Original Research

DHODH Alleviates Heart Failure via the Modulation of CoQ-Related Ferroptotic Inhibition

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Abstract

Background: Heart failure (HF) is a clinical syndrome that seriously endangers human health and quality of life as the terminal stage of cardiovascular diseases. Ferroptosis as a new iron-dependent programmed cell death mode that is closely related to the occurrence and development of cardiovascular diseases. Dihydroorotate dehydrogenase (DHODH) has been found to play a crucial role in inhibiting ferroptosis and improving mitochondrial function, and its expression can be upregulated by estradiol (E2). Recent studies have found that DHODH can inhibit ferroptosis by reducing coenzyme Q (CoQ) to CoQH2. Therefore, this study aims to explore the effect of up-regulation of DHODH on the pathological hypertrophy and fibrosis of heart failure and its mechanisms. Methods: The mouse heart failure model was established by transverse aortic constriction (TAC), surgery in mice. Two days after the operation, a subcutaneous injection of E2 or the same volume of sesame oil was given for 8 weeks. Then, the left ventricular systolic function related indicators of mice were measured by echocardiography, and the degree of myocardial fibrosis of mice was detected by histological analysis; the expression levels of heart failure markers were detected by quantitative polymerase chain reaction (q-PCR) and western blot (WB) analysis; the morphological changes of mitochondria in cardiac cells of mice were observed by transmission electron microscopy. Cell model were established by stimulating with phenylephrine for 96 hours. Ferroptosis markers were detected by kits and WB analysis. Mitochondrial function was verified by JC-1 fluorescent probe, and 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining. The knockdown results were detected by WB analysis after transfection of small interfering RNA (siRNA) of CoQ. Fer-I was added as a positive control to verify the ferroptosis-related changes of myocardial cells. Results: In the animal model, we found that E2 treatment alleviates TAC-induced cardiac hypertrophy and fibrosis and suppresses cardiomyocyte ferroptosis by promotes DHODH upregulation in murine cardiomyocytes. In the cell model, DHODH upregulation protects against phenylephrine-induced cardiomyocytes with failure. However, the effect on up-regulating DHODH was inhibited by transfection to down-regulate CoQ expression. Conclusions: The up-regulation of DHODH could effectively ameliorate the manifestations of heart failure such as myocardial hypertrophy and fibrosis in mice after TAC surgery, inhibit ferroptosis of cardiac myocytes, and ameliorate mitochondrial function. The mechanism involves CoQ-related biological processes.

Keywords: heart failure; ferroptosis; dihydroorotate dehydrogenase; estradiol; CoQ

1. Introduction

The latest Global Burden of Cardiovascular Disease report indicates that the number of deaths from cardiovascular disease has increased to 19.8 million in 2022 [1], revealing the impact of the current global population growth and aging trends on cardiovascular disease. Heart failure (HF) is the clinical end-stage of most forms of primary cardiovascular disease (CVD), and it is a leading global cause of patient morbidity and mortality. To facilitate the design of more effective targeted cardioprotective interventions, there is a clear need to further explore the pathological mechanisms that underlie cardiomyocyte injury in HF. HF patients tend to present with a range of serious symptoms, high rates of hospitalization and mortality, and a poor overall prognosis. While evidence-based treatment implementation can help protect against mortality among HF patients, rates of patient death remain very high. In patients with stable HF, the annual mortality rate is approximately 6–7%, while these rates can climb to 25% or higher in hospitalized patients with acute HF [2–4].

Cardiomyocytes are terminally differentiated cells, and they are thus unable to undergo further division or proliferation. Cardiomyocyte death thus inevitably results in a decline in the overall number of these cells, compromising cardiac structural and functional integrity in a manner that can aggravate HF development and progression. There is thus clear potential clinical significance to research focused on the pathways that regulate cardiomyocyte death, with the goal of designing new strategies to inhibit cardiomyocyte death and prevent CVD incidence by preserving over-
all heart health. The suppression of cardiomyocyte death offers a promising means of preserving heart function and thereby effectively preventing heart disease.

Dihydroorotate dehydrogenase (DHODH) is an iron-containing enzyme that plays an essential role in the process of ab initio pyrimidine synthesis. DHODH functions by catalyzing a redox reaction that processes dihydroorotate into oxalate, which is then further converted into uridine monophosphate that is used to generate RNA nucleotides, supporting ribosomal biosynthesis. As it plays key roles in pyrimidine synthesis and the functionality of mitochondria, inhibitors of DHODH were first employed for the treatment of rheumatoid arthritis and multiple sclerosis. DHODH can inhibit mitochondrial inner membrane-related ferroptotic induction via reducing pan-quinone coenzyme Q (CoQ) to pan-alcoholic CoQH₂ [5], which is an antioxidant that can trap free radicals and protect against the induction of ferroptosis. Several studies have demonstrated that mitochondrial dysfunction plays a contributing role in the onset of several forms of CVD, including diabetic cardiomyopathy, alcoholic cardiomyopathy, ischemia-reperfusion injury, septic cardiomyopathy, cardiovascular aging, atherosclerosis, cardiac hypertrophy, and HF [6–13]. In recent studies, E₂ supplementation has been demonstrated to protect against ferroptosis at least in part through the restoration of DHODH expression and the remediation of mitochondrial function [14].

Ferroptosis was first proposed as a mode of cell death in 2012 by Dixon et al. [15]. This unique cell death pathway is regulated by phospholipid hydroperoxidase (GPX4) signaling pathway and driven through the iron-dependent peroxidation of phospholipids and is subject to regulation by various pathways including mitochondrial activity, iron metabolism, redox homeostasis, and sugar, lipid, and amino acid metabolism pathways. A range of disease-related signaling pathways also regulate ferroptotic activity, which is distinct from other modes of cell death including necrosis, apoptosis, autophagy, and pyroptosis. Ferroptosis primarily occurs through a mechanism related to the dysregulation of iron metabolism, the disruption of amino acid antioxidant systems, and the aggregation of lipid peroxides. The initial disruption of normal homeostatic iron metabolism can result in a rise in the levels of free iron within cells, and divalent iron can then catalyze reactive oxygen species (ROS) generation via the Fenton reaction, further promoting lipid peroxidation such that these peroxides aggregate and trigger ferroptotic death [16]. Ferroptotic cells are characterized by morphological features including reduced mitochondrial volume, increased membrane density, damage to the mitochondrial outer membrane, and the reduction or absence of cristae [17], without any corresponding nuclear changes, chromatin condensation, or destruction of the cell membrane. Iron homeostasis is vital to normal mammalian cell function, particularly for the maintenance of cardiovascular health. HF is a leading cause of death associated with hemochromatosis and thalassemia [18]. Iron deficiency is also a common complication observed in HF patients, serving as a risk factor that is related to poor prognostic outcomes and independent of anemia complications. Under conditions of iron deficiency, myocardial iron content decreases, in turn limiting the oxygen transport capacity of patients and inducing erythropoietin resistance, with these pathological changes potentially culminating in HF. Ferritin serves as a vital mediator of cardiac iron homeostasis and normal cardiac function [19], with ferritin deficiency within myocardial cells contributing to elevated levels of ROS biogenesis. This can give rise to cardiac damage and contribute to greater susceptibility to iron overload-related ferroptosis and cardiomyopathy.

One research team identified DHODH as a ferroptosis-related factor that is independent of the classical GPX4 signaling pathway [20]. DHODH inactivation can trigger the extensive peroxidation of mitochondrial lipids and drive ferroptotic induction in cancer cells expressing low levels of GPX4 while also synergizing with other ferroptotic inducers to drive these effects in cancer cells expressing high levels of GPX4. Mechanistically, DHODH thus functions in parallel with mitochondrial GPX4 while remaining independent of cytoplasmic GPX4 or ferroptosis suppressor protein 1 (FSP1), inhibiting mitochondrial inner membrane-related ferroptotic activity via reducing panurea to pan-alcohol, which is an antioxidant with anti-ferroptosis activity. E₂ can influence mitochondrial dynamics and bioenergetics, with the dysregulation of these processes being thought to predispose patients to neurodegenerative diseases [21]. Estradiol (E₂) can stabilize MMP, inhibit ROS biogenesis, and improve ATP production and basal respiration in the context of aging [22]. These results support a potential hypothesis wherein E₂-mediated DHODH upregulation can improve the function of cardiomyocyte mitochondria, thereby protecting against HF. The results of this study have the potential to offer new guidance for clinical efforts to treat HF, thereby improving patient outcomes.

2. Material and Methods
2.1 Animal Model Experiments

Specific pathogen-free healthy male C57BL/6 mice (8 weeks old, 20–25 g) were obtained from the Laboratory Animal Center of Nantong University (Nantong, China). The Animal Ethics Committee of Nantong University approved all animal studies, which were consistent with the Guide for the Care and Use of Laboratory Animals. The animal model mice were raised in a standard laboratory environment that consisted of a 12-hour light/dark cycle, with a constant temperature of 25 °C. The mice had free access to animal feed and tap water. Mice were randomized into Sham+sesame oil (as a control for E₂), Sham+E₂, transverse aortic constriction (TAC)+sesame oil, and TAC+E₂ groups (n = 6/group). After inducing anesthesia with 4%
isoflurane, anesthetization was maintained via the inhalation of 1.5% isoflurane through a mask. After cutting open the sternum, a chest expander was used to open the chest so that the thymus could be separated, exposing the aortic arch and its branches. A 5-0 suture was passed through the soft tissue under the aortic arch using an L-shaped needle prepared in-house, after which a 27G probe was placed on the needle and ligated to narrow the vessel. In the sham group, the suture was placed through the soft tissue under the aortic arch without ligation, while all other steps were the same as in the experimental group. E₂ was subcutaneously injected for 6 consecutive weeks beginning 2 days after the procedure, using the same dose as in prior studies [14] (dissolving E₂ (20 μg/kg bw/day) dissolved in sesame oil (1 mL/kg), utilizing ethanol (10 μg/μL) as an adjuvant) or an equivalent volume of sesame oil.

2.2 Transthoracic Echocardiography

Information on the materials used in this study can be found in the Supplementary Material.

Measurements of the left ventricular end-diastolic diameter (LVIDd), left ventricular end-systolic diameter (LVIDs), left ventricular end-diastolic interventricular septum thickness (IVSd), and other indices were made along the long axis and short axis sections of the left ventricle. M-mode ultrasound measurements were processed to calculate indicators that reflect left ventricular systolic function, including left ventricular ejection fraction (LVEF) and fractional shortening (FS). Cardiac functional parameters were measured based on the average values from 3–5 cardiac cycles.

2.3 H&E Staining

Cardiac tissues were fixed for 24 h using 4% paraformaldehyde, dehydrated with an ethanol gradient (75%, 85%, 95%, 95%, 100%, 100%), treated with xylene, immersed for 3 h in pre-dissolved paraffin, and placed in an embedding box and frozen at –20°C. A microtome was then used to cut 5 μm sections that were stored at room temperature. For analysis, these sections were immersed twice in xylene (10 min each), rehydrated with an ethanol gradient (100%, 100%, 95%, 85%, and 75%; 5 min each), washed for 5 min with phosphate buffered saline (PBS), stained with hematoxylin, washed for 5 min with distilled water, and immersed in 1% HCl for 30 min for differentiation. Then, samples were incubated for 30 min in 0.1% ammonia in water, rinsed, immersed for 5 min in eosin staining solution, dehydrated using 90%, 95%, and 100% ethanol for 20 s each, incubated twice for 10 min in xylene, sealed using glue, allowed to air dry, and analyzed via microscopy.

2.4 Masson Staining

Place the sections in the following order in xylene I for 20 minutes, xylene II for 20 minutes, absolute ethanol I for 10 minutes, absolute ethanol II for 10 minutes, 95% alcohol for 5 minutes, 90% alcohol for 5 minutes, 80% alcohol for 5 minutes, 70% alcohol for 5 minutes, and then wash with distilled water. Stain the cell nuclei with hematoxylin: immerse the sections in Weigert’s hematoxylin stain from the Masson staining kit for 5 minutes, wash with tap water, differentiate with 1% hydrochloric acid alcohol for a few seconds, wash with tap water, and rinse with running water for several minutes to return blue. Stain with eosin and phloxine for 5–10 minutes using the Masson staining kit, and quickly wash with distilled water. Treat with phosphomolybdic acid: immerse the sections in the phosphomolybdic acid solution from the Masson staining kit for about 3–5 minutes. Do not wash with water, and directly stain with Fast Blue solution from the Masson staining kit for 5 minutes. Differentiate: treat with 1% acetic acid for 1 minute. Dehydrate and clear the sections by immersing them in 95% alcohol I for 5 minutes, 95% alcohol II for 5 minutes, absolute ethanol I for 5 minutes, and absolute ethanol II for 5 minutes, and then in xylene I and xylene II for 5 minutes. Remove the sections from xylene and allow them to air-dry slightly. Mount the sections with neutral resin and cover them with a cover slip. Examine the sections under a microscope and capture and analyze the images.

2.5 Extraction of Primary Mouse Cardiomyocytes

Hand-held suckling mice (1–2 days old), scrub the chest and abdomen with 75% alcohol, then use an ophthalmic scissor to cut open the sternum and extrude the heart, put it into a PBS dish and wash it repeatedly, then transfer it into a small glass bottle and fully cut it into a pulpy shape. The cut heart was transferred into a blue-covered wide-mouth bottle containing 10 mL collagenase, gently blow the tissue fragments, and place them into a constant temperature oscillator at 37 °C for 5 minutes. The cell suspension after oscillation was transferred to a 15 mL centrifuge tube, centrifuged at 1000 rpm for 5 minutes, and the rest of the heart tissue was added with 10 mL collagenase, gently blown, and then placed into a constant temperature oscillator at 37 °C for 5 minutes. After centrifugation, 2 mL of complete culture was added to the cells, and the cells were blown evenly and left standing. The suspension after centrifugation was digested again, and the cycle was repeated for 7 times in total to make the tissue completely digested. All the cells collected above were collected and transferred into a new centrifuge tube, and then centrifuged again, and the supernatant was discarded. Then add an appropriate amount of complete medium to the centrifuge tube and gently shake to re-suspend the cells. After filtration with a cell sieve, transfer the cells to a Petri dish. Add Brdu (5-bromo-2-deoxyuridine) to the medium and place it in an incubator for 1.5 hours at a differential speed. The cells that are adherent to the wall are fibroblasts, and the cells that are not adherent to the wall are cardiomyocytes. Take the supernatant and inoculate it in a Petri dish. Change to
serum-free medium after 48 hours, and starve for 24 hours before drug addition and transfection.

2.6 Cell Culture

All cell lines were validated by short tandem repeat (STR) profiling and tested negative for mycoplasma. Retrieve the cryovial labeled with H9C2 cells and thaw it rapidly in a 37 °C water bath. Cells were all cultured in a humidified incubator at 37 °C and 5% CO2. Using a pipette, carefully mix 2 mL of cell suspension containing 10% fetal bovine serum (FBS) from fully grown to frozen vial cells by gentle pipetting. Centrifuge at 1000 rpm for 5 minutes, disinfect the centrifuge tube with alcohol, and discard the supernatant in a laminar flow hood. Allow the cell sediment to be suspended in complete culture medium for 5 minutes. Transfer the cell mixture to a culture dish using a sterile pipette and place it in a 37 °C incubator for growth and incubation. Replace the culture medium after 24 hours of incubation and continue cultivation. Upon reaching 80%–90% confluence, perform a subculture at a ratio of 1:3. Discard old culture medium, wash three times with PBS buffer solution, add 1.5 mL of digestive solution (0.25% trypsin), then incubate cells at 37 °C for five minutes under microscopic observation. When cells assume round granular shape due to digestion termination, gently resuspend them using a pipette; subsequently centrifuge at 1000 rpm for 5 minutes, discard supernatant, add fresh complete culture medium to suspend cells before transferring them into a culture dish containing approximately four milliliters of complete culture medium.

2.7 Cell Counting Kit-8 (CCK-8) Experiment

The cells were inoculated into 96-well plates at a concentration of 5 × 10^4 cells/well and cultured for 24 hours at 37 °C, 95% air and 5% CO2 environment. The cells were treated with different concentrations (1, 5, 10, 25, 50, 100, 150, 200 µM) of E2, and 6 groups of parallel culture groups were set up for each concentration. After continued culture for 24 h, the cells were incubated with CCK-8 (10 µL/well) for 2 hours, and the spectrophotometric quantification was performed at 450 nm. Similarly, after 24 hours of cell culture, E2 was added in increasing concentrations (1, 5, 10, 25, 50, 100, 150, 200 µM), and 6 groups of parallel culture groups were set up for each concentration. After continued culture for 24 hours, the cell survival rate was determined by using a rapid and sensitive CCK-8 detection kit. Optical density (OD) value, which directly reflects the survival status and vitality of cells. The survival rate of cardiomyocytes in the control well was set as 100%, and the percentage of other wells was calculated accordingly.

2.8 Cardiomyocyte Transfection

Negative control siRNA (si-NC) and si-CoQ were synthesized by GenePharma (Suzhou, China). Use Lipo3000 transfection reagent to transfect cells according to the manufacturer’s scheme. Add an appropriate amount of Lipo3000 transfection reagent to a certain amount of Opti-Minimal essential medium (Opti-MEM) medium and mix it thoroughly; then use a certain amount of serum-free DMEM and an appropriate amount of si-NC or si-CoQ transfection reagent to mix it thoroughly; shake and mix the two dilutions that have been configured at a ratio of 1:1, and place them at room temperature for 20 min. Add the above mixture to the well plate containing cells one by one with a pipette gun, and then place it in the cell incubator for incubation. After 6 hours, replace it with fresh culture, and then culture for 24–48 hours.

2.9 Real-Time Fluorescence Quantitative Polymerase Chain Reaction (PCR) Method

Total RNA was extracted from the above-mentioned mouse heart tissues and cells. The reverse transcription reaction system was prepared by using the reverse transcription kit (G3337), gently mixed and centrifuged, and the reverse transcription program was set to complete the reverse transcription on the PCR instrument. Take 0.1 mL PCR reaction plate, prepare the following reaction system, and prepare 3 tubes for each reverse transcription product. After the sample was added, the PCR sealing plate membrane was used to complete the sealing with the sealing plate instrument, and the microwell plate centrifuge was used to complete the centrifugation. Then the amplification was completed on the fluorescence quantitative PCR instrument.

2.10 Western Blot Analysis

Total protein was extracted from mouse heart tissues or H9C2 cells. The bicinchoninic acid (BCA) protein detection kit (ThermoFisher, MI, USA) was used to measure the protein content. According to the measured protein concentration, 5× Loading Buffer was added in proportion, and the protein was cooked at 100 °C for 10 minutes after vortex oscillation and mixing. An equal amount of protein is loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was prepared by using Omni-Easy one-step PAGE gel rapid preparation kit (PG213, EpiZyme, China), and then prepare the electrophoresis solution, confirming that the positive and negative electrodes are connected correctly before electrophoresis. Set the program as follows: 80 V constant voltage for 40 minutes, then switch to 120 V until the bromophenol blue band reaches the bottom of the glass plate. The primary and secondary antibodies were incubated after being blocked by rapid blocking solution for 20 min. After washing the membrane with Tris-Borate-Sodium Tween-20 (TBST), the membrane was put into a chemiluminescence imaging system and a small amount of configured electrochemiluminescence (ECL) luminescence solution was added to the membrane to display the strip, and the image analysis software (Image J, Bethesda, MD, USA) was used for quantitative analysis. The following antibodies were used: DHODH Antibody, E9X8R, CellSignaling,
USA; CoQ Antibody, A15193, Abclonal, China; GPX4 Antibody, HY-P80450, MCE, China; SLC7A11 Antibody, D2M7A, CellSignaling, USA; FSP1 Antibody, A21808, Abclonal, China; GAPDH Antibody, A19056, Abclonal, China; PCBP1 Antibody, A19276, Abclonal, China; TFR Antibody, A5865, Abclonal, China; Ferritin Light Chain Antibody, A11241, Abclonal, China; IgG H&L Antibody, AB150801, Abcam, USA.

2.11 Measurement of Malondialdehyde (MDA) Level

Cell samples were homogenized or lysed using PBS or lysis buffer, and the supernatant was collected by centrifugation. Thiobarbituric acid (TBA) storage solution and MDA detection working solution were prepared and dissolved by heating. For the measurement, add the blank control, standard, or sample to the centrifuge tube, then add the MDA detection working solution. Mix well and heat for 15 minutes. Cool to room temperature, centrifuge, and collect the supernatant. Transfer the supernatant to a 96-well plate and measure the absorbance at 532 nm using a microplate reader. Calculate the molar concentration of MDA based on the standard curve.

2.12 Ferrous Ion Analysis

In accordance with the standard process, the cells were collected and cleaved, and then the BCA protein analysis kit was used for the accurate determination of protein concentration. At the same time, the ferrous ion colorimetric test kit was used for the scientific analysis of ferrous ion content: the probe was loaded with ferrous ions in the cells, and the optical density value (OD value) of the generated substance at the strong absorption peak of 593 nm wavelength was determined, and the corresponding calculation was performed, so as to determine the content of ferrous ions (Fe$^{2+}$) in the cells.

2.13 Glutathione (GSH) Determination

Fresh cell model was measured according to the standard procedure. The cells were washed twice with PBS and centrifuged. The supernatant was discarded, and protein removal reagent was added, followed by full eddy current oscillation. The samples were rapidly frozen-thawed twice with liquid nitrogen and 37 °C water bath, and then placed in a 4 °C refrigerator or ice bath for 5 minutes, and then centrifuged for 10 minutes at 4 °C. The supernatant was used for the determination of GSH. The absorbance was measured at 405 nm with an Enzyme-Linked Tmmunosorbent Assay (ELISA) kit, and compared with the standard curve.

2.14 Ghost Mushroom Ring Peptide Fluorescence Staining

Place the cells in a PBS solution containing 3% to 4% formaldehyde at room temperature for 10 to 30 minutes to fix them. Then, suck out the fixative solution and wash the cells with PBS 2 to 3 times. To quench excess formalde-
Fig. 1. Estradiol (E$_2$) treatment promotes Dihydroorotate dehydrogenase (DHODH) upregulation in murine cardiomyocytes and alleviates transverse aortic constriction (TAC)-induced cardiac hypertrophy and fibrosis. (A) Construction of a mouse model of heart failure and a timeline of drug treatments. (B,C) Representative Western blot and quantitative analysis showing DHODH expression in cardiomyocytes. (D–F) Left ventricular M-mode echocardiographic images and parameters, including ejection fraction (EF) and fractional shortening (FS) of the left ventricle short axis in mice. (G) Overall cardiac morphology and hematoxylin-eosin (H&E)-stained sections (40- and 200-fold) were used to assess cardiomyocyte morphology in mice. Scale bar: 1000 µm, 50 µm. (H,I) Bar graphs showing quantitative data on heart weight/body weight (HW/BW) and heart weight/tibial length (HW/TL). (J) Detection of the degree of cardiomyocyte fibrosis and corresponding quantification results by Masson staining. Scale bar: 50 µm. (K) Wheat germ agglutinin (WGA) staining and cell surface area quantifications showing cardiomyocyte cross-sectional area (CSA). Scale bar: 20 µm. (L) Q-PCR detection of mRNA expression levels of cardiomyocyte fibrosis markers ANP, BNP, β-MHC, Acta1, CTGF, and COL1a1. Data are shown as mean ± SEM. ****p < 0.0001, ***p < 0.0005, **p < 0.005, *p < 0.05, n = 3 per group.
the TAC group (Fig. 1H). Masson and WGA staining approaches additionally revealed significant increases in the cross-sectional cardiomyocyte area in the TAC group, with E2 treatment having significantly reversed these changes (Fig. 1J,K). These data suggest that E2 can have a beneficial effect in TAC model mice, partially alleviating cardiac hypertrophy and fibrosis. The mRNA levels of HF and myocardial fibrosis-associated markers including ANP, BNP, β-MHC, Acta1, CTGF, and COL1a1 were also significantly increased following TAC surgery, while their upregulation was suppressed in the cardiac tissue of TAC model mice treated using E2 (Fig. 1L).

3.2 E2 Suppresses Cardiomyocyte Ferroptosis in TAC Model Mice

As the above results support the successful establishment of the murine TAC model system, analyses of iron levels in murine cardiomyocytes were next conducted. A significant increase in these iron levels was observed in mice from the TAC group, whereas iron concentrations in mice from the TAC+E2 group were reduced as compared to mice from the TAC group (Fig. 2A). Significantly elevated MDA levels were observed in the cardiac tissue of mice from the TAC group as compared to the control group, while these levels were restored to baseline following treatment with E2. Conversely, TAC mice presented with significant reductions in cardiac GSH levels, while this change was reversed in animals that had undergone E2 treatment (Fig. 2B,C).

Western blotting was next used to analyze the expression of ferroptosis-associated factors in tissue samples from these mice including DHODH, GPX4, SLC7A11, FSP1, CoQ and PCBP1, with the goal of further confirming the anti-ferroptotic effects of E2 in vivo. These analyses revealed that relative to the TAC group, GPX4, SLC7A11, FSP1, and CoQ were upregulated, whereas the opposite was true for the ferroptosis marker PCBP1. Treatment with E2 was sufficient to partially reverse these effects (Fig. 2D,E). These data thus underscore the value of further studies focused on the mechanisms underlying these phenotypic outcomes.

3.3 DHODH Upregulation Protects against phenylephrine (PE)-Induced Cardiomyocytes with Failure

To probe the mechanistic role of DHODH in cardiomyocytes, a series of in vitro experiments was next conducted. Initially, H9C2 cardiomyocytes were treated for 24 h with a range of E2 concentrations (1–200 µm), and a CCK-8 kit was used to quantify cell survival. No toxic effects of E2 treatment were observed within this dose range. In light of prior studies [14], E2 was selected for use at a 10 µm concentration in further experiments (Fig. 3A,B). A PE dose of 50 µm was also selected based upon prior research [23]. The impact of E2 on the morphological changes associated with PE-induced cardiomyocyte hypertrophy was next assessed via a ghost pen cyclic peptide fluorescence staining approach. Primary cardiomyocytes that had not undergone any treatment were fusiform and exhibited a uniform distribution, whereas following PE stimulation there was a significant increase in cell volume and greater morphological irregularity. This suggests that PE can induce cardiomyocyte failure. Relative to PE-treated primary cardiomyocytes, those cells that underwent E2 treatment exhibited more normal morphological characteristics and a significant reduction in volume (Fig. 3C,D). E2 treatment can thus significantly inhibit PE-induced primary cardiomyocyte failure.

Further analyses of Fe2+ levels and the lipid peroxidation markers GSH and MDA revealed significant increases in Fe2+ and MDA levels in cardiomyocytes treated with PE, whereas there was a significant decrease in GSH levels. E2 significantly reversed these observed changes in PE-treated hypertrophic cardiomyocytes (Fig. 3E–G).

Subsequently, a ferroptosis-specific inhibitor Ferrostatin-1 (Fer-1) was added as a positive control. Analysis of protein levels of DHODH, Ferritin light chain and transferrin receptor (TFR), which are downregulated in ferroptosis, and ferroptosis marker PCBP1 further showed that these three proteins were significantly upregulated in PE-induced primary cardiomyocytes with failure, but returned to baseline levels after E2 treatment, which was consistent with the results of the Fer-1 group. In summary, these experiments demonstrated the ability of E2 to resist ferroptosis (Fig. 3H,I).

3.4 DHODH Protects against PE-Induced Ferroptosis in Cardiomyocytes via Enhancing Mitochondrial Function

To further interrogate the effects that DHODH has on mitochondria, transmission electron microscopy was employed to assess mitochondrial changes in the cardiomyocytes of TAC model mice. These analyses revealed significantly reduced mitochondria in TAC model mice, with the remaining mitochondria exhibiting disorderly arrangement, outer membrane rupture, and the partial or total loss of cristae. In contrast, TAC+E2 treatment was associated with regularly arranged mitochondria with more cristae and substantially reduced mitochondrial outer membrane rupture (Fig. 4A).

The impact of E2 on mitochondrial membrane potential in H9C2 cardiomyocytes that had been treated with PE was next evaluated via JC-1 fluorescent staining. JC-1 polymerizes within the mitochondrial matrix under normal conditions and emits red fluorescence, whereas it remains in a monomeric state in cells with damaged mitochondria owing to a drop in mitochondrial membrane potential. Cardiomyocytes in the control and E2 treatment groups exhibited a strong red fluorescent signal, whereas PE treatment was associated with a significant increase in green fluores-
Fig. 2. E2 suppresses cardiomyocyte ferroptosis in TAC model mice. (A–C) Measurement of iron (A), Malondialdehyde (MDA) (B), and Glutathione (GSH) (C) levels in mouse cardiomyocytes using commercial kits. (D,E) Representative Western blot and quantitative analysis showing the expression of phospholipid hydroperoxidase (GPX4), solute carrier family 7 member 11 (SLC7A11), ferroptosis suppressor protein 1 (FSP1), coenzyme Q (CoQ), and Poly(rC) Binding Protein 1 (PCBP1) in cardiomyocytes. Data are shown as mean ± SEM. ****p < 0.0001, ***p < 0.0005, **p < 0.005, *p < 0.05, n = 3 per group.

ience. The simultaneous treatment of cells with PE and E2 significantly reduced this induction of green fluorescence. E2 treatment can thus protect against the loss of mitochondrial membrane potential (Fig. 4B).

To explore the in vitro inhibition of ferroptotic cell death by E2, 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was employed to detect ROS levels in H9C2 cells. These analyses indicate that relative to the control or E2 treatment groups, ROS levels were significantly elevated following PE treatment for 48 h, while E2 was sufficient to reverse this PE-induced ROS production (Fig. 4C,D).

Mitochondrial function was analyzed by detecting ATP concentrations in H9C2 cells via ELISA. A significant decline in ATP levels was detected in PE-treated H9C2 cells, while treatment with both PE and E2 led to an increase in ATP concentrations (Fig. 4E). This suggests that DHODH upregulation can have reparative effects on damaged mitochondria in cardiomyocytes.
Fig. 3. DHODH upregulation protects against phenylephrine (PE)-induced cardiomyocytes with failure. (A,B) Effects of different concentrations of E2 on the optical density values and cell viability of H9C2 cells. (C,D) Representative images of ghost pen peptide fluorescence staining and cell surface area quantification of primary cardiomyocytes. Scale bar: 50 µm. (E–G) Determination of Fe^{2+} (E), MDA (F) and GSH (G) levels in primary mouse cardiomyocytes using commercial kits. (H,I) Western blotting and quantitative analysis showing the expression levels of DHODH, Ferritin light chain, transferrin receptor (TFR) and PCBP1 in H9C2 cells. Data are shown as mean ± SEM. ****p < 0.0001, ***p < 0.0005, *p < 0.05, compared with PE group; ns, not significant. n = 3 per group, and the cell survival rate in control group was set at 100%.
Fig. 4. DHODH protects against PE-induced ferroptosis in cardiomyocytes via enhancing mitochondrial function. (A) Representative image of mitochondrial ultrastructure morphology in mouse cardiomyocytes. Scale bar: 5 µm, 2 µm, 500 nm. (B) Representative JC-1 green/red fluorescence image. Scale bar: 50 µm. (C, D) Detection of intracellular reactive oxygen species (ROS) levels in H9C2 cells using DCFH-DA fluorescence probe. Scale bar: 100 µm. (E) Detection of ATP concentration in H9C2 cells using mouse adenosine triphosphate (ATP) ELISA kit. Data are shown as mean ± SEM. ****p < 0.0001, ***p < 0.0005, n = 3 per group.
Fig. 5. DHODH regulates CoQ to exert cardioprotective effects and prevent ferroptotic induction. (A,B) Western blotting and quantitative analysis of DHODH, CoQ, Ferritin light chain and TFR expression in H9C2 cells. (C,D) Determination of ROS levels in H9C2 cells using DCFH-DA fluorescent probe. Scale bar: 100 μm. (E) Detection of ATP concentration in H9C2 cells using mouse adenosine triphosphate (ATP) ELISA kit. (F–H) Determination of Fe^{2+}, MDA and GSH levels in H9C2 cells using commercial kits; Data shown as mean ± SEM; ns, not significant. ****p < 0.0001, ***p < 0.0005, n = 3 per group.
3.5 DHODH Regulates CoQ to Exert Cardioprotective Effects and Prevent Ferroptotic Induction

To confirm the functional role that CoQ plays as a regulator of the anti-ferroptotic effects of DHODH, the knockdown of CoQ in H9C2 cells was performed prior to PE treatment, with Western blotting being used to confirm successful CoQ knockdown following si-CoQ transfection. Cells treated with PE also exhibited lower levels of ferroptosis-related proteins including DHODH, Ferritin light chain, and TFR as compared to control levels, with the expression of these proteins being significantly elevated in the PE+E2 group relative to the PE-treated group. However, CoQ knockdown was sufficient to disrupt the upregulation of these genes (Fig. 5A,B). CoQ knockdown in the PE+E2+si-CoQ treatment group also prevented E2-mediated reductions in ROS levels (Fig. 5C,D). When an ELISA approach was employed to detect ATP levels in H9C2 cells, PE treatment was found to significantly reduce ATP concentrations. PE+E2 treatment was associated with higher ATP concentrations, while the silencing of CoQ reversed this increase (Fig. 5E).

Fe$^{2+}$ concentrations, as well as levels of MDA and GSH, were also analyzed to explore the link between CoQ and ferroptosis, revealing that the knockdown of CoQ was sufficient to attenuate the anti-ferroptotic effects associated with DHODH upregulation (Fig. 5F–H).

4. Discussion

DHODH is a rate-limiting enzyme located in the mitochondria, responsible for de novo synthesis of pyrimidine nucleotides, which can associate pyrimidine nucleotide synthesis with the electron transport chain (Electron transport chain, ETC) of mitochondrial complex III through CoQ, playing an important role in cellular energy metabolism [24]. A recent study has found that [5] inhibiting DHODH gene expression helps promote ferroptosis in tumor cells, and DHODH-related research has shown significant clinical significance in the treatment of blood system diseases, liver tumors, and small cell lung cancer [25–27]. However, the role of DHODH in cardiovascular diseases has not yet been widely recognized. Some studies have shown that DHODH-specific inhibitor Teriflunomide can exacerbate rat cardiac ischemia-reperfusion injury [28]. In this study, we selected E2 as a DHODH agonist and conducted in-depth exploration of the role of DHODH in heart failure in both in vivo and in vitro environments. We found that TAC mice showed excessive Fe$^{2+}$ and abnormal expression of myocardial fibrosis markers, and the expression level of DHODH was significantly lower after TAC surgery, accompanied by increased myocardial hypertrophy, fibrosis, and deterioration of cardiac function. PE stimulation resulted in significant enlargement of the volume and irregular shape of mouse primary cardiomyocytes. These changes were significantly reversed after E2-induced upregulation of DHODH expression. Ferroptosis is a novel iron-dependent programmed cell death mechanism that exhibits distinct features from traditional cell death modes such as necrosis, apoptosis, pyroptosis, and autophagy. Since its discovery, ferroptosis has been extensively studied in various fields, including tumors, neurodegenerative diseases, and cerebral ischemia-reperfusion [29]. Research has shown that [30] ferroptosis is highly correlated with cardiomyocyte death. After cardiac ischemia-reperfusion in adult mice, iron accumulates in the myocardial scar tissue surrounding cardiomyocytes. Recent studies have found that ferroptosis plays an important role in cardiovascular diseases such as myocardial infarction, ischemia-reperfusion injury, and heart failure [31]. In this study, we evaluated the expression of MDA, GSH, and Fe$^{2+}$ in TAC mouse heart tissue and primary cardiomyocytes in vivo and in vitro, and verified the expression levels of ferroptosis-related factors in H9C2 cells using Western blot immunoblotting, which were consistent with the results obtained using an ferroptosis inhibitor Fer-1, indicating that upregulation of DHODH improves mouse heart failure by inhibiting cardiomyocyte ferroptosis.

The mitochondria, as the cell’s energy center, are involved in various physiological processes, including programmed cell death, apoptosis, autophagy, metabolism, calcium flux, and innate immunity [32–35]. Because of its high energy demand, the heart is the organ with the most mitochondria [36]. In the development of the heart, mitochondria play a crucial role, especially in the development stage of the fetal heart, the differentiation process of cardiac muscle cells, and maintaining the contractile function of the heart [37–39]. The role of mitochondria is indispensable. Mitochondrial damage can break the metabolic balance and further produce excessive ROS, thereby triggering more severe cell damage and death [40]. A study has found that [41] mitochondrial targeted therapy significantly reduces the cardiac damage induced by ferroptosis. Therefore, maintaining mitochondrial homeostasis is of vital importance to cells, and these regulatory mechanisms are collectively referred to as mitochondrial quality control (MQC) [42]. Studies have shown that [43] knocking down DHODH increases the production of reactive oxygen species and lowers the mitochondrial membrane potential. We have proven by transmission electron microscopy observation of mitochondrial morphology in mouse cardiomyocytes, JC-1 staining to detect the mitochondrial membrane potential of cardiomyocytes, ELISA to detect ATP concentration, ROS fluorescence detection, and other methods that upregulating DHODH can improve mitochondrial function in cardiomyocytes. Our study also found that the expression of CoQ was significantly downregulated in TAC-induced heart failure mouse models and PE-induced primary mouse cardiomyocyte models. To further investigate the relationship between CoQ expression and ferroptosis, we downregulated CoQ expression in H9C2 cells using si-CoQ. The results showed that the downregulation of CoQ
partially counteracted the benefits brought by the upregulation of DHODH using E2. This suggests that DHODH may achieve resistance to myocardial cell ferroptosis through a CoQ-related pathway, thereby protecting mouse cardiac function.

There are certain limitations to this experiment. We will continuously improve and refine our experimental protocol to obtain more accurate results. We only evaluated mice 8 weeks after TAC surgery and did not compare with earlier time points, which is a limitation of this study. The sample size for animal experiments is inadequate, and it will be expanded in subsequent experiments. Hemodynamic measurements were not conducted in the various groups of mice in this study and will be addressed in future research. In addition, this study only validated the relationship between DHODH and CoQ, but did not explore the specific action pathways in more depth. This still needs to be further explored and studied in subsequent experiments.

5. Conclusions

This study found that dihydroorotate dehydrogenase (DHODH) plays a crucial role in alleviating heart failure through a CoQ10 (CoQ)-related ferroptosis inhibition mechanism. Key findings include: upregulation of DHODH significantly improves myocardial hypertrophy and fibrosis caused by transverse aortic constriction (TAC) surgery, while inhibiting cardiomyocyte ferroptosis and improving mitochondrial function. Estradiol (E2) also alleviates TAC-induced myocardial damage by upregulating DHODH expression. In cell experiments, upregulation of DHODH protects against phenylephrine (PE)-induced cardiomyocyte failure, but this protective effect is inhibited by downregulating CoQ expression. The study highlights that although there is strong evidence supporting the potential of DHODH in treating heart failure, limitations such as small sample sizes need to be addressed in future research. Overall, ferroptosis plays a key role in the development and progression of heart failure, and DHODH and CoQ offer new strategies for personalized treatment of heart failure.

Availability of Data and Materials

The original data supporting the conclusions of this paper will be provided by the authors without reservation upon reasonable request.

Author Contributions

CW, CC, JZ, QL and SW selected the topic, prepared the initial manuscript draft, searched the literature, generated all figures and revised the manuscript critically for important intellectual content. JS and HS contributed to collection and assembly of data. JL, PG and XW performed statistical analysis of all data. All authors contributed to editorial changes in the manuscript. The final manuscript has been approved by all corresponding author, who agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experiments involving animals were approved by the Animal Ethics Committee of Nantong University (Permit Number: S20220310-010).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

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References


