptotic cells. ****P < 0.01** and **##P < 0.01**, one-way ANOVA, n = 4. F Changes in mitochondrial membrane permeability were used to detect the stage of cell apoptosis. ****P < 0.01** and **##P < 0.01**, one-way ANOVA, n = 4.

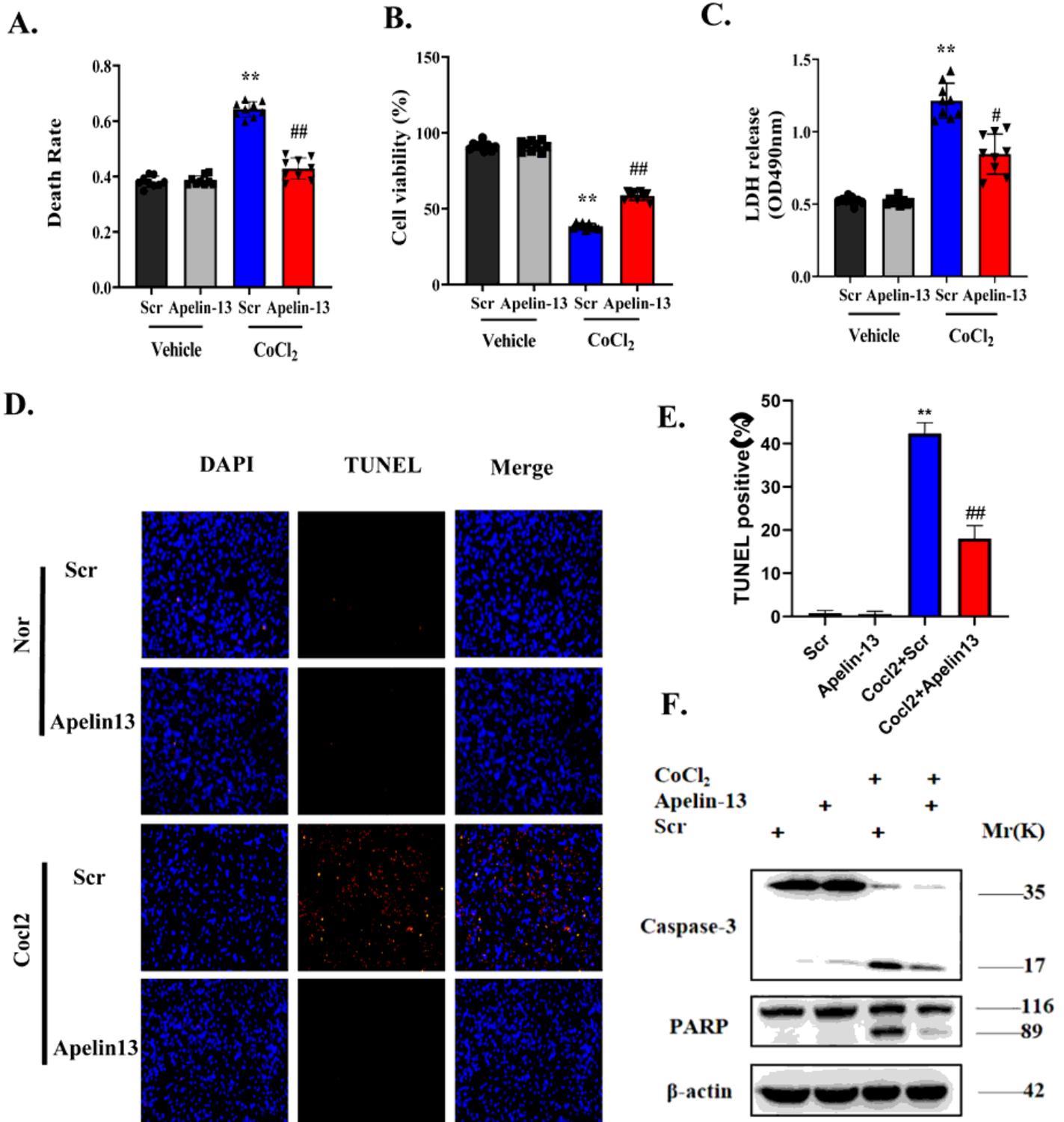


Figure 4

The effect of apelin-13 on the chemically induced hypoxic model in rat primary cells. A Apelin-13 effectively reduced cell death rate compared to treatment with CoCl₂. **P < 0.01 and ###P < 0.01, one-way ANOVA, n = 4. B Apelin-13 effectively enhanced cell survival. **P < 0.01 and ###P < 0.01, one-way ANOVA, n = 4. C Apelin-13 significantly reduced LDH release. **P < 0.01 and ###P < 0.01, one-way ANOVA, n = 4. D The number of apoptotic cells were reduced by apelin-13. **P < 0.01 and ###P < 0.01, one-way ANOVA, n = 4. E apoptotic cells rated were decreased by apelin-13 compared with CoCl₂ and Sramble peptide. **P < 0.01 and ###P < 0.01, one-way ANOVA, n = 4. F The effect of apelin-13 on apoptosis was observed by western blot analysis.

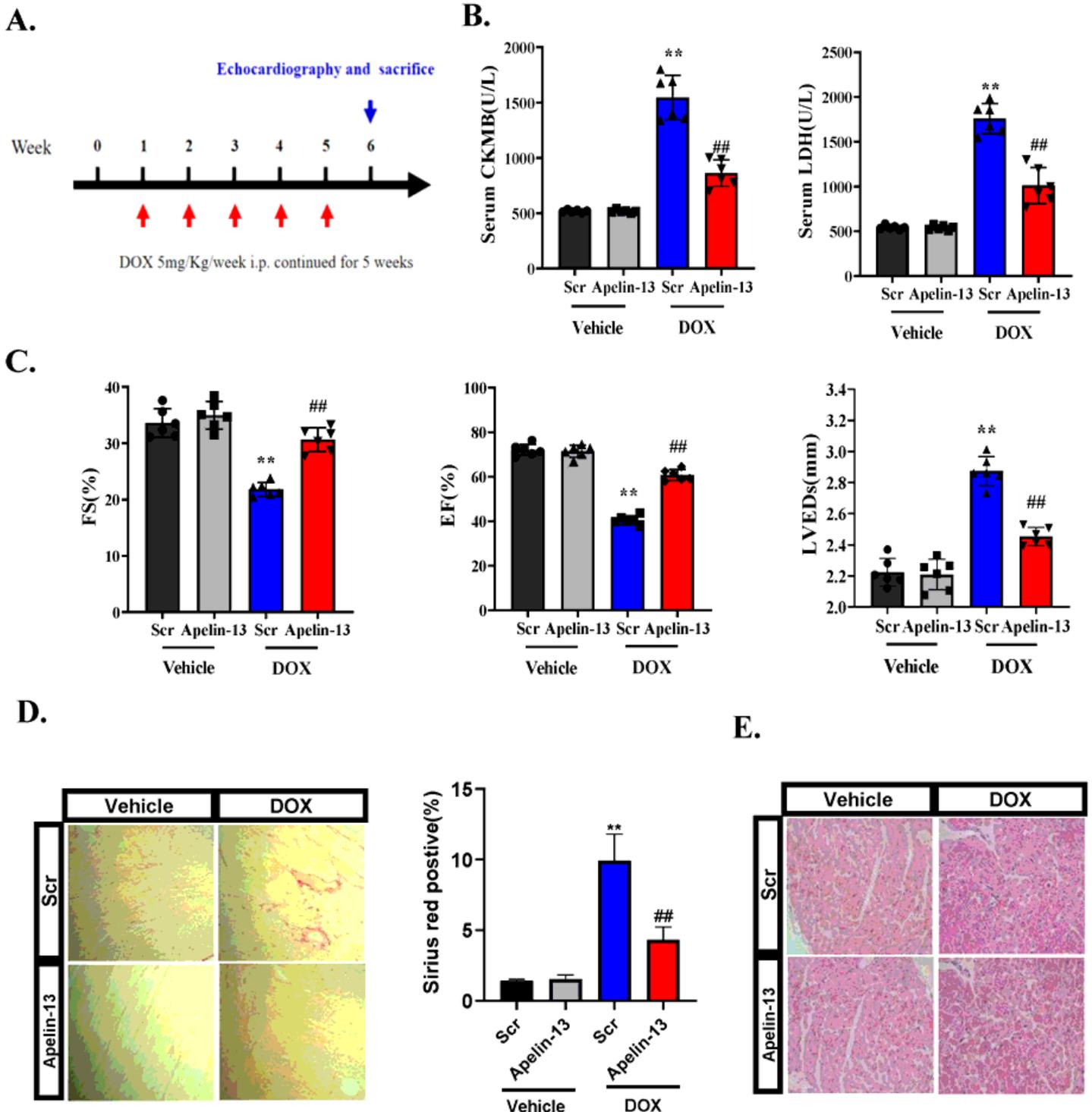


Figure 5

Apelin-13 significantly ameliorated DOX-induced heart failure in vivo. A Mice were injected with 5 mg/kg/week doxorubicin for 5 weeks, and echocardiography was performed before sacrifice. B Serum CKMB of the mice treated with apelin-13 was significantly decreased ($p < 0.01$). $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$; The release of serum LDH upon apelin-13 treatment was decreased compared with the level released by the experimental group mice. $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$. C The EF and FS rates were increased ($p < 0.01$), and the LVED values were decreased significantly ($p < 0.01$). $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$. D Apelin-13 decreased fibrosis formation by sirius red staining significantly ($p < 0.01$). $**P < 0.01$ and $##P < 0.01$, one-way ANOVA, $n = 4$. E Apelin-13 increased the quantity of apoptotic cells as assessed by HE staining.

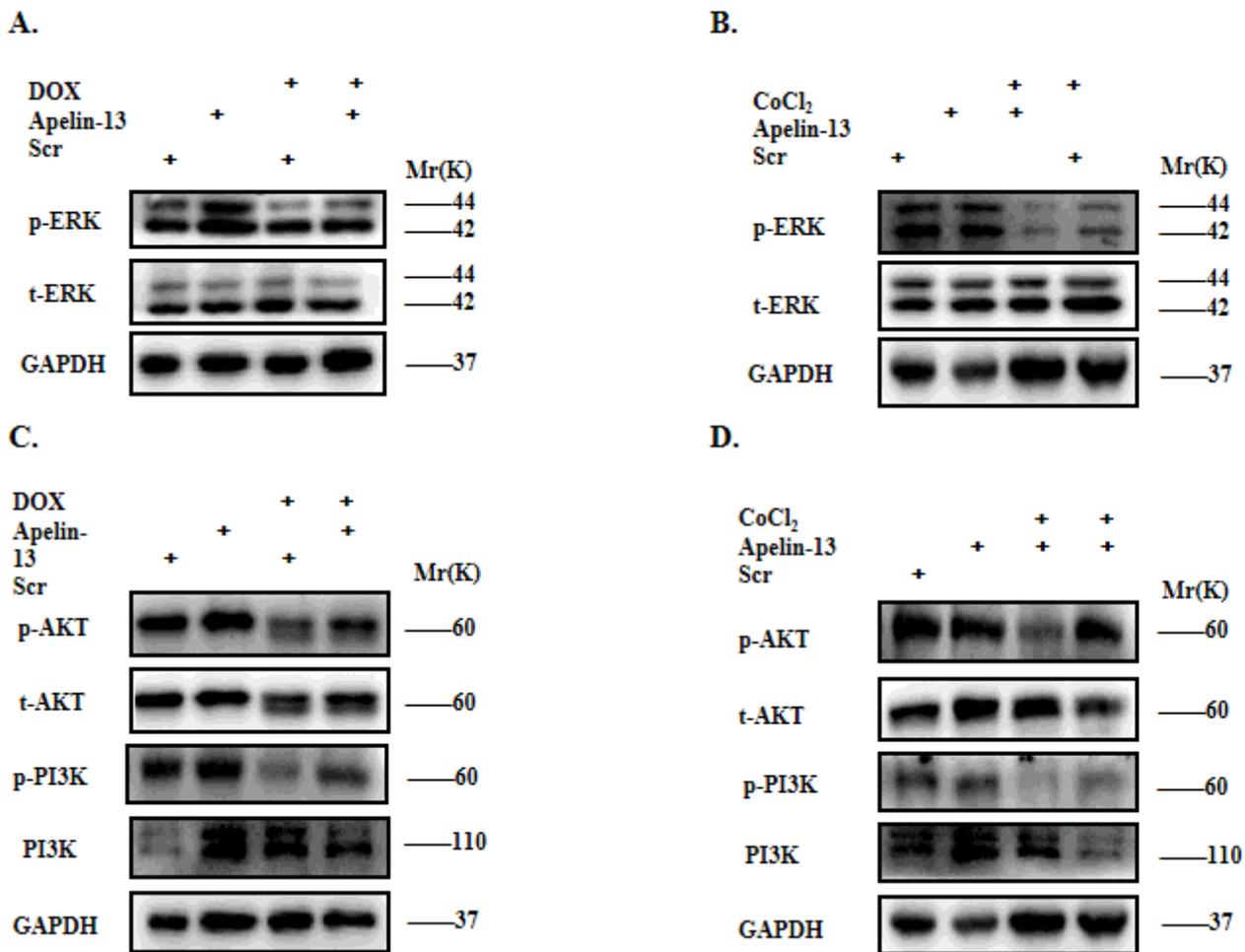


Figure 6

Apelin-13 is involved in the ERK/MAPK and PI3K/AKT signaling pathways. A. Apelin-13 activated phosphorylated ERK protein expression than DOX in the experimental groups; B. phosphorylated ERK was activated by apelin-13 in CoCl₂-treated cells; C. phosphorylated PI3K and AKT protein were activated after treatment with DOX; D. the expression of phosphorylated PI3K and AKT proteins were activated by apelin-13 after CoCl₂ administration.

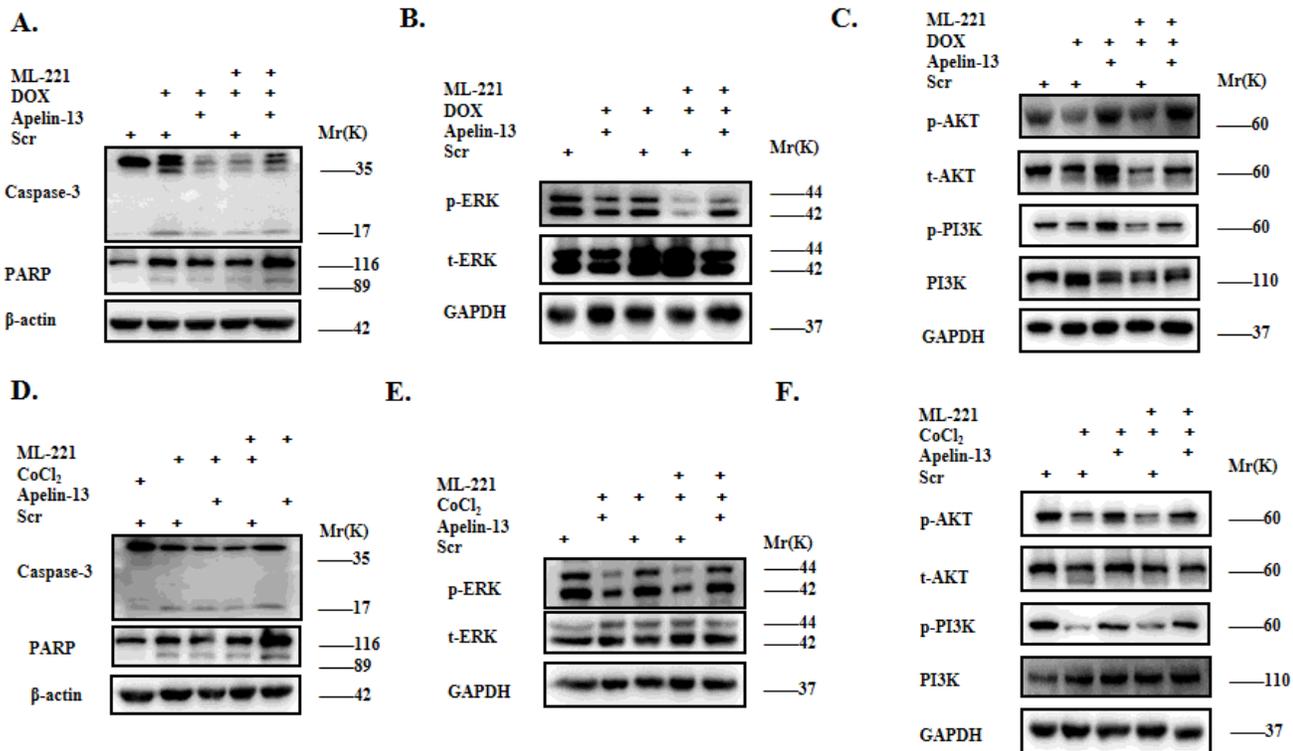


Figure 7

Apelin-13 protects cardiac function by binding APJ. A ML-221 treatment inhibited the upregulated the expression of cleaved caspase-3. B the expression of ERK phosphorylation was downregulated by ML-221. C ERK/MAPK and PI3K/AKT signaling pathway were also inhibited by ML-221 when administered with DOX. D ML-221 upregulated the expression of cleaved caspase 3 when treated with CoCl₂. E ML-221 with CoCl₂ treatment inhibited the expression of the phosphorylated ERK protein. F p-PI3K and p-AKT protein expression were inhibited by treatment with ML-221 and CoCl₂.

Supplementary Files

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