Oxidative Medicine and Cellular Longevity

The Role of Anthracyclines in Cardio-Oncology: Oxidative Stress, Inflammation, and Autophagy

Lead Guest Editor: Leonardo Schirone
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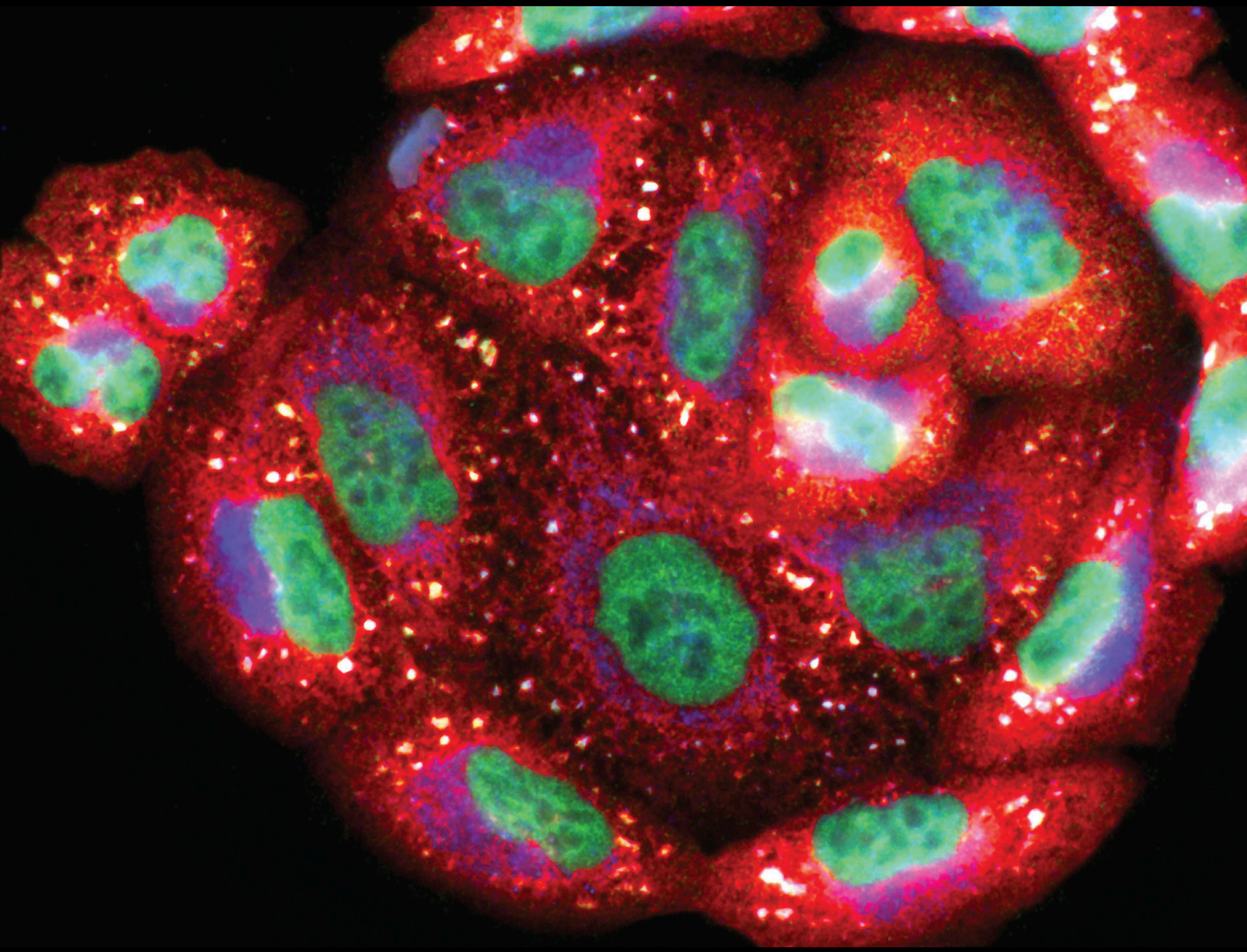

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Editorial

The Role of Anthracyclines in Cardio-Oncology: Oxidative Stress, Inflammation, and Autophagy

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In past decades, much effort was put to define the molecular mechanisms underlying the development of anthracycline-induced cardiomyopathy. As a result, administration protocols were refined and dosages were lowered according to new guidelines, whenever it was possible. To manage the delicate balance between tumor eradication and cardiac health of the patients, an increasing number of multidisciplinary cardio-oncology wards were created in past years in several hospitals. This new synergistic discipline is aimed at transferring from the bench to the bedside the novel approaches that come from basic and translational research.

Among the anthracycline, doxorubicin (DOX) is one of the most studied due to its wide use as a chemotherapeutic agent in a number of malignancies and due to the frequent development of adverse side effects. These include doxorubicin-induced cardiomyopathy (DCM), a potentially lethal condition that may manifest both acutely or chronically and fail to respond to current therapies used to treat cardiovascular disease [1]. Acute cardiotoxicity occurs within 2-3 days from the administration of the drug and results in the development of myopericarditis, palpitations due to tachycardia, electrocardiographic changes (e.g., non-specific ST-T alterations), and premature atrial and ventricular beats [2]. Rarely, acute left ventricular (LV) failure may develop. Despite the fact that the mechanisms for these alterations are not clear, several lines of evidence focused on a role for doxorubicin-induced myocardial edema, which is reversible and may be treated [1]. The incidence of acute DCM is approximately 11%, while chronic DCM is estimated at 1.7% of all treated patients [1]. The lack of accurate epidemiological data is mainly due to the extensive time range (from 30 days to more than 10 years) in which chronic DCM may emerge from the moment of doxorubicin administration. Moreover, the incidence varies largely depending on the dose that is used, varying from 1.7% (below 500 mg/m^2) to 36% (over 600 mg/m^2) [3]. Lastly, being very young or old and having a history of cardiovascular disease are risk factors for congestive heart failure (CHF), a condition that has a very poor prognosis (50% of the affected patients die within 1 year from congestive heart failure) [4].

The development of neglected side effects in a tissue with very low (and controversial) regenerative properties like the myocardium has always induced scientists to find therapeutic targets to achieve cardioprotection without altering the antineoplastic activity of doxorubicin. The toxicity exerted on proliferating cells is probably based on completely different mechanisms compared than those affecting cardiomyocytes. Curiously, dozens of possible mechanisms have been proposed in past decades and can be clustered in oxidative stress-based, gene expression-based, and cell death-based.

Anthracyclines strongly affect mitochondrial respiration and integrity in cardiomyocytes, favoring the reactive

oxygen species (ROS) production. In this special issue, Doroshow and colleagues studied the effect of different chemotherapeutic drugs on cyanide-resistant oxygen consumption and ROS production in an *in vitro* model of cultured adult primary rat cardiomyocytes. Apart from 5-iminodaunorubicin and mitoxantrone, all the tested drugs increased the oxygen consumption of cardiomyocytes. Considering that a ROS scavenging treatment with catalase or acetylated cytochrome c reduced oxygen consumption and that the author detected increased hydrogen peroxide production in response to DOX, it is likely that the mechanisms underlying the cardiotoxicity of all the hereby tested anticancer quinones include ROS overproduction [5].

In this special issue, Carrasco et al. reviewed the latest findings on the role of oxidative stress-mediated molecular mechanisms underlying the development of doxorubicin-induced cardiomyopathy. Oxidative stress is the first and most widely accepted mechanism of doxorubicin cardiotoxicity. Doxorubicin can be reduced to an unstable semiquinone metabolite that targets the cardiomyocytes due to its high affinity to cardiolipin, a phospholipid whose density is exceptionally high in mitochondria, which are impressively numerous in cardiomyocytes, due to their high energy demand [6]. In mitochondria, the molecule cycles and produces free radicals that damage these organelles, facilitated by the fact that antioxidant enzymes and molecules are poorly expressed in cardiomyocytes [7]. Damage to mitochondria may in turn lead to permeability issues to the mitochondrial membranes impairing the electron transport chain, causing a positive feedback that further increases the production of ROS and promotes apoptosis [8].

DOX is also known to interfere with the three isoforms of the nitric oxide synthase (NOS) enzyme, which catalyze the formation of NO from L-arginine and O_2, as studied by Wang et al. (see below).

Besides, many studies in past decades focused on the ability of anthracyclines to chelate free iron, forming a complex that reacts with molecular oxygen and triggers ROS production [9]. However, the iron-DOX complex is nowadays considered to have only a minor role in the pathology of DCM, while free iron accumulation in the myocardium has been proven to trigger apoptosis through mechanisms that are independent of oxidative stress [10].

In their work, Carrasco et al. critically review the different mechanisms defined in past decades and cluster most of them as oxidative stress-related [8]. The authors highlight that many of the cardiovascular risk factors associated with anthracycline-induced cardiomyopathy correlate with increased susceptibility to oxidative stress. Coherently, most of the current preventive and mitigative pharmacological strategies that target the development of this life-threatening condition positively affect the patient's redox status. These include statins, ACE inhibitors, beta-blockers (carvedilol, nebivolol) and polyunsaturated fatty acids. The authors underline that the past trials aimed at directly scavenging ROS likely failed because of their simplistic design, compared to the novel approaches that counteract ROS by stimulating the native antioxidant response, e.g., activating the long-lasting Keap1/Nrf2/ARE pathway. Moreover, genetic variability may account for the interindividual different susceptibility to develop anthracycline-induced cardiomyopathy, as detailed by Yang et al. in this special issue (see below).

Following this line of evidence, the work from Zhang et al. provides new insight into the ROS-scavenging approach to mitigate the effects of DOX administration. In this study, mice were treated for 10 days (5 days before and 5 days after acute DOX treatment) with O-methylated flavone oroxylin A (OA), which is known to be beneficial against inflammation and cancer. OA administration prevented the DOX-associated myocardial atrophy, systolic derangements, and cell death. The authors linked the protective effects exerted by OA administration to preserved levels of expression of sirtuin 1 (Sirt1), a critical deacetylase that is downregulated by DOX. They reported that OA is not protective in cardiac-restricted Sirt1 KO mice, showing that OA relies on Sirt1 activity to prevent acute DOX-induced cardiotoxicity [11].

A different approach was used by Wang et al, who used electroacupuncture (EA) at Neiguan acupoint (PC6) to prevent the development of DOX-induced cardiomyopathy in iNOS-deficient and cardiac-specific arginase 2- (Arg2-) deficient mice. In this study, the authors found that DOX stimulates the production of nitrogen monoxide (NO) and that its levels correlate with iNOS upregulation and Arg2 downregulation. However, EA at PC6 impaired DOX-induced NO upregulation and exerted protective cardiac effects in treated mice, by reducing cardiac dysfunction and hypertrophy. These functional benefits were mechanistically linked to the activity of iNOS and Arg2: iNOS-deficient mice displayed a better heart function than the wild type after DOX treatment but EA did not further improve their phenotype; conversely, cardiac-specific Arg2-KO mice developed a worse heart function and did not benefit from EA. Together, these findings demonstrate that EA at PC6 may represent a novel approach for alleviating DOX-induced cardiomyopathy by preventing NO overproduction [12].

Besides oxidative stress, Yang et al. reviewed the genetic variability that has been associated with increased risk of developing DCM, which involves mutations in genes implicated in metabolism, autophagy, ROS scavenging, mitochondrial function, DNA damage, endoplasmic reticulum stress, inflammation, and apoptosis. These include CYBA, GSTA1, NCF4, RAC2, ABCC1, ABCC2, CAT, UVRAG, GCN2, TCL1A, TLRS, C282Y, Hmox1, CBRs, MYH7, TNNT2, and TTNtv. This work brings together the findings of different genetic clinical studies and, despite studies from larger cohorts being still needed, represents a useful starting point for future research projects on the subject. Defining genomic combinations of polymorphisms that predict an increased risk of developing anthracycline-induced cardiomyopathy may represent a cornerstone to guide clinicians to personalized rational drug use and, eventually, to the use of combined cardioprotective strategies.

Nowadays, much attention is being paid to avoid reaching a critical life-long dose of anthracycline. However, at the state of the art, clinicians are often cornered between the

urgency of treating aggressive cancers and the risk of causing iatrogenic heart disease. This is particularly true for those patients that received chemotherapy in pediatric age and that face a second oncologic disease in their adulthood. In the future, merging traditional ROS scavenging approaches, the new mechanistic molecular insights beneath anthracycline-induced cardiomyopathy and genetic screening will hopefully result in personalized therapies that will help vulnerable patients to be safely cured of cancer.

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Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

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Research Article

Protection against Doxorubicin-Induced Cardiotoxicity through Modulating iNOS/ARG 2 Balance by Electroacupuncture at PC6

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Background. Doxorubicin (DOX) is a commonly used chemotherapeutic drug but is limited in clinical applications by its cardiotoxicity. *Neiguan* acupoint (PC6) is a well-recognized acupoint for the treatment of cardiothoracic disease. However, whether acupuncture at PC6 could be effective in preventing DOX-induced cardiotoxicity is still unknown. *Methods*. A set of experiments were performed with myocardial cells, wild type, inducible nitric oxide synthase knockout (iNOS-/-), and myocardial-specific ablation arginase 2 (Myh6-ARG 2-/-) mice. We investigated the protective effect and the underlying mechanisms for electroacupuncture (EA) against DOX-induced cardiotoxicity by echocardiography, immunostaining, biochemical analysis, and molecular biotechnology in vivo and in vitro analysis. *Results*. We found that DOX-mediated nitric oxide (NO) production was positively correlated with the iNOS level but has a negative correlation with the arginase 2 (ARG 2) level in both myocardial cells and tissues. Meanwhile, EA at PC6 alleviated cardiac dysfunction and cardiac hypertrophy in DOX-treated mice. EA at PC6 blocked the upregulation of NO production in accompanied with the downregulated iNOS and upregulated ARG 2 levels in myocardial tissue induced by DOX. Furthermore, knockout iNOS prevented cardiotoxicity and EA treatment did not cause the further improvement of cardiac function in iNOS-/- mice treated by DOX. In contrast, deficiency of myocardial ARG 2 aggravated DOX-induced cardiotoxicity and reduced EA protective effect. *Conclusion*. These results suggest that EA treatment at PC6 can prevent DOX-induced cardiotoxicity through modulating NO production by modulating the iNOS/ARG 2 balance in myocardial cells.

1. Introduction

Doxorubicin (DOX) is an anthracycline antibiotic that is widely used to treat leukemias, Hodgkin's lymphoma, cancers of the bladder and breast, multiple myeloma, and other cancers [1, 2]. However, DOX, like other anthracyclines, can damage the heart irreversibly. Cardiotoxicity characterized by decreased left ventricular ejection fraction, cardiomyopathy, and heart failure is a major side effect that can develop years after successful cancer therapy. At present, dexrazoxane is the only FDA-approved drug available to protect the heart against the cardiotoxic side effects of anthracyclines [3]. However, dexrazoxane is not used with the initiation of anthracycline therapy and is not approved

for use in children or adolescents [4]. Thus, there is an urgent need for the development of effective and safe alternative therapies that can be used especially in earlier time windows or young patients to prevent anthracycline-induced cardiotoxicity.

Acupuncture including electroacupuncture (EA) is one of the most widely used and accepted complementary and alternative medical treatments. For cancer patients, many clinical studies have shown that acupuncture is helpful in alleviating side effects caused by chemotherapeutics, including pain, vomiting, fatigue, anxiety, insomnia, and postoperative intestinal obstruction [5–8]. The *Neiguan* acupoint (PC6) is located at the flexor aspect of the forearm between the tendons of the palmaris longus and flexor carpi radialis, overlying the median nerve [9]. PC6 stimulation has been used to improve symptoms of angina, palpitation, and left cardiac function in patients with heart diseases [10, 11]. Furthermore, experimental studies have reported that PC6 stimulation can effectively limit heart damage in various animal models including ischemia/reperfusion injury, myocardial ischemia, hypertension, hypertrophy, and bupivacaine-induced cardiotoxicity [12–15]. However, whether acupuncture at PC6 could be effective in preventing DOX-induced cardiotoxicity has not been reported yet.

The molecular mechanisms of DOX-induced cardiotoxicity include oxidative stress, calcium overload, lipid peroxidation, and mitochondrial dysfunction [16, 17]. Previous studies have suggested that increased nitric oxide (NO) production is involved in DOX-induced increases in nitrosative stress [18]. The production of NO is catalyzed by nitric oxide synthases (NOS) from L-arginine, and arginase competes with NOS for common substrates, L-arginine, to produce L-ornithine and urea [19]. In pathological conditions, the abnormal activity of inducible nitric oxide synthase (iNOS) or arginase disturbs the balance and causes the abnormal level of NO production [20]. Studies in models of DOX-induced cardiotoxicity have shown increased tissue levels of iNOS [21, 22]. Both two arginase isoforms, including arginases 1 and 2, are involved in the NO production in various cardiovascular diseases such as atherosclerosis and myocardial ischemia-reperfusion injury [23, 24]. Arginase 2 (ARG 2) is the predominant isoform expressed in cardiac tissue [25, 26]. However, the role of ARG 2 has not been reported in cardiomyocytes and further study is needed.

Some studies reported that EA can effectively alleviate hypertension and cardiac hypertrophy through regulating the NO level [27, 28]. Moreover, stimulation of PC6 can upregulate myocardial NO and NOS for relieving myocardial injury in myocardial ischemic reperfusion injury rats [29]. Therefore, we hypothesized that EA can provide cardioprotection against DOX-induced cardiotoxicity by regulating NO signaling. Here, we demonstrated DOX-induced cardiac injury was prevented by EA at PC6 through modulating the balance between iNOS and ARG 2 levels. These results strongly suggested that EA at PC6 should be seriously applied as an effective alternative therapy for protection against DOX-induced cardiotoxicity.

2. Materials and Methods

2.1. Experimental Animals. Eight-week-old male C57BL/6J mice were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China). Knockout iNOS mice (iNOS-/-) were purchased from Jackson Laboratory (B6.129P2-Nos2tm1Lau/J, stock number: 002609). Myh6-CreER mice were purchased from Jackson Laboratory (stock number: 005657, B6129-Tg (Myh6-cre/Esr1)1Jmk/J). Floxed ARG 2 (ARG $2^{f/f}$) mice were generated by Shanghai Model Organisms Center, Inc. The mice were maintained in a 12 h light/dark cycle at 22 ± 2°C with relative humidity of 60% ± 5%. Food and water were supplied ad libitum. All experiments and animal care in this study were approved by the Institutional Animal Care and Use Committee of the Guangzhou University of Chinese Medicine.

2.2. DOX Administration. The DOX-induced cardiotoxicity animal model was established as previously described [30]. Briefly, mice were injected intraperitoneally with 3 mg/kg of DOX (D1515, Sigma, St. Louis, MO) for five continuous days; the vehicle control group received an equal volume of 0.9% NaCl.

2.3. Tamoxifen Administration. Tamoxifen administration was performed as we previously described [31]. Myh6-CreER mice were crossed with ARG $2^{f/f}$ mice to get Myh6-CreER; ARG $2^{loxp/loxp}$ (Myh6-ARG 2-/-). Tamoxifen (Sigma-Aldrich) was prepared in sesame oil (Sigma-Aldrich) and was intragastrically administered to the adult mice (100 mg/kg body weight/day, 8 weeks old) for 5 consecutive days.

2.4. EA Treatment. The intensity of EA was 2 mA, and the duration was 20 min per day for 7 continuous days, as described previously [32]. For EA treatment, mice were anesthetized with isoflurane (2%) and needles (0.18 mm × 7 mm, Suzhou Acupuncture & Moxibustion Appliance Co. Ltd., China) were inserted 2-3 mm into the left and right acupoints. Three groups of mice received EA. The frequency of the electric current of the EA therapeutic apparatus was set at 2, 50, or 100 Hz. To control for the influence of anesthesia in the EA groups, mice in the control and DOX groups were also given isoflurane (2%) inhalation anesthesia.

2.5. Experimental Design. To explore the effect of EA at PC6, mice were randomly divided into five groups as follows: the vehicle control group (Veh), DOX treatment group (DOX), and DOX with EA treatment group (DOX+2 Hz EA; DOX +50 Hz EA; DOX+100 Hz EA). Following treatments, mice were subjected to behavioral assessments and echocardiographic measurement. Then, mice were sacrificed to collect blood and heart tissue for biochemical tests. In some experiments, L-arginine (30 mg/mL, Sigma, USA) was administered in drinking water (130 mg/kg of body weight/day) as previously described [33]. For experiments using iNOS-/- mice, mice were randomly divided into five groups: control littermates with Veh and DOX treatments and iNOS-/- mice with Veh, DOX, and DOX+EA treatments. Experiments using ARG $2^{f/f}$ mice and Myh6-ARG $2^{f/f}$ mice were,

respectively, divided into four groups: ARG $2^{f/f}$ with DOX treatment, ARG $2^{f/f}$ with DOX+EA treatment, Myh6-ARG 2-/- mice with DOX treatment, and Myh6-ARG 2-/- mice with DOX+EA treatment. The number and division of animals are shown in Supplementary Table 1-4.

2.6. Echocardiography. Echocardiography was performed as previously described [34]. Briefly, transthoracic echocardiography was performed using a high-resolution echo machine with a 30 MHz probe. Animals were anesthetized with 3% isoflurane, their chests were shaved, and temperature-controlled anesthesia was maintained with 1.5% isoflurane. The probe was situated perpendicular to the heart to determine the position, and then, the probe was rotated clockwise 30-45 degrees to determine the left ventricle of the heart. The following parameters were measured from the M-mode images and two-dimensional images obtained in the short-axis views by the corresponding matching software (Vevo 2100 high-resolution small animal ultrasound system, VisualSonics, Canada): the percentage of fractional shortening (FS, %), ejection fraction (EF, %), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV). Stroke volume (SV) was calculated using the formula: LVEDV − LVESV (Figure S1).

2.7. Rotarod Test. The rotarod test was performed as previously described [35]. Briefly, 12 days after DOX treatment, motor function was tested with a rotarod test (Shanghai Jiliang Software Technology Co. Ltd., China). The rotarod test was performed by placing a mouse on a rotating rod and measuring the time and distance it was able to maintain its balance. Mice were trained for 5 min three times in a day before the formal trial. The speed of the rotarod accelerated from 4 to 40 rpm over the 5 min period. To allow the mice to adapt to the accelerating rod, we put them back on the rod once they dropped from it during the training periods. In the formal trial, each mouse was placed on the rotating rod once and returned to the home cage after dropping from the rod. After an interval of 30 min, each mouse was subjected to another trial, for three trials in total.

2.8. Histological Analysis. Histological analysis was performed as previously described [36]. Hearts were excised after echocardiography, fixed overnight with 4% formalin/PBS-buffered, and embedded in paraffin. Transverse sections at a thickness of 5 μm were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining to evaluate gross morphology. Glass slides were dewaxed with xylene, followed by absolute alcohol and 95%, 85%, and 75% alcohol. The H&E staining sequence was as follows: hematoxylin for 6-8 min, washed by running water for 1 min; 0.2% hydrochloric alcohol for 3 s, washed by running water for another 1 min and soaked in double-distilled water for 10 min; eosin for 20-30 s, washed by xylene, absolute alcohol, and 75%, 85%, and 95% alcohol. To quantitate individual myocyte size, heart tissue sections were stained with FITC-conjugated wheat germ agglutinin (WGA) (Invitrogen, Carlsbad, CA, U.S.A.). The WGA staining sequence was as follows: incubation with WGA (10 μg/mL) at room temperature for 20 min in darkness, washed in PBS 3 times for 5 min each; stained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole) for 10 min in darkness, washed in PBS 3 times for 5 min. For image analysis, the data value for each mouse was calculated from 5 sections/mouse and the number of mice in each group is 3.

2.9. Assessment of NO Production, Malondialdehyde (MDA), and 3-Nitrotyrosine (3-NT) Levels. Assessment of NO production was performed as previously described [37]. Pieces of frozen heart (~20 mg) were homogenized in precooled normal saline. The homogenate was centrifuged at 2000 r/min for 15 min at 4°C, and the supernatant was collected. NO production in the cardiac tissue was quantitated by evaluating its oxidation products (nitrate and nitrite) using the nitrate reductase method with a Total Nitric Oxide Assay Kit (No: S0024, Beyotime Institute of Biotechnology, Beijing, China) following the kit instructions. Briefly, the standard curve was obtained with $NaNO_2$ at concentrations of 0, 1, 2, 5, 10, 20, 60, and 100 μM. Samples (50 μL/well) were mixed thoroughly with prewarmed Griess Reagent I and II (50 μL of each reagent/well) in a 96-well plate. Then, the absorbance of each sample was determined at the wavelength of 540 nm. Total NO content (μmol/g protein) was determined using a standard curve. MDA levels were determined using a MDA assay kit (Nanjing Jiancheng Bioengineering Institute Co., Ltd.; cat. no. A003-1-2) as previously described [38]. Simply, heart tissues were uniformly weighed and added corresponding reagents of the kit. Then, tubes were heated at 95°C for 40 min and centrifugated (4000 rpm, 10 min at 4°C). The OD values were measured at 532 nm. The concentrations of 3-NT were determined using a 3-NT assay kit (Elabscience Biotechnology Co., Ltd.) as previously described [39]. The OD values were measured at 450 nm. There are ten mice per group in animal experiments. All samples were assayed in triplicate.

2.10. Real-Time Quantitative PCR. Quantitative real-time PCR was performed as we previously described [40]. Briefly, total RNA was extracted from the heart tissue using Trizol reagent (9109, Takara, Japan) and complementary cDNA was synthesized using a PrimeScript™ RT reagent kit (RR047A, Takara, Japan). Gene expression of iNOS (forward, 5′-CGA GGA GGC TGC CTG CAG ACT TGG-3′ and 3′ reverse, 5′-CTG GGA GGA GCT GAT GGA GTA GTA-3′), endothelial nitric oxide synthase (eNOS) (forward, 5′-TCA GCC ATC ACA GTG TTC CC-3′ and reverse, 5′-ATA GCC CGC ATA GCG TAT CAG-3′), neuronal nitric oxide synthase (nNOS) (forward, ACCCAACGTCATTTCT GTCC and reverse, AAGGTGGTCTCCAGGTGTGT), TNF-α (forward, 5′-ACTCAACAAACTGCCCTTCTGAG-3′ and reverse, 5′-TTACAGCTG GTTTCGATCCATTT-3′), IL-1β (forward, 5′-TGTGGCTGTGGAGAAGCTGT-3′ and reverse, 5′-CAGCTCATATGGGTCCGAGA-3′), and IL-10 (5′-GTTGCCAAGCCTTATCGG GAA-3′ and 5′-CCAGGG AATTCAAATGCTCCT-3′) was determined by quantitative PCR with SYBR Green Dye Gene Expression Assays, performed on an ABI7500 system (Applied Biosystems, Carlsbad,

CA, USA). The reaction conditions were as follows: 30 s polymerase activation at 95°C and 40 cycles at 95°C for 5 s, and 60°C for 31 s. β-Actin was used as internal control for normalization (forward, 5′-CTGACACCTTCACCATTCCAG-3′ and reverse, 5′-ATTGCTGACAGGATGCAG AAG-3′). The probes of TaqMan assay (Invitrogen) were used to detect ARG 2 and hypoxanthine phosphonbosyltransferase (HPRT) as internal control (Mm00477592_m1 and Mm00446968_m1). Data were normalized to HPRT, and the fold change between levels of different transcripts was calculated by the CT method. The number of mice in each group is 6, and each sample was tested in triplicate.

2.11. Arginase Activity. Heart tissues were used for arginase activity assay as described [40]. Briefly, 10 mM $MnCl_2$ were added to the samples and heated at 57°C for 10 min to activate arginase. L-arginine (0.5 mol/L) was added and incubated at 37°C for 1 h. And the hydrolysis reaction was stopped with acid solution mixture (H_2SO_4: H_3PO_4: H_2O). The solution of a-isonitrosopropiophenone (9%, a-ISPF in EtOH, Sigma, No: 13502) was added, and the mixture was heated at 100°C for 45 min. All samples were kept in the dark at room temperature for 10 min, and absorbance was measured by absorbance at 540 nm. There are ten mice per group in animal experiments and 6 samples per group in cell studies. All samples were assayed in triplicate.

2.12. Western Blot. Western blot was performed as we previously described [40]. Briefly, frozen cardiac samples were washed twice with cold PBS and resuspended in RIPA buffer. Equal amounts of total protein (30 μg) were separated by SDS-PAGE. Then, gels were transferred to PVDF membranes and blocked for 1 h in blocking solution at room temperature. The membranes were incubated overnight at 4°C with primary antibodies (iNOS: 1 : 1000, ARG 2: 1 : 1000, Cell Signaling Technology, Danvers, MA, USA) followed by treatment with anti-rabbit secondary antibodies (1 : 5000, Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature. β-Tubulin (1 : 5000, Arigo Biolaboratories, Hsinchu City, Taiwan) was used as an internal control. An enhanced chemiluminescence ECL Plus system (Tanon, Shanghai, China) was used for visualization. There are six mice per group in animal experiments. All samples were assayed in triplicate.

2.13. Cell Culture and Treatment. Cell culture and treatment were performed as described [41]. Briefly, rat primary cardiomyocytes isolated from neonatal rat hearts (RAT-iCell-c001; iCell Bioscience, Shanghai, China) were cultured in a primary cardiomyocyte culture medium (PriMed-iCell-022; iCell Bioscience) in the incubator of 5% CO_2 at 37°C. Rat primary cardiomyocytes were seeded at a density of 6.6×10^4 cells/cm^2 to incubate with DOX (0, 1.25, 2.5, 5, and 10 μmol/L) for 24 h in darkness. Supernatant collection and protein extraction were performed after incubation for subsequent experiments. The measurement methods of NO production, arginase activity, ARG 2, and iNOS protein expression are the same as these of heart tissues.

2.14. Cell Viability. Cell viability was performed as described [42]. Rat primary cardiomyocytes were cultured in 96-well plates, and 10 μL CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well at a 1/10 dilution, followed by a further 4 h incubation in the incubator. Absorbance was measured at 450 nm with a microplate reader (EPOCH, Bio-Tek Winooski, Vermont, USA). Three wells in the indicated groups were used to calculate the percentage of cell viability according to the following formula: percentage of cell viability = (OD treatment group − OD blank control group)/(OD control group − OD blank control) × 100%. There are six samples per group, and experiments were repeated five times.

2.15. Statistical Analyses. All data were expressed as mean ± SEM. Statistical differences were determined using analysis of variance (ANOVA) followed by Turkey's post hoc test. The nonparametric Spearman rank correlation was calculated for NO production between iNOS and ARG 2 protein in cells. Comparisons were performed using SPSS (version 21.0). The number of experiments is indicated by "n," and P values < 0.05 were taken as significant.

3. Results

3.1. DOX-Induced Cardiac Dysfunction Was Ameliorated by EA. To investigate whether EA protected against DOX-induced cardiac dysfunction, we conducted echocardiography to measure the cardiac function in DOX-treated mice (Figure S1). Three different frequencies of EA stimulation at PC6 were performed for seven consecutive days (Figures 1(a) and 1(b)). As shown in Figures 1(c)–1(f), the values of EF%, FS%, and SV of the DOX group were significantly decreased, compared with the Veh group. Treatment of 2 Hz EA exhibited the best protection based on the values of EF%, FS%, and SV, compared with 50 Hz EA and 100 Hz EA treated groups (Figures 1(c)–1(f)). Previous studies have shown that impaired exercise ability is a manifestation of heart dysfunction [43]. To measure the motor function of mice, a rotarod test was used. As shown in Figures 1(g) and 1(h), the fall-off time and total distance on the rod decreased in the DOX group compared with the Veh group. EA at 2 Hz improved their motor function, but not 50 Hz EA or 100 Hz EA treatment. Taken together, these results suggested that DOX-induced cardiac dysfunction and impaired motor function were ameliorated by EA at 2 Hz.

3.2. DOX-Induced Cardiac Hypertrophy Was Alleviated by EA. Cardiac hypertrophy is one of the manifestations of DOX-induced cardiotoxicity [44]. To investigate the impact of DOX and EA treatment on pathological changes of the heart, we first measured heart weight and body weight. As shown in Figures 2(a)–2(c), the ratio of heart to body weight and heart weight to tibia length in the DOX group was increased compared with other groups. This result suggested that EA treatment prevented the DOX-induced cardiac hypertrophy. To further evaluate the effects of EA on DOX-induced cardiac hypertrophy, heart specimens were harvested at 20 days after DOX treatment for H&E and WGA

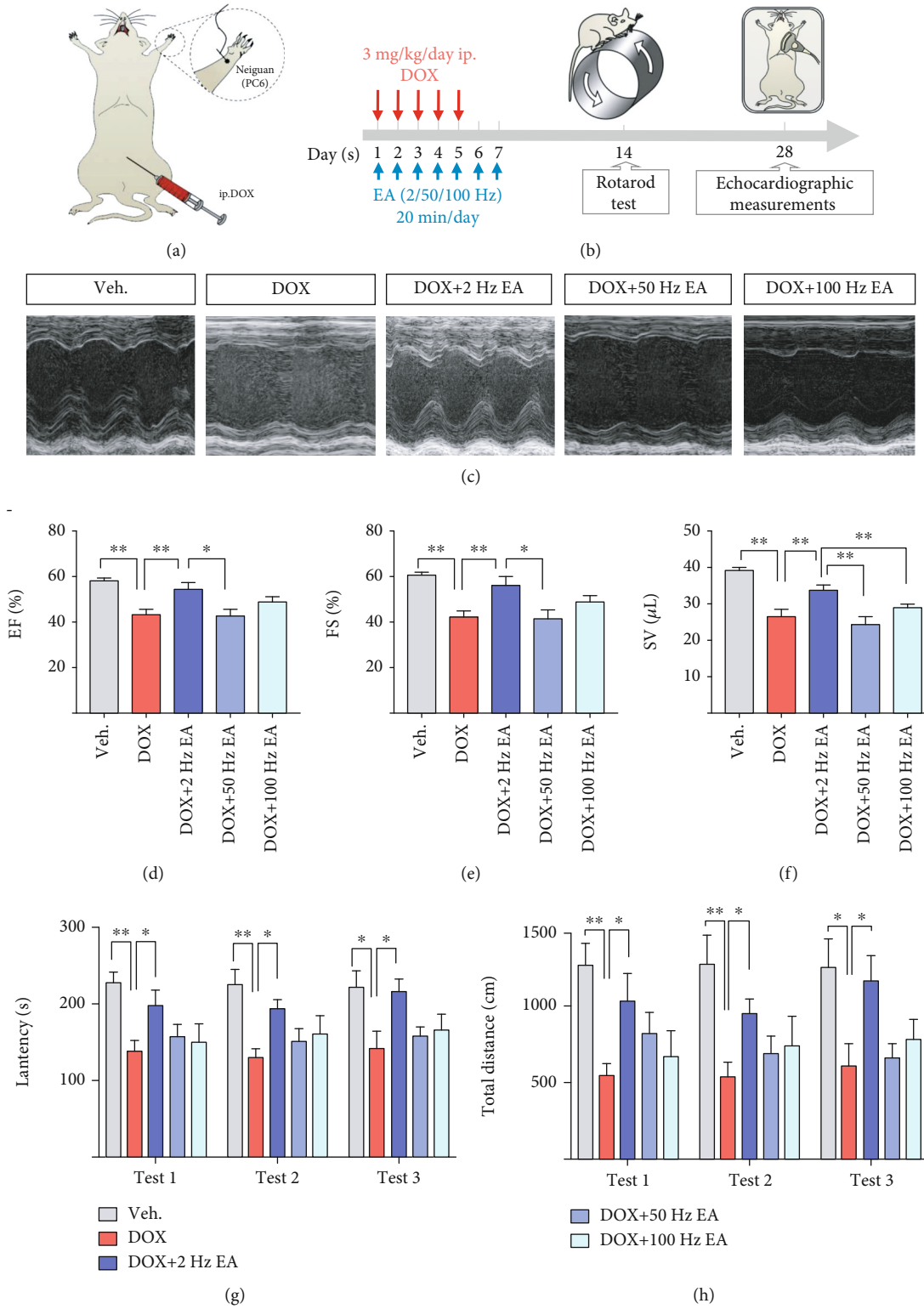


Figure 1: EA ameliorated DOX-induced heart dysfunction and impaired motor activity. (a) Illustration of the location of acupoint (*Neiguan*, PC6) and establishment of DOX-induced cardiotoxicity mice. (b) The experimental schedule of the DOX-induced cardiotoxicity model, EA treatment, rotarod test, and echocardiography. (c) Representative M-mode echocardiographic images. (d–f) Echocardiographic measurement of EF%, FS%, and SV. (g) Latency to fall from the rod (sec) and (h) total distance on the rod (cm) of the rotarod test in Veh and DOX-induced mice with and without EA treatment. Veh: mice treated with 0.9% NaCl; DOX: mice treated with DOX; DOX+2 Hz EA: DOX-induced mice treated with 2 Hz EA treatment; DOX+50 Hz EA: DOX-induced mice treated with 50 Hz EA treatment; DOX+100 Hz EA: DOX-induced mice treated with 100 Hz EA treatment; PC6: *Neiguan* acupoint; EA: electroacupuncture; DOX: doxorubicin; FS: fractional shortening; EF: ejection fraction; SV: stroke volume. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$, $n = 8$-10 mice/group.

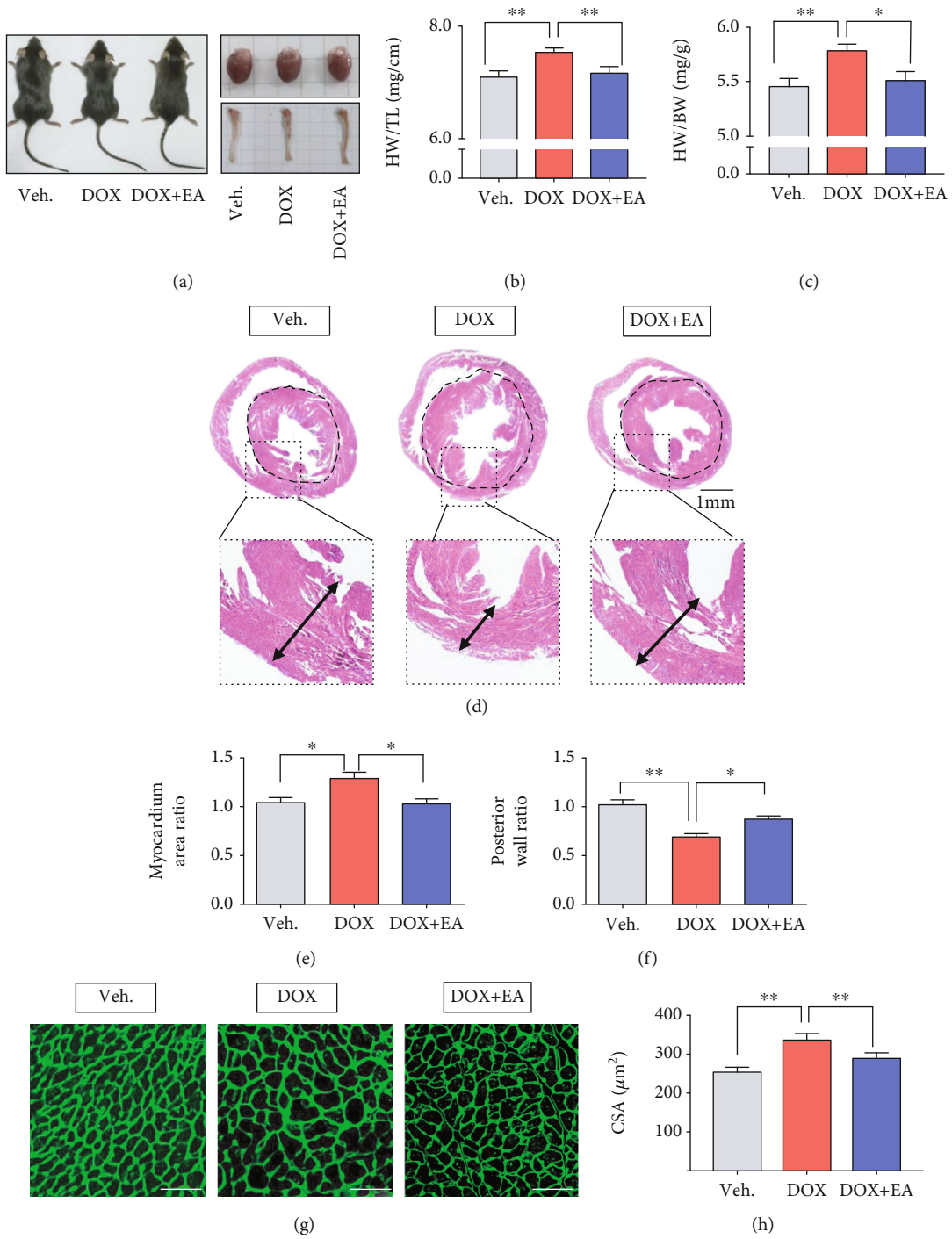


Figure 2: EA attenuated DOX-induced cardiac hypertrophy. (a) Photos of representative mice, heart and tibia samples of Veh, DOX, and DOX+EA groups. (b) The ratios of heart weight to tibia length (HW/TL) in each group. (c) The ratios of heart to body weight (HW/BW) in each group. (d) Representative images of heat sections by hematoxylin and eosin (H&E) staining in each group (scale bar = 1 mm). (e, f) Quantification of myocardium area ratio and posterior wall ratio of each heart section. (g) Representative images of heart sections by wheat germ agglutinin (WGA) staining in each group (scale bar = 50 μm). (h) Quantification of cardiomyocyte size. Veh: mice treated with 0.9% NaCl; DOX: mice treated with DOX; DOX+EA: DOX-induced mice treated with 2 Hz EA treatment. Values are presented as mean ± SEM, $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 10$ mice/group in a ratio of HW/TL and HW/BW. $n = 3$ mice/group in H&E and WGA staining.

staining. As shown in Figures 2(d)–2(f), the left ventricle chamber was larger and the ventricular posterior wall was thinner in the DOX group as compared with the control group. The EA treatment prevented these DOX-induced alterations. Consistently, DOX increased the size of the cardiomyocytes and this was also blocked by EA treatment (Figures 2(g) and 2(h)). These data indicated that DOX-induced hypertrophy was alleviated by EA at PC6.

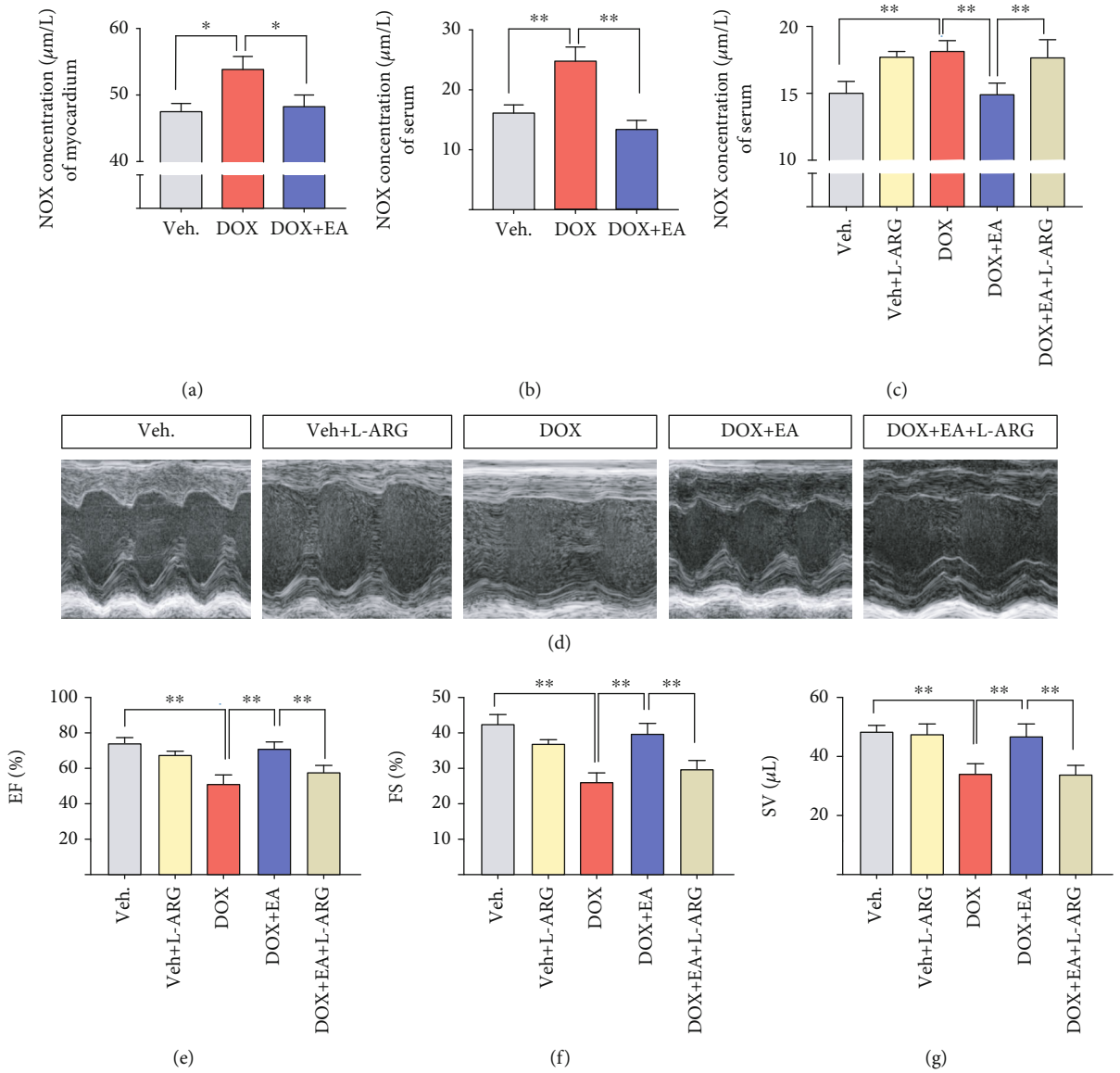


Figure 3: EA prevented DOX-induced cardiotoxicity through regulating the NO level. (a, b) NO concentration in heart tissue and serum of Veh, DOX, and DOX+EA groups. (c) NO concentration in serum of Veh, Veh+L-arginine (L-ARG), DOX, DOX+EA, and DOX+EA+L-ARG groups. (d) Representative M-mode echocardiographic images of the heart. (e–g) Echocardiographic measurement of cardiac EF%, FS%, and SV values in each group. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$. $n = 6$-10 mice/group.

3.3. EA Prevented DOX-Induced Cardiac Dysfunction through Regulation of NO Production. It has been reported that DOX-induced cardiotoxicity can be alleviated by modulating NO levels in the myocardium [45]. To determine whether EA treatment prevents DOX-induced heart dysfunction through an effect on NO, we first measured NO levels in serum and myocardium. We found that NO levels in both serum and heart were markedly increased in the DOX-treated group compared to the control group and this increase was blocked in the EA treatment group, suggesting that EA at PC6 prevented the DOX-induced increases in NO production (Figures 3(a) and 3(b)). Since L-arginine is the substrate used by NOS in the process of NO production [19], we treated mice with L-arginine supplement in DOX +EA groups. The purpose of this experiment is to determine whether the protective effect of EA was achieved by regulating the NO levels. First, we examined the impact of L-arginine on the increased NO level and cardiotoxicity induced by DOX. NO serum levels were analyzed in the five groups, which showed that the two groups with the administration of L-arginine had increased the NO level, compared with control and DOX+EA groups (Figure 3(c)). Next, we found that the values of FS%, EF%, and SV were decreased in the DOX+EA+L-ARG group, compared to the DOX+EA group, which suggested that the protective effect of EA was blocked by the increased NO production following the L-arginine administration (Figures 3(d)–3(g)). It was reported that NO was involved in the generation of oxidative/nitrosative stress [21]. Thus, we measured the levels of MDA and 3-NT in the heart tissues. Both levels of MDA and 3-NT were

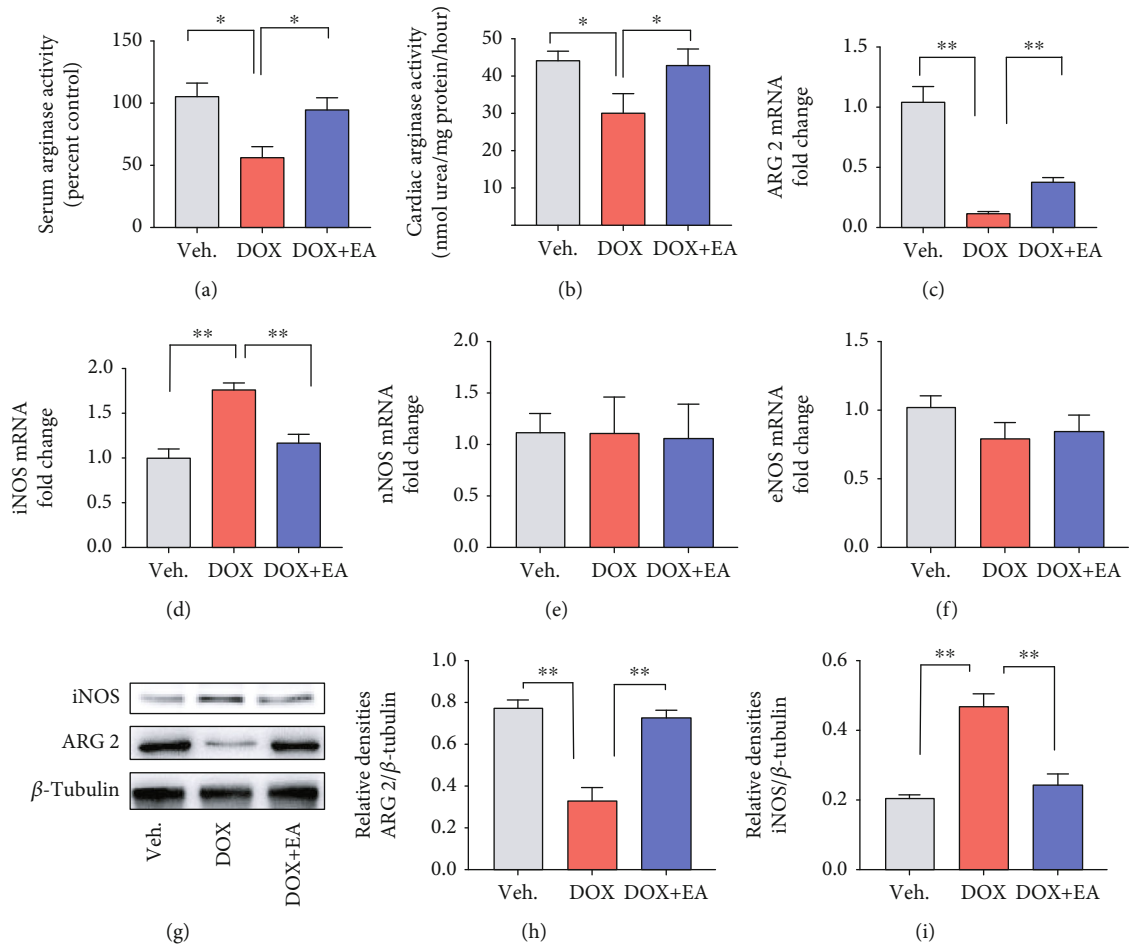


Figure 4: EA enhanced arginase activity and ARG 2 level in DOX-treated mice with reduced iNOS level. (a, b) The arginase activity in serum and cardiac tissues of Veh, DOX, and DOX+EA groups. (c–f) mRNA levels of ARG 2, iNOS, nNOS, and eNOS in cardiac tissues of Veh, DOX, and DOX+EA groups. (g) Representative images of ARG 2 and iNOS protein expression in cardiac tissues of each group. (h, i) Quantification of ARG 2 and iNOS protein expression in each group. β-Tubulin was used as the loading control. iNOS: inducible nitric oxide synthase; ARG 2: arginase 2; eNOS: endothelial nitric oxide synthase; nNOS: neuronal nitric oxide synthase. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$. $n = 6$ mice/group.

increased in DOX-induced mice significantly, which were prevented by EA treatment at PC6 (Figure S2). Increased levels of cardiac NO in DOX-induced heart may be activated by iNOS via inflammatory cytokines [46]. Next, we found increased cardiac inflammation including the changes of mRNA levels of TNF-α, IL-1β, and IL-10 in DOX-induced heart tissues, which were ameliorated by EA treatment as well (Figure S3). Together, these results indicated that a reduction in NO production is required for the therapeutic benefits of the EA treatment.

3.4. EA Modulated DOX-Induced Abnormal Levels of iNOS and ARG 2. To further examine the role of NOS and arginase signaling in the effect of EA treatment, we next analyzed the arginase activity, expression levels of ARG 2, and three NOS isoforms including iNOS, eNOS, and nNOS in myocardial tissue. Both arginase activities in serum and myocardium were decreased significantly in the DOX group (Figures 4(a) and 4(b)), along with the reduced mRNA level of ARG 2 (Figure 4(c)). In contrast, the level of iNOS mRNA was increased in the DOX group compared to the control group (Figure 4(d)), but eNOS and nNOS were not altered (Figures 4(e) and 4(f)). Consistent with the changed mRNA level, there were lower ARG 2 levels and higher protein levels of iNOS in myocardial tissues of the DOX group (Figures 4(g)–4(i)). Furthermore, EA treatment at PC6 reversed the DOX-induced abnormal levels of iNOS and ARG 2 (Figures 4(a)–4(d) and 4(g)–4(i)). These results indicated that DOX administration caused both abnormal levels of iNOS and ARG 2, which can be restored by EA at PC6.

3.5. DOX Induced the Increased iNOS but Decreased ARG 2 Levels in Myocardial Cells. To further clarify whether cardiomyocyte ARG 2 and iNOS are involved in the DOX-induced cytotoxicity, we performed a set of experiments in rat primary cardiomyocytes (Figure 5(a)). Exposure of myocardial cells to the different concentrations of DOX (0, 1.25, 2.5, 5, and 10 μM) for 24 h caused a significant reduction of cell viability and arginase activity with the enhancement of NO production in a dose-dependent way (Figures 5(b)–5(d)).

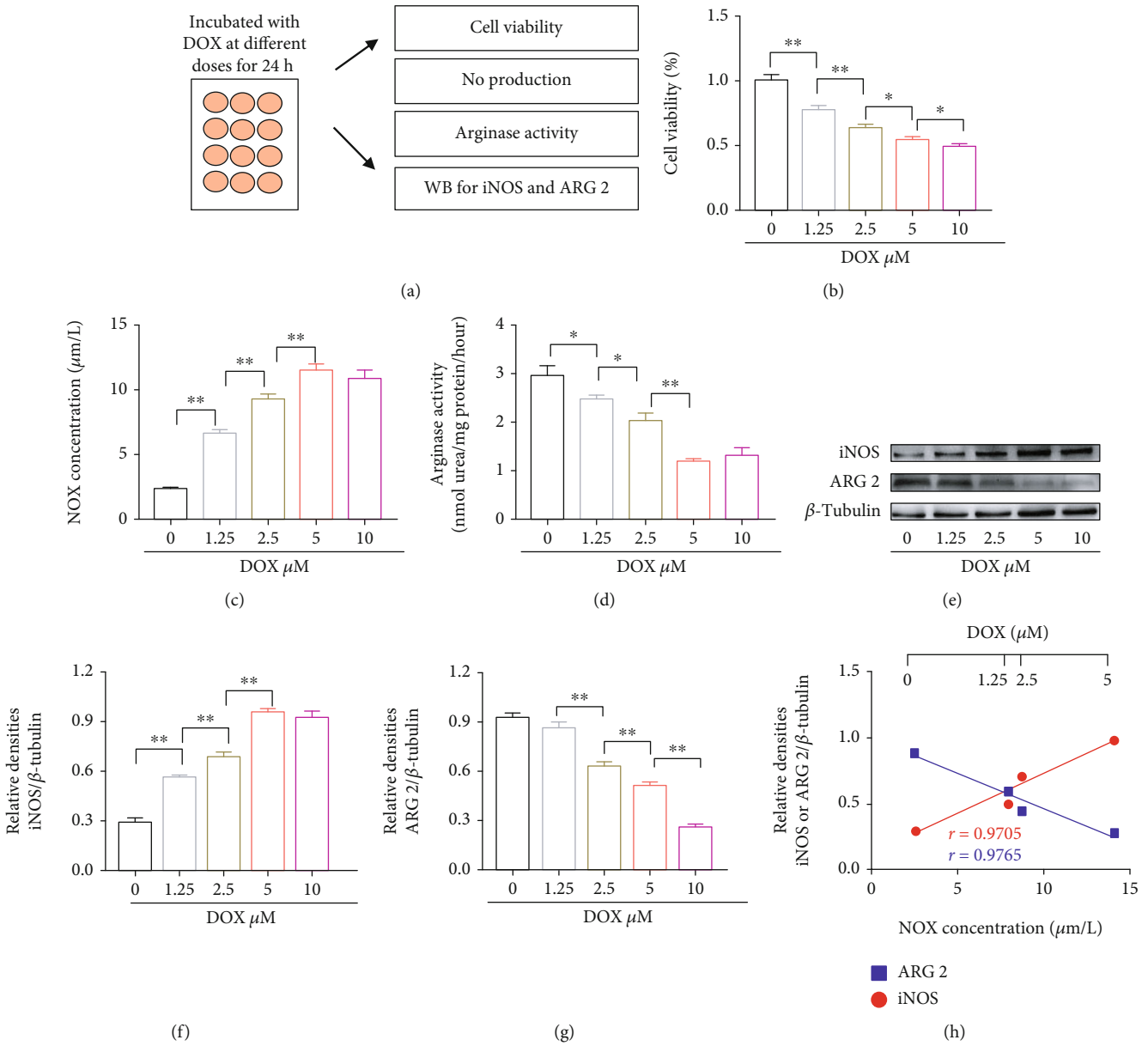
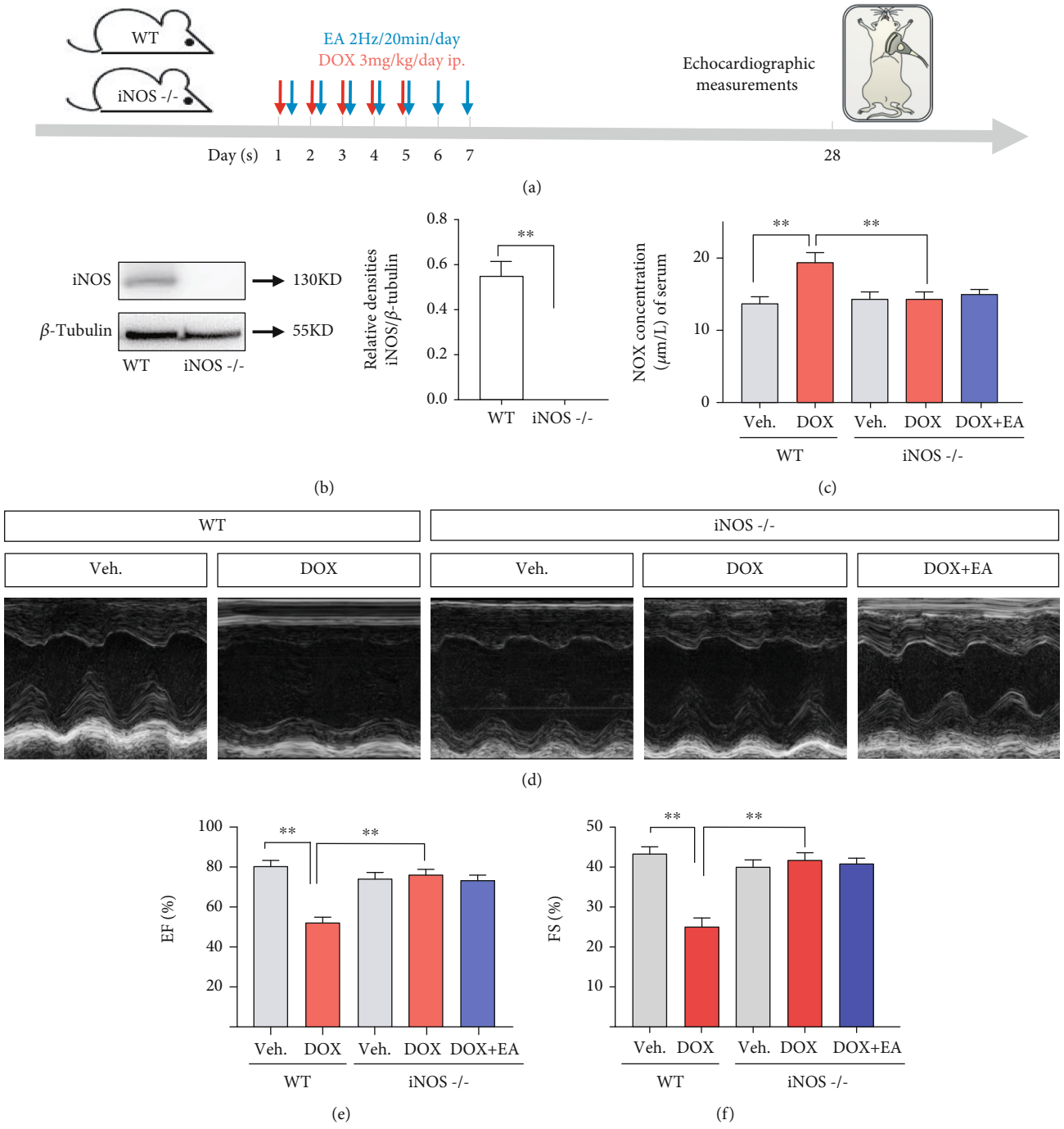


Figure 5: Both iNOS and ARG 2 are implicated in the DOX-induced cytotoxicity in myocardial cells. (a) The schematic diagram of rat embryonic ventricular myocardial cells with DOX inducement. (b) The value of cell viability, (c) NO production, and (d) arginase activity under the various concentrations of DOX inducement (1.25, 2.5, 5, and 10 μM) in rat primary cardiomyocytes. (e) Presentative images of iNOS and ARG 2 protein expression in rat primary cardiomyocytes with and without DOX inducement (0, 1.25, 2.5, 5, and 10 μM), and β-tubulin was used as the loading control. (f, g) Quantification of iNOS and ARG 2 protein expression in all groups. (h) Correlation analysis of iNOS and ARG 2 protein expression to NO production, respectively, in all groups. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$. $n = 6$ samples/group.

Furthermore, all different concentrations of DOX significantly increased iNOS and reduced ARG 2 protein levels of myocardial cells compared with controls (Figures 5(e)–5(g)). As shown in Figure 5(h), the DOX-mediated level of NO production was positively correlated with iNOS levels ($r = 0.9705$), but negatively correlated with ARG 2 protein levels ($r = 0.9765$) at different concentrations of DOX stimulation (0, 1.25, 2.5, and 5 μM). Together, the above results suggested that both alterations of cardiomyocyte iNOS and ARG 2 levels can contribute to the abnormal NO production induced by DOX.

3.6. iNOS Was Critical for Protection of EA against DOX-Induced Cardiac Dysfunction. To further examine the role of iNOS in DOX-induced heart dysfunction and EA treatment, we performed experiments using iNOS-/- mice (Figure 6(a)). First, we confirmed that the iNOS protein level was undetectable in iNOS-/- mice (Figure 6(b)). As shown in Figure 6(c), knocking out of iNOS blocked the NOX-induced increase in NO production. We also found that EA treatment did not alter the levels of NO in the iNOS-/- mice with and without DOX administration. Next, we performed echocardiographic measurement to evaluate heart function in the



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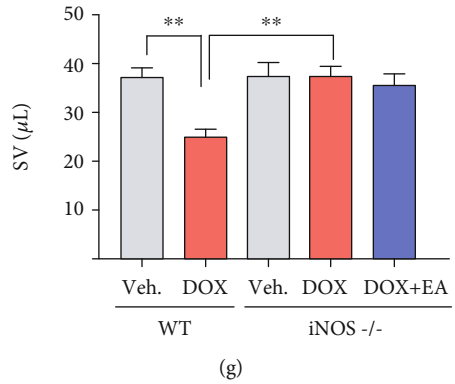


Figure 6: Prevention of EA in DOX-induced cardiotoxicity was blocked by knocking out iNOS. (a) The experimental schedule of DOX-induced cardiotoxicity model, EA treatment, and echocardiography measurement in wild-type (WT) mice and iNOS knocking out (iNOS-/-) mice. (b) The protein expression of iNOS in WT and iNOS-/- hearts; (c) NO concentration of serum in Veh and DOX-induced WT and iNOS-/- mice with and without EA treatment; (d) representative M-mode echocardiographic images of hearts; (e–g) echocardiographic measurement of cardiac EF%, FS%, and SV values in each group. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$, $n = 6$ mice/group.

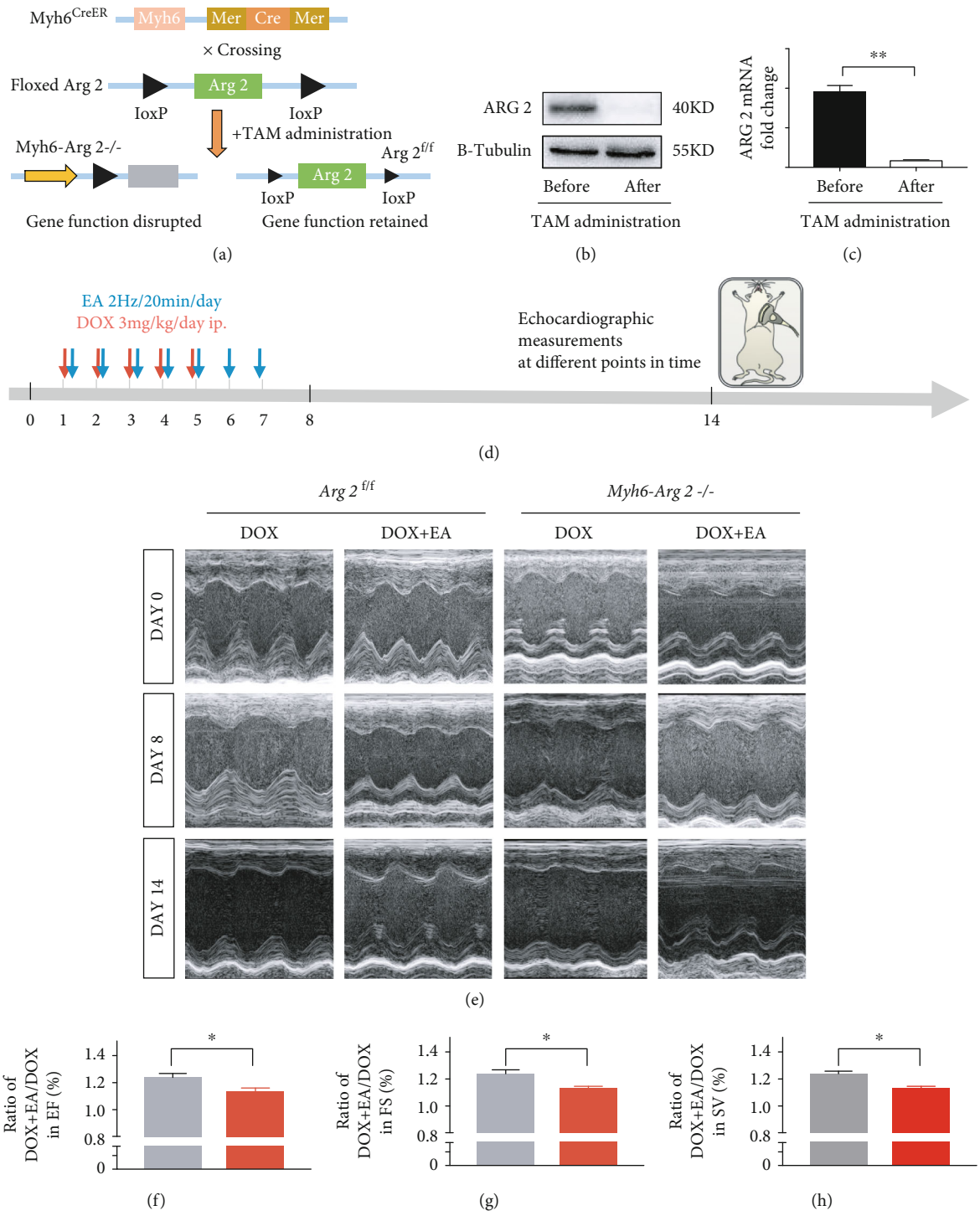
different groups. Unlike the DOX-treated wildtype control which showed significant decreases in EF%, FS%, and SV, iNOS-/- mice with the DOX treatment showed no changes in the above three test indexes. This data suggests that knocking out iNOS can prevent the DOX-induced cardiac dysfunction (Figures 6(d)–6(g)). Considering that both EA treatment and iNOS knockout prevent DOX-induced heart impairment, we then determined whether they act via the same mechanism. If so, EA should not be able to further improve the heart function in the DOX-treated iNOS-/- mice. Consistent with this hypothesis, EA at PC6 failed to further increase the values of EF%, FS%, and SV in iNOS-/- mice, compared with DOX-treated iNOS-/- mice (Figures 6(d)–6(g)). Together, these results indicated that iNOS is a therapeutic target for the prevention of EA treatment against DOX-induced cardiotoxicity.

3.7. Ablation of Cardiomyocyte-Specific ARG 2 Exacerbated DOX-Induced Cardiac Dysfunction and Weakened the Protective Effect of EA Treatment. The change of ARG 2 and iNOS levels presented an opposite trend in both DOX-induced cardiomyocytes and cardiac tissues (Figures 4 and 5). Meanwhile, arginase negatively regulated NO production by competing with NOS for their common substrate L-arginine. Therefore, we hypothesized that DOX induced cardiotoxicity through breaking the ARG 2/iNOS balance. If so, ablation of cardiomyocyte-specific ARG 2 should exacerbate DOX-induced cardiac dysfunction and weaken the protective effect of EA treatment. To this end, ARG $2^{f/f}$ mice were crossed with Myh6-CreER mice to generated mice lacking ARG 2 in myocardial cells until TAM administration (Myh6-ARG 2-/-) (Figure 7(a)). As shown in Figures 7(b) and 7(c), ARG 2 protein and mRNA level in myocardial tissues were markedly reduced in Myh6-ARG 2-/- mice, compared to ARG $2^{f/f}$ mice. Both ARG $2^{f/f}$ and Myh6-ARG 2-/-mice have received DOX injection for 5 days and EA treatment for 7 days from the same day; echocardiographic measurements were performed at 0, 8, and 14 days of DOX injection (Figure 7(d)). As Figures 7(i)–7(k) show, Myh6-ARG 2-/- mice exhibited the same cardiac function before DOX treatment and the significant impairment of heart function at 14 days from the first DOX-injection, compared with ARG $2^{f/f}$ mice. Furthermore, it is worth noting that the deficiency of ARG 2 in myocardial cells reduced the degree of improvement of EA on DOX-induced cardiac dysfunction, which was presented by the lower ratios of DOX +EA to DOX in the values of EF%, FS%, and SV from Myh6-ARG 2-/- mice, compared with ARG $2^{f/f}$ mice (Figures 7(f)–7(h)). All results suggested that myocardial ARG 2 is also critical in DOX-induced heart dysfunction and in the protection of EA against DOX-induced cardiotoxicity.

4. Discussion

Neiguan (PC6) acupoints have been reported to effectively improve cardiac function in various diseases [10]. In this study, EA at PC6 significantly improved left ventricular systolic dysfunction and cardiac hypertrophy and increased motor ability in mice with DOX-induced cardiotoxicity (Figures 1 and 2). Consistent with our results, one study showed that puncturing acupoints PC6 and PC4 (*Ximen*) enhanced the contractility of the left ventricle wall and increased the stroke volume of the heart in patients with coronary heart disease [47]. In addition, a randomized clinical trial showed that EA pretreatment at PC6 and PC4 reduced myocardial injury after postpercutaneous coronary intervention in patients with coronary artery diseases [48]. Moreover, acupuncture at PC6 improved cardiac function in a myocardial ischemia rat model by increasing left ventricular diastolic and systolic function [14]. Similarly, acupuncture at PC6 prevented myocardial hypertrophy [49] and improved the value of FS% in mice with cardiac hypertrophy [13]. Our results provide evidence for the first time that stimulation at PC6 is an effective therapy to prevent DOX-induced cardiotoxicity.

The appropriate frequency of EA stimulation at PC6 for treatment of DOX-induced cardiotoxicity is still unclear.



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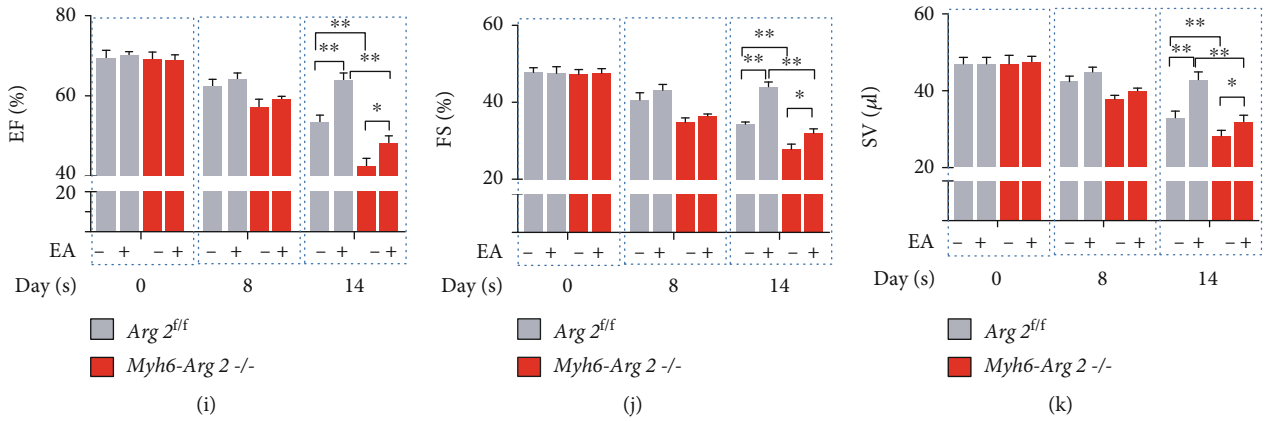

Figure 7: Deficiency of ARG 2 in the heart weakened the protective effect of EA in DOX-induced cardiac dysfunction. (a) Mating strategy of Myh6-ARG 2-/- mice from ARG $2^{f/f}$ and Myh6-CreER mice; (b, c) ARG 2 protein and mRNA levels in myocardial tissue of ARG $2^{f/f}$ and Myh6-ARG 2-/-mice; (d) the experimental schedule of DOX administration, EA treatment, and echocardiographic measurement in ARG $2^{f/f}$ and Myh6-ARG 2-/- mice; (e) representative M-mode echocardiographic images in DOX-induced ARG $2^{f/f}$ and Myh6-ARG 2-/- mice with and without EA treatment at 0, 8, and 14 days of DOX injection; (f–h) ratios of DOX+EA to DOX in the values of EF%, FS%, and SV in DOX-induced ARG $2^{f/f}$ and Myh6-ARG 2-/- hearts; (i–k) echocardiographic measurement of EF%, FS%, and SV values at three different points of time in DOX-induced ARG $2^{f/f}$ and Myh6-ARG 2-/- mice with and without EA treatment. Values are presented as mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$, $n = 8$-12 mice/group.

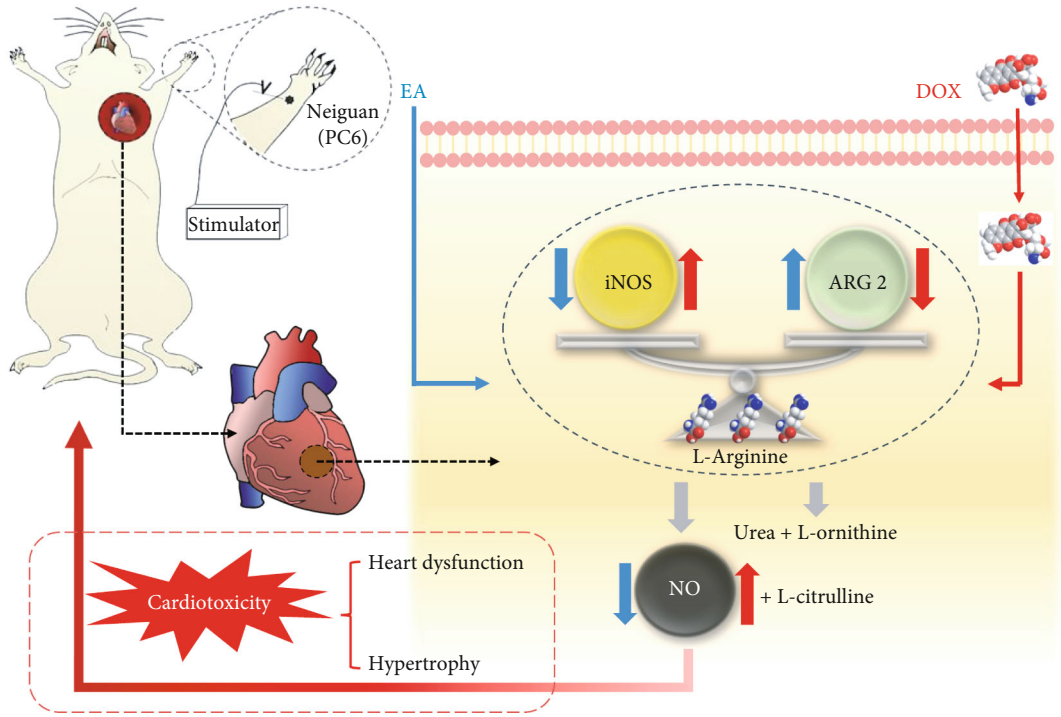

Figure 8: A proposed mechanism underlying DOX-induced cardiotoxicity and EA treatment. DOX inducement caused the abnormal NO signaling including increased ARG 2 and decreased iNOS levels in myocardial cells. EA prevented against DOX-induced heart dysfunction and hypertrophy by regulating cardiomyocyte iNOS/ARG 2 balance.

Our results provide evidence to show that 2 Hz is the most effective level of EA in DOX-treated mice compared with 50 Hz and 100 Hz (Figure 1). Similarly, it was reported that the application of low-frequency EA significantly improved heart rate variability, sympathetic stress, and parasympathetic vagal tone in patients [50]. Compared with the stimulation frequency of 40 or 100 Hz, percutaneous EA at the PC5 and PC6 acupoints with 2 Hz protected against the stress-induced myocardial ischemia [51]. In addition, low-frequency (1-5 Hz) EA inhibited the reflex cardiovascular pressor response via inhibiting sympathetic excitation [52]. The underlying mechanism for this was unknown, but one study showed that the somatic sensory nerve fiber stimulation underlies acupuncture's cardiovascular actions and

low-frequency EA can lead to much greater activation of afferent fibers than higher stimulation frequencies [53]. In other diseases including chronic pain and erectile dysfunction in a rat model, low-frequency EA also proved more effective in treating different diseases [54, 55].

NO is a small signaling molecule that is critically involved in cell growth, differentiation, and apoptosis. Our results showed that DOX-induced cardiotoxicity in mice is associated with increased levels of NO, oxidative/nitrosative stress, and inflammation of myocardium (Figure 3, Figures S2 and S3), consisting with the conclusion in another study that DOX-induced cytotoxicity is due to an increase in oxidative/nitrosative stress and inflammation [18]. Furthermore, our study found that the EA at PC6 treatment improved heart function in DOX-treated mice by a mechanism involving reductions in NO levels (Figure 3). Consistent with our results, decreased NO serum level resisted DOX-induced cardiotoxicity [56]. Studies have shown that the DOX-induced damage of cardiac tissue can be prevented through reductions in activities of NOS [57]. The expression of the inducible NOS isoform iNOS is frequently associated with inflammation and malignant diseases [58]. Previous studies have validated the cardioprotective effect of iNOS inhibition in DOX-mediated cardiotoxicity [21, 22]. Consistently, we found that EA at PC6 decreased the expression of iNOS in heart tissue in DOX mice (Figure 4). Our data also showed that knocking out iNOS can protect against DOX-induced cardiotoxicity. However, the protective effect protection was not further improved by combination with EA treatment (Figure 6). This suggests that the protective effect of EA at PC6 on DOX-induced cardiotoxicity occurs via reducing the expression of iNOS.

Previous studies supported that the treatment of diseases in TCM is through the downregulation of "hyperactive" signaling pathways and the upregulation of "deficient" signaling pathways, which can balance and restore the normal status [59]. Excessive arginase is highly involved in the regulation of NO production by competing with NOS for L-arginine, causing NOS uncoupling in several disease models [60]. Similarly, we found that the abnormal increasing NO in DOX-induced cardiomyocytes was caused by the imbalance between the expression of iNOS and ARG 2 in this study (Figure 8). Consistently, other studies reported that the competition of the common substrate between arginase and eNOS affected NO production and further caused endothelial dysfunction condition, which can be improved by adjusting both ARG 2 and eNOS signaling [61, 62]. Additionally, it was worthy to point out that EA treatment still ameliorated the damage of the heart due to the ablation of myocardial ARG 2, which suggested EA ameliorating DOX-induced cardiotoxicity through other signaling besides the ARG 2 pathway, which needs to be further investigated.

5. Conclusions

In the current study, we found that EA at PC6 with 2 Hz effectively protected against DOX-induced cardiotoxicity and overactivated iNOS-NO signaling. Second, cardiomyocyte-specific ARG 2 exacerbated DOX-induced cardiotoxicity accompanied with abnormal NO production and iNOS increased. Third, genetically intervention iNOS reduced the DOX-induced cardiotoxicity and EA treatment in iNOS-/- mice did not cause the further improvement on the impaired cardiac function. In contrast, ablation myocardial ARG 2 exacerbated DOX-induced cardiac dysfunction and weakened the protective effect of EA treatment. These results revealed that the cardioprotection of EA treatment against DOX-induced cardiotoxicity is through modulating the iNOS/ARG 2 balance and restoring the impaired NO production in cardiomyocytes.

Data Availability

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

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

YJ Chen and L Yao conceived and designed the protocol. JY Wang, XL Wu, Q Guo, SX Sun, and J Li performed the experiments. JY Wang and XL Wu analyzed the data. JY Wang, L Yao, and YJ Chen wrote the paper. RB Caldwell and RW Caldwell provided the iNOS-/- mice and helped to review the manuscript. All the authors reviewed and approved the submitted version of the paper. Jingya Wang and Lin Yao contributed equally to this work.

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Supplementary Materials

Figure S1: schematic diagram of the measurement of mouse left ventricle function by ultrasound system and the calculation of parameters. An illustration on the left is B-mode ultrasound of the short axis section in the left ventricle, and an orange single scan line is placed next to the papillary muscles. An illustration on the right is M-mode ultrasound recording the left ventricular motion curve in the area marked by an orange single scan line. The measurement parameters included left ventricular end-diastolic diameter (LVID; d) and end-systolic diameter (LVID; s), left ventricular end-diastolic anterior wall thickness (LVAW; d), end-

systolic anterior wall thickness (LVAW; s), left ventricular end-diastolic posterior wall thickness (LVPW; d), and left ventricular end-systolic posterior wall thickness (LVPW; s). The above parameters are calculated by Vevo 2100 to get left ventricular ejection fraction (EF%), shortening rate of left ventricular short axis (FS%), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV). Stroke volume (SV) was calculated as follows: SV = LVEDV − LVESV. Figure S2: EA treatment prevented abnormal levels of cardiac oxidative/nitrosative stress in DOX-induced mice. (A) The cardiac MDA level in each group; (B) the cardiac 3-NT level in each group; Veh: mice treated with 0.9% NaCl; DOX: mice treated with DOX; DOX +EA: DOX-induced mice treated with 2 Hz EA treatment. MDA: malondialdehyde; 3-NT: 3-nitrotyrosine.Values are presented as mean ± SEM, $^{**}P < 0.01$. $n = 10$ mice/group. Figure S3: EA treatment reduced cardiac inflammation levels in DOX-induced mice. (A-C) mRNA levels of TNF-α, IL-1β, and IL-10 in heart tissues of Veh, DOX, and DOX+EA groups. Veh: mice treated with 0.9% NaCl; DOX: mice treated with DOX; DOX+EA: DOX-induced mice treated with 2 Hz EA treatment. Values are presented as mean ± SEM, $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 10$ mice/group. Supplementary Table 1: experiment for investigating the best frequency of EA treatment. Supplementary Table 2: experiment for investigating the production of NO in the efficacy of EA. Supplementary Table 3: experiment for investigating the role of iNOS in the efficacy of EA. Supplementary Table 4: experiment for investigating the role of ARG 2 in the efficacy of EA. *(Supplementary Materials)*

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Review Article

Role of Oxidative Stress in the Mechanisms of Anthracycline-Induced Cardiotoxicity: Effects of Preventive Strategies

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Anthracycline-induced cardiotoxicity (AIC) persists as a significant cause of morbidity and mortality in cancer survivors. Although many protective strategies have been evaluated, cardiotoxicity remains an ongoing threat. The mechanisms of AIC remain unclear; however, several pathways have been proposed, suggesting a multifactorial origin. When the central role of topoisomerase 2β in the pathophysiology of AIC was described some years ago, the classical reactive oxygen species (ROS) hypothesis shifted to a secondary position. However, new insights have reemphasized the importance of the role of oxidative stress-mediated signaling as a common pathway and a critical modulator of the different mechanisms involved in AIC. A better understanding of the mechanisms of cardiotoxicity is crucial for the development of treatment strategies. It has been suggested that the available therapeutic interventions for AIC could act on the modulation of oxidative balance, leading to a reduction in oxidative stress injury. These indirect antioxidant effects make them an option for the primary prevention of AIC. In this review, our objective is to provide an update of the accumulated knowledge on the role of oxidative stress in AIC and the modulation of the redox balance by potential preventive strategies.

1. Introduction

Over the past two decades, there have been significant improvements in the early detection and pharmacological treatment of cancer, leading to a dramatic increase in survivorship [1] [2]. However, this improvement in the life expectancy of cancer patients has also led to an increase in the pool of patients at risk of experiencing long-term chemotherapy-related side effects. Chemotherapy-induced cardiotoxicity is a common complication of many cancer therapeutics and a frequent cause of morbidity and mortality in cancer survivors. Amongst cancer therapeutics, anthracycline compounds contribute to a significant proportion of the cardiovascular disease burden.

Anthracyclines are specific cytostatic antibiotics, and they represent one of the most used chemotherapeutic agents to treat many solid cancer tumors and hematological malignancies [3]. Its clinical use has, however, been limited by the development of cardiotoxicity in a cumulative dose-dependent manner [4]. This side effect impacts the long-term prognosis of patients treated successfully from an oncological point of view [5]. The effects of anthracycline-induced cardiotoxicity (AIC) have become more apparent for several reasons, including a stricter clinical follow-up and improvements in cardiovascular diagnostic methods [6].

A meta-analysis by Lotriionte et al. evaluated the late incidence of AIC after a median of 9 years follow-up, finding an occurrence of clinically evident cardiotoxicity in 6% and

subclinical cardiotoxicity in 18% [7]. More recently, Cardinale et al. prospectively followed adult patients treated with anthracyclines and found an incidence of AIC of 9%. AIC was detected within the first year after completion of treatment in 98% of cases [8]. An important finding was that close monitoring of cardiac function during this period allowed early detection and treatment of cardiotoxicity, with significant left ventricular ejection fraction (LVEF) recovery in most cases [8]. Current clinical interventions are focused on prompt detection of subclinical damage through cardiac imaging and biomarker techniques; however, these interventions are focused on damage control rather than a preventative approach. Unfortunately, despite decades of research efforts to improve clinical strategies of primary prevention of AIC, there is still no satisfactory therapy to avoid this complication. Therefore, a better understanding of the mechanisms of cardiotoxicity could offer new opportunities to provide optimal primary prevention strategies.

The objective of this review is to provide an update on the accumulated knowledge regarding the early and critical role of oxidative stress in the damage mechanisms of AIC and discuss the potential benefits of preventive strategies that reduce oxidative stress damage through lifestyle changes, physical exercise, and pharmacological therapies to reduce risk factors and environmental stressors.

2. Mechanisms of Anthracycline-Induced Cardiotoxicity

Anthracyclines tend to accumulate in the mitochondria, which partly explains their tendency to accumulate in myocardial tissue, which is characterized by a high mitochondrial density due to its high energetic demand [9]. Specifically, in the heart, cardiomyocytes have classically been considered the primary cellular target of the toxic anthracycline effect. However, other cell types such as cardiac progenitor cells, cardiac fibroblasts, and endothelial cells have also been recognized as potential anthracycline targets, which through paracrine effects mediated by microRNA (miRNA) and other cells signals could also be involved in cardiomyocyte injury [10, 11]. Several pathways have been proposed to explain the development of AIC, such as the potential generation of oxidative stress, inhibition of topoisomerase 2β (Top2β), changes in iron metabolism, and Ca2+ signaling [10]. However, the precise reason as to why only some patients develop AIC remains unclear, suggesting a multifactorial origin that could comprise complex interactions between the different involved pathways [12].

2.1. Anthracycline Accumulation in the Heart. One of the most significant determinants of the development of AIC is the cumulative dose of anthracyclines in cardiac tissue [13], which is also related to the magnitude of redox imbalance. For this reason, the cumulative dose remains the leading risk factor for AIC [7]. Furthermore, anthracyclines are more retained within cardiomyocytes than in cells of other noncardiac tissues [14]. The primary process that determines heart accumulation is the liver biotransformation to secondary alcohol metabolites. These metabolites are more polar than original compounds and exhibit a higher entry rate and reduced elimination rate from cardiac tissue [15, 16]. Doxorubicinol, the most crucial alcohol metabolite of doxorubicin, has been implicated in the cardiotoxicity observed in doxorubicin-treated patients [17, 18]. The NADPH-dependent reduction of doxorubicin to doxorubicinol is catalyzed by carbonyl reductase 1 (CBR1), a well-characterized monomeric enzyme present at high basal levels in the liver [19] and carbonyl reductase 3 (CBR3) less characterized, present in the liver at low basal levels [20]. Animal models have shown that high metabolizer mice develop an accelerated cardiotoxicity course through an increased heart accumulation of secondary alcohol metabolites [21]. Therefore, hepatic biotransformation of anthracyclines represents a potential research focus to establish higher risk groups and new targets for pharmacological interventions. At a subcellular level, the accumulation of anthracycline secondary alcohol metabolites in cardiomyocytes is especially pronounced in the mitochondria, affecting the mitochondrial transmembrane potential, and inhibiting the complex I respiratory chain, which causes an impairment of mitochondrial metabolism and subsequent mitochondrial dysfunction [22].

2.2. Convergence of "Redox Cycling" and "Topoisomerase 2β" Hypotheses: ROS Generation and AIC. Despite the multiple mechanisms of AIC described, there is consensus in considering "Redox Cycling" and the "Top2β inhibition" as the two main mechanisms. Several preclinical and clinical trials, as well as genetic studies, have shown that oxidative stress generated by reactive oxygen species (ROS) accumulation is the crucial step in the development and progression of AIC [23–26]. Initially, it was suggested that early ROS would be produced by a direct anthracycline interaction with the mitochondrial electron transport chain [9] [27]. The enzymatic antioxidant defences are lower in the cardiac tissue compared with other organs (liver and kidney); this can make the heart particularly vulnerable to free radical damage [12].

Later studies suggested that interaction with topoisomerase 2β (Top2β) would be the initiating trigger for cardiotoxicity [24]. Top2β is an enzyme involved in nuclear and mitochondrial DNA replication, which plays a crucial role in AIC for the formation of anthracycline–DNA-topoisomerase 2β complexes [24]. Some mechanisms studied as possible mediators of Top2β-dependent cardiotoxicity include p53 activation, modulation of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and -1β (PGC-1β), and modulation of antioxidant enzyme gene transcription [24]. Both PGC-1α and PGC-1β are highly expressed in the heart, playing a critical role in mitochondrial biogenesis regulating oxidative metabolism [28, 29]. They have also been associated with heart failure pathogenesis [30, 31]. Top2β inactivation by anthracycline accumulation heavily reduced the expression of PGC-1α and PGC-1β in rat cardiomyocytes [24]. Nevertheless, the same data also indicate that anthracycline interaction with Top2β leads to mitochondrial dysfunction with the subsequent generation of ROS-mediated oxidative stress [24]. This triggers a progressive disruption in Ca+2 homeostasis, inflammation, and the inhibition of ATP generation [32],

which in turn promotes apoptosis and cardiac remodeling, critical events in the development of AIC [24, 33].

According to the time of presentation, AIC was previously classified as acute, subacute, or chronic. Recent findings challenge this old classification, suggesting that AIC is a continuous phenomenon, starting at the myocardial subcellular level, followed by a progressive functional decline, which could lead to overt heart failure [10]. In all patients who develop AIC, the damage associated with oxidative stress starts very early at the molecular level, which causes subcellular dysfunction through various mechanisms [34]. If antioxidant defences are rapidly overwhelmed, prompt damage could occur, leading to a rare short-term event of acute AIC [34]. These acute AIC events are infrequent but associated with high mortality risk, and their manifestations may include pericarditis, arrhythmias, and left ventricular systolic dysfunction (LVSD) [35] [13]. However, it should be noted that the majority of patients with AIC do not manifest a clinically evident acute cardiotoxicity; they are usually asymptomatic and present some signs of acute subclinical cardiotoxicity evidenced exclusively by functional alterations in the left ventricle (changes in longitudinal strain or LVEF) or by an increase in cardiac biomarkers [36, 37].

2.3. - Mediators of Increased Susceptibility to Oxidative Stress Injury and AIC

2.3.1. Overview of Oxidative Stress in the Cardiovascular System. Since oxidative stress plays a crucial role in AIC, it is important to understand how ROS are generated and countered in the cardiovascular system. Oxidative stress can be defined as an imbalance between the generation and detoxification of ROS [38]. At physiological levels, a slight increase in reactive oxygen species (ROS) could induce protective effects through triggering redox signaling, for example, via improving adaptive antioxidative response by activating of Keap1/Nrf2/ARE pathway. If the ROS generation outweighs the antioxidative capacity, then at higher ROS levels, cell damage and endothelial dysfunction arise contributing to the development of atherosclerosis and heart injury [39].

Aging, genetic predisposition, traditional cardiovascular risk factors, and environmental factors can induce oxidative stress, particularly in the heart and vessels [39]. At the cardiac cellular level, enzymatic sources for ROS, such as the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), uncoupled nitric oxide (NO) synthase, and mitochondria, are all considered relevant sources of ROS that contribute to the development of vascular and cardiac dysfunction [38, 39]. Importantly, mitochondria amplify ROS derived from NOX and may thereby function as a "redox hub" in cardiac pathophysiology [40]. ROS determines myocardial calcium (Ca2+) overload, an event that plays a causal role in contractile dysfunction, arrhythmias, and the maladaptive cardiac remodeling process by inducing hypertrophic signaling, apoptosis, necrosis, and autophagy [41].

2.3.2. Cardiovascular Risk Factors Are Associated with a Significant Susceptibility to Oxidative Stress Injury. The pathophysiological effects of traditional cardiovascular risk factors on the cardiovascular system are driven by oxidative stress. Therefore, it is not surprising that many of the risk factors for AIC such as age > 65 years, valvular heart disease, baseline left ventricular dysfunction, arterial hypertension, African-American ancestry, renal failure, concomitant exposure to radiation and/or trastuzumab, iron overload, and genetic factors [3] [42] have oxidative stress as a common element. Abdel-Qadir et al. developed and validated a multivariable risk prediction model for major adverse cardiovascular events (MACE) in patients with early-stage breast cancer, where age, hypertension, diabetes, ischemic heart disease, atrial fibrillation, HF, cerebrovascular disease, peripheral vascular disease, chronic obstructive pulmonary disease, and chronic kidney disease were significantly associated with MACE [43]. Again, many of the risk factors for MACE in this study have oxidative stress as a common pathophysiological mechanism for cardiovascular disease and hence increase susceptibility to AIC.

2.3.3. Genetic Susceptibility to Oxidative Stress Injury. Susceptibility due to inherited genetic, variation could partially explain the high interindividual variability in risk of AIC [44, 45]. In this sense, a genetic approach has been used to identify patients at increased risk. Several polymorphisms in candidate genes have been proposed, some of them relate to the accumulation or biotransformation of anthracyclines, but most of them related to redox balance, either associated with antioxidant defence or ROS generation [44]. Some of these candidate genes are CBR1 and 3, NAD(P)H quinone oxidoreductase, which have been the most described and are discussed in more detail below, and also glutathione S-transferase and multidrug resistance proteins 1 and 2 [46].

(1) Carbonyl Reductase 1 and 3. As previously described, CBR1 and CBR3 catalyze the NADPH-dependent reduction of doxorubicin to doxorubicinol. Therefore, polymorphisms that contribute to higher levels of hepatic transformation result in a greater accumulation of toxic metabolites within the heart [21, 47]. Preliminary epidemiological data have shown that the human CBR3 polymorphisms, but not CBR1, are associated with differential cardiac outcomes in doxorubicin-treated patients [47, 48]. For example, in childhood cancer patients treated with doxorubicin, a relatively common polymorphism in the CBR3 gene (present in 30% of Caucasians) that encodes for a nonsynonymous amino acid change (V244M) was associated with a decreased risk of developing cardiomyopathy [47]. Furthermore, another CBR3 variant (11 $G > A$) has been shown to influence the relative expression of CBR3—and subsequent doxorubicinol formation—in a cohort of Southeast Asian breast cancer patients [48].

(2) NADPH Oxidase (NOX). NOX has been suggested as one of the most important sources of ROS in the cardiovascular system. Five NOX isoforms have been identified; in particular, NOX2 and NOX4 play a significant role in the heart signaling, as they are bound to the sarcolemma of the cardiomyocytes [49]. Due to the importance of NOX in

ROS generation, a possible association of some of their polymorphisms with the development of AIC has been studied. Thus, individuals with less active genetic variants of NOX might be protected from heart damage and fibrosis induced by anthracyclines [49].

This hypothesis has been supported by a case-control clinical study of doxorubicin-induced cardiotoxicity, showing that NADPH genetic variations can modulate the risk for acute and chronic cardiac events [50]. Also, another study confirmed the predictive value of the three NADPH oxidase polymorphisms (rs1883112, rs4673, and rs13058338), although only one of them (rs1883112) was significant in the multivariable analysis [51]. Finally, the role for the genetic variants in the generation of cardiac lesions was demonstrated in a retrospective case-control study that evaluated cardiac histological lesions and three different NADPH genotypes (rs1883112, rs4673, and rs13058338) in 97 consecutive decedent patients with cancer diagnosis (48 treated with anthracyclines) [52]. One polymorphism of the subunit p40phox of NADPH oxidase was strongly associated with increased myocardial interstitial fibrosis, which could be explained by the higher level of the regulatory subunit p40 in NOX2. On the other hand, other polymorphisms were associated with a lesser degree of oxidative stress, apoptosis, and myocardial damage after anthracycline treatment, which might be explained by lower activity or expression levels of NOX2 and NOX4. Thus, the findings of this study provide a possible mechanistic link between NADPH functional SNPs and cardiac dysfunction [52].

(3) Other Candidate Genes. A meta-analysis assessed the role of genetic polymorphisms in AIC based on 28 studies examining 84 different genes. This analysis revealed that polymorphisms in three genes were significantly associated with an increased odds of cardiotoxicity in individuals treated with anthracyclines, and two of them were associated with oxidative stress: CYBA and RAC2 genes [53]. Genetic variants in CYBA altered the NADH/NADPH oxidase activity and may be associated with the excessive production of ROS [54]. Rac2, encoded by the RAC2 gene, is a mitochondrial protein that is required in the electron transfer reaction of NADPH oxidase during the formation of ROS [55]. Alteration of the RAC2 gene results in mitochondrial dysfunction and, thus, an increase in ROS production [53]. Despite all the existing data, we must emphasize that the individual risk provided by these candidate genes was only moderate, so new prospective studies are still needed in order to validate these genetic biomarkers for clinical application [53]. Therefore, currently, the potential role of these genes for a pharmacogenomic screening approach in routine clinical practice before anthracyclines therapy remains limited.

2.4. Second-Hit Hypothesis. The second-hit hypothesis suggests that the ability of the heart to adapt to new stress conditions is impaired after exposure to anthracyclines [56]. This means that the cardiac tissue of patients previously treated with anthracyclines, even with no previous evidence of measurable subclinical damage, may have a decreased resistance to new injuries, resulting in an increased risk of developing heart failure [57]. Progenitor cell impairment secondary to anthracyclines, concomitant or subsequent treatments with other antineoplastic drugs, and genetic predisposition may play a role in the mechanism to explain the second-hit hypothesis [56]. However, the second-hit events are more likely to relate to the development of new pathological conditions, mainly cardiovascular risk factors that are associated with an oxidative imbalance, for example, hypertension, diabetes mellitus, obesity, or atrial fibrillation [58]. Furthermore, the development of coronary artery disease has also been found to be related with the late development of left ventricular dysfunction in patients treated with anthracyclines [8].

From a clinical point of view, as cancer survivors are at higher risk for other noncommunicable diseases [59], second-hits will not be infrequent in anthracycline-treated patients. It is important to highlight that cardiovascular risk factors could contribute as much as a cancer treatment to the development of diastolic and systolic dysfunction in childhood cancer survivors [60]. For instance, survivors with metabolic syndrome are more likely to have abnormal longitudinal strain and diastolic dysfunction [61]. Survivors also have a higher prevalence and a more premature presentation of hypertension and dyslipidemia [62]. In fact, it has been described that childhood cancer survivors are 15 times more likely to develop congestive heart failure and 10 times more likely to have coronary artery disease compared to their siblings [63]. Cardiovascular risk factors are known to be more frequent in survivors of breast, prostate, colorectal, and gynaecologic cancers compared to age-matched individuals, with a reported prevalence of overweight/obesity, diabetes, and hypertension of 62%, 21%, and 55%, respectively [64]. A recent study showed that older age (>60 years) or preexisting chronic diseases like hypertension and diabetes were present in the majority of patients with heart failure hospital presentations after the diagnosis of early-stage breast cancer [65], again emphasizing the importance of concomitant cardiovascular risk factors. Therefore, the development of adequate surveillance follow-up programs in cancer survivors to promote a healthy lifestyle and the early detection, assessment, and management of cardiovascular risk factors are essential [66].

3. Effects of Cardioprotective Strategies on the Redox Balance for the Prevention of Anthracycline-Induced Cardiotoxicity

3.1. Overview of Antioxidant Strategies in Cardiovascular Diseases. For many decades, researchers have tried to elucidate the role of oxidative stress in cardiovascular disease, establishing that a redox imbalance with subsequent oxidative stress is essential for the development of many cardiovascular diseases, including atherosclerosis, hypertension, and congestive heart failure [67]. However, a wide variety of antioxidant strategies to prevent cardiovascular disease has not yielded positive results with respect to their clinical efficacy [68–70].

Multiple reasons could explain these poor results, such as inadequate choice of drugs, dosage, and duration of antioxidants interventions [67, 71–73]. All this could be summarized by stating that the strategies that have been studied may be too simple by trying to restore the redox balance, generally using antioxidants with direct effects [52–54] [48], instead of strategies with indirect antioxidants, which have better preclinical evidence of effectiveness [55]. To better understand this, it is also essential to recognize that there are two types of small-molecule antioxidants which provide cellular protection against oxidative stress: (i) direct antioxidants, which are redox-active, short-lived because they are sacrificed during the process of their antioxidant actions and need to be replenished or regenerated, and may evoke prooxidant effects; and (ii) indirect antioxidants, which activate the Keap1/Nrf2/ARE pathway resulting in transcriptional induction of a battery of phase 2 enzymes, that act catalytically, are not consumed, have long half-lives, and are unlikely to evoke prooxidant effects [74]. Another factor could be the influence of the genetic factors previously explained, which raises the possibility that only some clusters of patients will benefit from antioxidant treatment [75].

3.2. Statins. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors are well known for their lipid-lowering capacity, but also their anti-inflammatory and pleiotropic antioxidant effects. Treatment with statins has been proposed as an option for primary prevention in the setting of anthracycline-induced cardiomyopathy. In the clinical setting, a previous study that included 40 patients undergoing anthracycline therapy randomized them to receive statin therapy versus placebo for six months. The decrease in the mean left ventricular ejection fraction after the completion of treatment was significant in the control group as compared with the statin group, and the mean increase in left ventricular end-diastolic diameter and left ventricular end-systolic diameter was significantly lower in the statin group as compared with controls [76]. An observational cohort study of breast cancer patients showed that uninterrupted statin use during anthracycline chemotherapy was associated with a significantly lower risk of incident heart failure [77].

Previous studies in animal models have demonstrated the cardioprotective effects of statins in anthracycline-induced cardiomyopathy, and they have also allowed a better knowledge of the intracellular pathways involved that explain this effect [78].

From a mechanistic point of view, statins have been shown to reduce the doxorubicin-induced cardiac inflammatory response and oxidative stress and to attenuate mitochondrial apoptotic pathways in animal models [79, 80]. However, we must consider that several of the pleiotropic effects of statins to prevent AIC are mediated by a reduction of oxidative injury. First, statins have been found to preserve mitochondrial membrane potential in response to oxidative stress. This effect could be mediated by NO, activating mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}), which results in cardioprotection [81]. Second, Riad et al. investigated the cardioprotective effects of fluvastatin in doxorubicin-induced cardiomyopathy in a mouse model. In this study, statin treatment improved cardiac function, associated with an increase of the expression of the antioxidative enzyme SOD2 and secondarily decreasing tumor necrosis factor α (TNF-α) levels, suppressing doxorubicin-induced overexpression of the proapoptotic protein Bax, decreasing cardiac nitrotyrosine production, and activated mitochondrial-located antioxidative and antiapoptotic mechanisms [80].

Another mechanism by which statins have shown efficacy in AIC prevention is through downstream inhibition of Rac1 [79, 82]; however, this effect is also related to an antioxidant effect. Cholesterol-independent cardioprotective effects of statins have been traced back to the inhibition of Rho GTPase Rac1 signaling [83]. At the same time, the known antioxidative effects of statins [84] could be explained by this inhibition of Rac1, which could lead to a reduced intrinsic generation of ROS since Rac1 regulates the NADPH oxidase complex [85].

Pharmacological characteristics of statins, such as a fixed dosage and lack of hemodynamic effects, make them an attractive option for primary cardiotoxicity prevention [86]. Ongoing prospective randomized studies are investigating the potential role of statins in the primary prevention of anthracycline-induced cardiomyopathy.

3.3. ACE Inhibitors and Aldosterone Antagonists. Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) are part of the standard pharmacologic therapy used in patients with heart failure and reduced left ventricular ejection fraction (HFrEF) due to their well-proven effect on cardiac remodeling and, consequently, mortality reduction in this population [87, 88]. The potential effect of ACE inhibitors in the treatment of AIC patients was first demonstrated more than twenty years ago by Jensen et al. in a small observational study of patients with AIC [89]. Subsequently, secondary prevention with ACE inhibitors is now well established, with early detection and treatment of cardiotoxicity results in at least partial LVEF recovery in most cases [8].

The evidence for primary prevention of AIC with these agents is mainly supported by small observational studies and single-center randomized clinical trials [90]. ACE inhibitors are often used in combination with other interventions (such as beta-blockers), and there are only a few clinical trials specifically designed to study the use of an ACE inhibitor alone (enalapril) for AIC prevention [91, 92]. Evaluation of the evidence in two meta-analyses has suggested a potential role as a prophylactic intervention [90], and that neurohormonal therapies in single or combination strategies are associated with higher LVEF in follow-up, although absolute changes in LVEF are small and could be within intertest variability for the LVEF measurement [93]. However, recently, a multicenter randomized trial (ICOS-One Trial) compared two strategies for the prevention of AIC with enalapril: primary prevention versus a biomarker-guided strategy during treatment with anthracyclines [94]. No differences were found between primary prevention with enalapril versus treatment with enalapril guided by early detection of troponin elevation [94]. These results suggest that primary

prevention with enalapril would not be superior to early treatment with enalapril when subclinical damage (elevation of biomarkers) is detected [94].

Aldosterone antagonists act by blocking the final step of the renin-angiotensin-aldosterone system in different organs. Several in vitro, preclinical, and clinical studies have established the importance of this target in heart failure and cardiac remodeling [95–97]. In the original report, Jensen et al. reported that spironolactone could enhance the effects of ACE inhibitors in AIC treatment [89]. However, this study encouraged the use of these two clinical interventions simultaneously to ensure the prevention of AIC [89]. The evidence on using aldosterone antagonist alone (spironolactone and eplerenone) is limited [10].

Considering the scarce evidence for aldosterone alone for AIC prevention and that the most robust evidence among ACE inhibitors is associated with enalapril [91, 92], which has shown an *in-vivo* capacity to inhibit oxidative stress, one could deduce that an antioxidant effect might be involved beyond the block of the renin-angiotensin-aldosterone axis [98, 99] [100]. From a redox point of view, we could also hypothesize a potential synergistic mechanism between enalapril and spironolactone, associated with two independent potential antioxidant mechanisms more than due to a specific receptor-mediated response. According to basic and preclinical studies, the spironolactone antioxidant effects have been associated with NADPH oxidase inhibition [101, 102], and enalapril oxidative stress abrogation has been associated with an enhancement of intracellular antioxidant defences (glutathione GSH-dependent antioxidant defences) [99, 100].

3.4. Beta-Blockers with Antioxidant Properties: Carvedilol and Nebivolol. Beta-blockers promote autonomic and neurohormonal regulation in the presence of cardiac dysfunction, leading to a positive impact on the cardiac remodeling of the left ventricle, resulting in reduced mortality from heart failure [88, 103]. With respect to the properties of the different beta-blockers, only carvedilol and nebivolol have antioxidant effects, potentially giving them some comparative advantages over other beta-blockers.

3.4.1. Carvedilol. Although all beta-blockers could have a preventive effect in AIC, carvedilol has been one of the most studied in this setting [93]. Its potent antioxidant property distinguishes it from other β adrenergic receptor antagonists [104]. In this sense, carvedilol is superior to atenolol (which represents an antagonist of β adrenergic receptors, but without antioxidant properties) in reducing the negative impact induced by doxorubicin in systolic function, as well as the increase in lipoperoxidation (a product of oxidative stress injury in biological membranes) [105]. Carvedilol is a β-blocker with unique ROS-suppressive properties, even at subtherapeutic doses [106]. There is still uncertainty about its clinical benefit in primary prevention of AIC, based on mixed results from clinical [107–110] [111] and observational studies [112]. The CECCY trial, the most contemporary clinical study, was a randomized, double-blind, placebo-controlled protocol of carvedilol in 200 anthracycline-treated women with HER2 negative breast cancer. This study failed to prevent a ≥10% reduction in LVEF at six months. Nevertheless, in that protocol, carvedilol was able to prevent other manifestations of cardiotoxicity, reducing the number of patients experiencing increases in serum Troponin I (TnI) levels and attenuating its peak levels. There was also a trend towards a lower increase in left ventricular diastolic diameter and a reduction in the percentage of patients with diastolic dysfunction [111]. *In vitro* studies in cardiomyocytes have suggested that the cardioprotective effect of carvedilol is driven by its antioxidant properties [113], which have also been suggested after some clinical trials conducted [114], and as other β-blockers evaluated have not shown such a significant attenuation of AIC in clinical settings [115].

The variability of carvedilol in AIC prevention and its magnitude between different studies could be explained by intrinsic and extrinsic factors [93].

Carvedilol's antioxidative properties could be associated with its effects against mitochondrial dysfunction, which is one of the mechanisms associated with AIC and is characterized by a secondary ROS generation [116]. The specific mechanism to prevent mitochondrial dysfunction could be mediated by the stimulation of mitochondrial biogenesis by carvedilol, which results in a functional gain of the mitochondria [116]. Finally, increased expression of PGC-1α and mitochondrial biogenesis induced by carvedilol might suggest a new mechanism of the therapeutic effects of carvedilol in heart failure and AIC [116].

Therefore, it is currently unclear whether the potential protective effect of carvedilol is due to its antioxidant activity and reduction in lipid peroxidation or whether it is due to its β-blocker properties [117].

Interestingly, antioxidants at standard oral doses are not able to induce enough local heart effects to appreciate clinical benefits in cardiac conditions associated with oxidative stress. However, since carvedilol has an affinity for cardiac tissue, it can show local effects that are impossible to appreciate with generic antioxidants, but even this appears to be insufficient to prevent AIC. Nonintrinsic carvedilol factors could include a variable cumulative dose of anthracycline within the different studies, high individual variability in anthracyclines bioavailability [48], population heterogeneity, differences in risk factors profiles [7], and variability of chemotherapy protocols. These factors can determine the expected AIC incidences for a particular study and, therefore, determine a greater or lesser carvedilol efficacy in that protocol when compared to groups of patients with a standard risk. It is expected that populations with higher incidences of AIC are more prone to benefit from cardioprotective interventions than lower-risk populations.

3.4.2. Nebivolol. Nebivolol is a highly selective β1 receptor beta-blocker drug, which is approximately 3.5 times more β1-selective than bisoprolol [118]. Unlike other beta-blockers with vasodilator effects (carvedilol and labetalol), which are mediated by blocking alpha-adrenergic receptors, nebivolol induces nitric oxide-dependent vasodilation, mediated by its agonist effect on endothelial β3 receptors that

stimulate the enzyme nitric oxide synthase [119–121] and activate the NO/cGMP/PKG signaling pathway [122]. These endothelium-dependent vasodilation properties have been associated with a more significant blood pressure reduction in mechanistic studies than other beta-blockers [117]. Nebivolol is also characterized by antiproliferative, anti-inflammatory, and antioxidant properties, which would give additional value to its already indicated antihypertensive and endothelial effect [123]. At the subcellular level, the increase in nitric oxide formation following treatment with nebivolol has shown to increase cytosolic free zinc at the cardiomyocytes, with inhibition of intracellular and mitochondrial calcium overload and consequent protection against the effects of ROS and lipid peroxidation involved in hypertensive heart disease [124, 125].

With respect to the potential properties of nebivolol to prevent AIC, experimental studies in rats have shown antiapoptotic effects on cardiomyocytes and a reduction of ventricular dysfunction [126]. An antioxidant reinforcing effect would be involved as one of the protective mechanisms after treatment with nebivolol, which would be explained by an increase in the activities of glutathione peroxidase and Mn-superoxide dismutase, in addition to a release of nitrite/nitrate in cardiac tissue, which has been evidenced in both in vivo and ex vivo models [126, 127]. The antioxidant reinforcement at subcellular and cellular levels by the previously mentioned mechanisms would achieve an attenuation of oxidative stress injury secondary to anthracyclines. This was evidenced by a decrease in the production of mitochondrial H_2O_2 and in the peroxidation of membrane lipids expressed in lower concentrations of 8-isoprostanes in both the mitochondria as in cardiomyocyte membranes, which would also be associated with a reduction in microscopic scarring and tissue collagen [124, 125].

With respect to clinical evidence, a prospective study in 60 breast cancer patients on anthracyclines treatment showed that nebivolol had cardioprotective effects in the short term (6 months). The nebivolol group prevented diastolic dysfunction and had a lower reduction in global longitudinal strain compared with the control group [128, 129]. Therefore, basic and some clinical evidence supports a potential cardioprotective effect of nebivolol against AIC based on its pleiotropic properties beyond its beta-blocker effects.

3.5. Dexrazoxane: Topoisomerase 2β Target and Iron Chelators. Dexrazoxane is the only approved agent for the AIC prevention and is used intravenously in conjunction with the anthracycline to decrease the incidence of cardiomyopathy and congestive heart failure in a variety of cancer types in children and adults [130, 131].

In 2013, a meta-analysis showed a significant decrease in cardiac events for patients pretreated with dexrazoxane with no prior history of heart failure [90]. More recently, another new meta-analysis in patients with breast cancer treated with anthracyclines evaluated the efficacy of dexrazoxane of nine trials [132]. In this latter study, dexrazoxane reduced the risk of clinical heart failure and cardiac events in patients with anthracycline chemotherapy with or without trastuzumab and did not significantly impact cancer outcomes. However, the authors concluded that the quality of available evidence remains low, and further new randomized trials are warranted before a systematic implementation of this treatment in clinical practice [132]. In this sense, dexrazoxane treatment does not eliminate the risk of AIC, so it is necessary to continue clinical and cardiac function monitoring before and during therapy [130]. Moreover, dexrazoxane can be responsible for different adverse effects such as a reversible elevation of hepatic transaminases as well as some myelotoxicity (neutropenia and thrombocytopenia), limiting the dose given to the patient [133]. Dexrazoxane has two main mechanisms to ameliorate the AIC: (i) to chelate redox-active iron, thereby decreasing the formation of anthracycline-iron complexes preventing Fenton reaction and subsequently decreasing the ROS generation, which is harmful to the surrounding cardiac tissue [23, 130]; (ii) to act as a DNA topoisomerase II inhibitor, which happens to be the same target of the DNA Top2 anticancer agent (anthracyclines), antagonizing the formation of the Top2 cleavage complex and also rapidly degrading Top2β [134]. This does not induce harmful breaks in the double-strands of DNA in the heart as the anthracyclines [135]. However, given that other iron chelators have not shown a cardioprotective benefit after anthracycline treatments, it is possible that the primary protective mechanism would be through the inhibition of TOP 2β [23].

From the oxidative stress point of view, both mechanisms decrease ROS generation; first, directly for inhibition of Fenton reaction; second, indirectly because when it prevents the binding between anthracyclines and Top2β, it stops the next steps (mitochondrial dysfunction and ROS generation) [23, 130, 134].

Despite the plausible mechanisms and limited data, dexrazoxane is still not used routinely in clinical practice and is only FDA approved in the metastatic breast cancer population. Initially, there were concerns that dexrazoxane could attenuate the antitumor effects of anthracyclines and increase the occurrence of secondary malignancies [10], given its inhibition of Top2α, which is the anthracycline target in cancer cells [136]. However, dexrazoxane is currently considered not to be associated with a reduction in antitumor efficacy or survival or a relevant increased risk of second primary malignancies [132].

3.6. New Potential Interventions: Strategies Based on Non-ischemic Pharmacological Preconditioning: Omega 3 LCPUFA (DHA/EPA). In recent years, other new strategies to prevent AIC are being evaluated. Some of these strategies proposed to prevent AIC are based on cardiac preconditioning, both ischemic and non-ischemic. Ischemic preconditioning has a broad preclinical base in cardiology, but it is usually complex to implement in cancer patients, and its efficacy would probably be limited. To our knowledge, currently, only one study (NCT02471885) is evaluating this type of strategy in AIC prevention [137]. However, for non-ischemic cardiac preconditioning, exercise has been proposed, based on preclinical evidence [138–140]. This kind of preconditioning can also be complex to apply in most cancer patients, but it is currently being tested by in a clinical trial (NCT02471053) [141].

Strategies based on non-ischemic pharmacological preconditioning have not been previously reported in clinical trials. However, a potential benefit of these interventions has been recently suggested by Serini et al., who hypothesized that n-3 Long-chain polyunsaturated fatty acids (LCPUFAs) could serve as cardio-protectors in AIC based on several preclinical models [142]. LCPUFAs have shown some evidence of having a role in the prevention and control of some cardiovascular diseases [143]. Specifically, EPA plus DHA has been shown to be efficacious in attenuating oxidative stress related to supraventricular arrhythmias in clinical trials [144–146]. Even though these effects have been associated with classical n-3 LCPUFAs properties, including anti-inflammatory activities, antiplatelet mechanisms, and biological membranes stabilization [147], more recent data suggest that indirect antioxidant properties could be more important [146]. It has been proposed that a high integration of n-3 LCPUFA into cardiomyocyte cell membranes would induce moderate lipid peroxidation, too weak to generate deleterious oxidative stress, but enough to activate the redox-sensitive transcription factor Nrf2. The activation of this factor upregulates antioxidant enzymes, thus, generating the pharmacological nonhypoxic myocardial preconditioning [148]. The activation of the Nrf2 pathway through n-3 LCPUFA and subsequent induction of antioxidant enzymes in cardiomyocytes have been described in both cellular [149] and preclinical models [150]. Regarding AIC, preclinical studies suggest that the Nrf2 pathway could play a role in physiological cardioprotection [151], as well as in the ability of n-3 LCPUFA to prevent doxorubicin-induced ROS production and the subsequent mitochondrial damage [152, 153]. Previously, a randomized controlled trial reported that n-3 LCPUFA nonhypoxic cardiac preconditioning was able to prevent postoperative atrial fibrillation trough enhancing endogenous heart antioxidant capacity [146].

Regarding the safety of high doses of n-3 LCPUFA in breast cancer patients treated with doxorubicin, there has been no clinical evidence of any harm or negative interactions. A randomized controlled trial reported no adverse effects in metastatic patients exposed to 1800 mg of DHA used as chemotherapy adjuvant, starting with a loading dose 7-10 days before initiating the first cycle of doxorubicin, and then maintaining the dose for five months [154]. Additionally, a 3-arm pilot double-blind placebo-controlled protocol was performed in patients with localized breast cancer undergoing first-time doxorubicin chemotherapy without finding any adverse effects in the n-3 LCPUFA arm [155]. In this arm, eleven patients were exposed to 2 g per day of n-3 LCPUFA (EPA + DHA) from 7 days before to 7 days after the first chemotherapy cycle, without any side effects associated with these fatty acids. Interestingly, the n-3 LCPUFA inhibited the expected NT-ProBNP plasma elevation after doxorubicin chemotherapy (48 hours), suggesting that the intervention was able to attenuate subclinical cardiotoxicity. Also, in the n-3 LCPUFA-treated group, there was a nonsignificant but lower echocardiography measured LVEF decline compared to double placebo patients at 10-12 months follow-up. The lack of statistical significance in this outcome is likely due to the small sample size and the high variability of echocardiography based LVEF measurements. Interestingly, despite these limitations, in the third arm, the eleven patients exposed to 12.5 mg of carvedilol every 12 hours showed a significantly lower reduction in LVEF at 10-12 months, compared to the double placebo group. Unexpectedly, in this study arm, carvedilol did not impact the levels of NT-ProBNP [155]. It is important to note that in this study, the population had a high prevalence of cardiovascular risk factors such as arterial hypertension (31%), dyslipidemia (22%), and smoking (42%) [155].

Currently, an ongoing clinical trial (CarDHA trial; ISRCTN69560410) in breast cancer patients receiving anthracyclines is designed to assess whether non-ischemic preconditioning with DHA plus carvedilol a week before the first chemotherapy cycle and, during 90 days after, would have a better capacity to limit subclinical AIC, compared with similar patients exposed to double placebo [156]. This study will evaluate any subclinical AIC manifestation in biomarkers, electrocardiographic alterations, LVEF by cardiac magnetic resonance, and global longitudinal strain by 2D echocardiography. Also, from a mechanistic point of view, the CarDHA trial is evaluating as secondary endpoint biomarkers of oxidative stress damage (plasma lipoperoxidation levels), as well as parameters of antioxidant balance (Erythrocyte Thiol Index (GSH/GSSG)). Therefore, this study will also enable the understanding of the impact of combined interventions (using as target two unrelated antioxidant pathways) on the inhibition or attenuation of the oxidative stress damage associated with AIC [156].

3.7. Combined Strategies: Classical (ACEi/ARBs plus Beta-Blockers) and New Combinations Targeting Different Unrelated Antioxidant Pathways (Omega 3 LCPUFA (DHA/EPA) plus Carvedilol). Combined strategies have been previously poorly evaluated, and only two clinical trials have used dual interventions [109] [157]. However, the pathophysiological focus of these dual strategies has been the blockade of neuroendocrine systems (sympathetic nervous and the renin-angiotensin-aldosterone system) to try to modulate the remodeling process that occurs following a myocardial injury [109, 157].

In the OVERCOME (prevention of left-ventricular dysfunction with enalapril and carvedilol) trial, the combination of enalapril and carvedilol vs. no treatment was tested in 90 patients diagnosed with malignant hemopathies treated with anthracyclines. LVEF did not change in the intervention group but decreased significantly in controls after six months. Also, the intervention group had a lower incidence of final LVEF of <45 and heart failure [109].

Also, a combined beta-adrenergic and angiotensin blockade approach had been evaluated in one of the arms of the PRADA trial (Prevention of Cardiac Dysfunction during Adjuvant Breast Cancer Therapy) using a 2×2 factorial trial with metoprolol and candesartan during anthracyclines treatment [157]. This study showed that candesartan, but not metoprolol, can protect against an early decline in LVEF, assessed with cardiac MRI. It is also important to note that in this study they did not think there was a protective

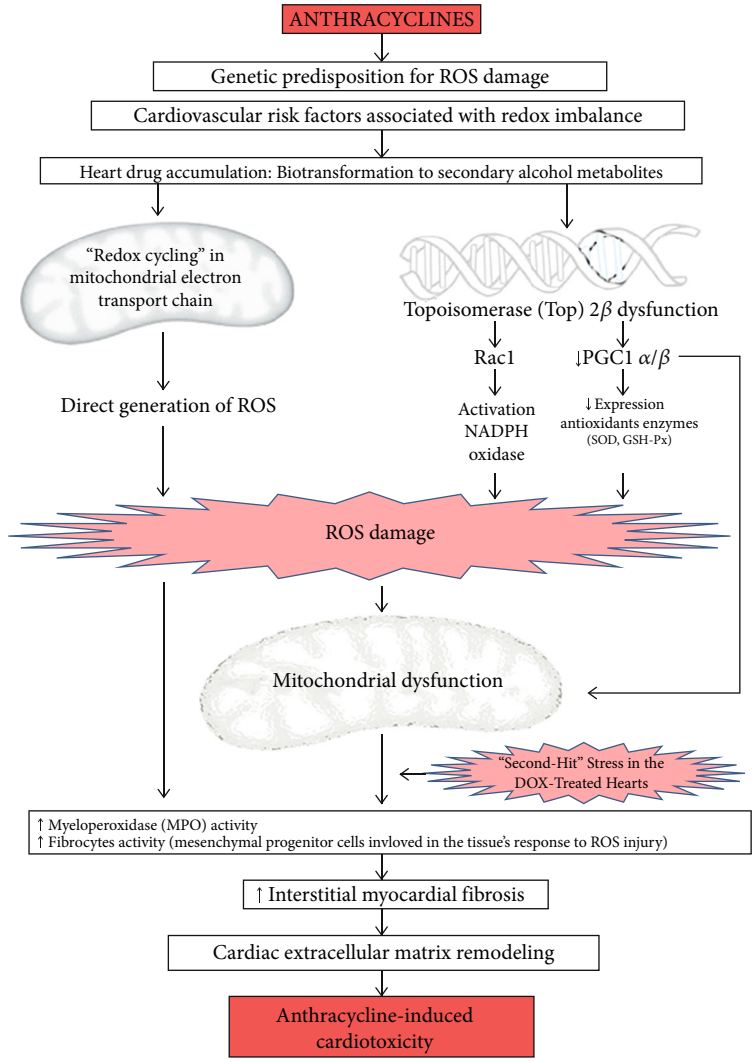

Figure 1: Mechanisms of anthracycline-induced cardiotoxicity and the role of oxidative stress. DOX: doxorubicin; GSH-Px: glutathione peroxidase; MPO: myeloperoxidase; PGC1 α/β: peroxisome proliferator-activated receptor-γ coactivator 1-α and 1-β; Rac1: a subunit of NADPH oxidase; ROS: reactive oxygen species; SOD: superoxide dismutase.

interaction between metoprolol and candesartan, due to combination was not better [157].

Unlike what was previously shown, new insights into the redox mechanism of damage in AIC allow consideration of new combined strategies. These new strategies with combined interventions based on the inhibition or attenuation of the oxidative stress injury associated with AIC by two unrelated antioxidant pathways could be more efficient than the potential for attenuation through treatments based on either one of the pathways alone.

An ongoing prospective randomized study by Carrasco et al. is investigating the potential role of a strategy based on two interventions with a focus on two different redox pathways for primary prevention of AIC [156].

Unlike other primary prevention protocols of AIC based on carvedilol alone, the CarDHA trial (ISRCTN69560410) is the first designed cardio-oncology study, based on using the antioxidant carvedilol properties associated with another antioxidant intervention as part of a dual therapeutic strategy focusing on attenuating the oxidative stress damage [156]. This clinical trial uses a sequential regimen. First, a DHA treatment is started one week before the anthracyclines to increase the antioxidant enzymatic activity in the myocardium, and then when the anthracycline is started, carvedilol is added, which provides direct antioxidant effects [156].

4. Conclusions

The multiple mechanisms proposed for anthracycline cardiotoxicity could be grouped under the umbrella of a unifying downstream mechanism: "oxidative stress" (Figure 1). Therefore, most of the interventions that could be beneficial in the primary prevention of AIC have potential antioxidant effects as a common theme (Figure 2).

Due to this crucial role of oxidative stress in AIC, the screening of cardiovascular risk factors associated with oxidative imbalance is essential to identify the subgroup of patients that could benefit the most from a primary

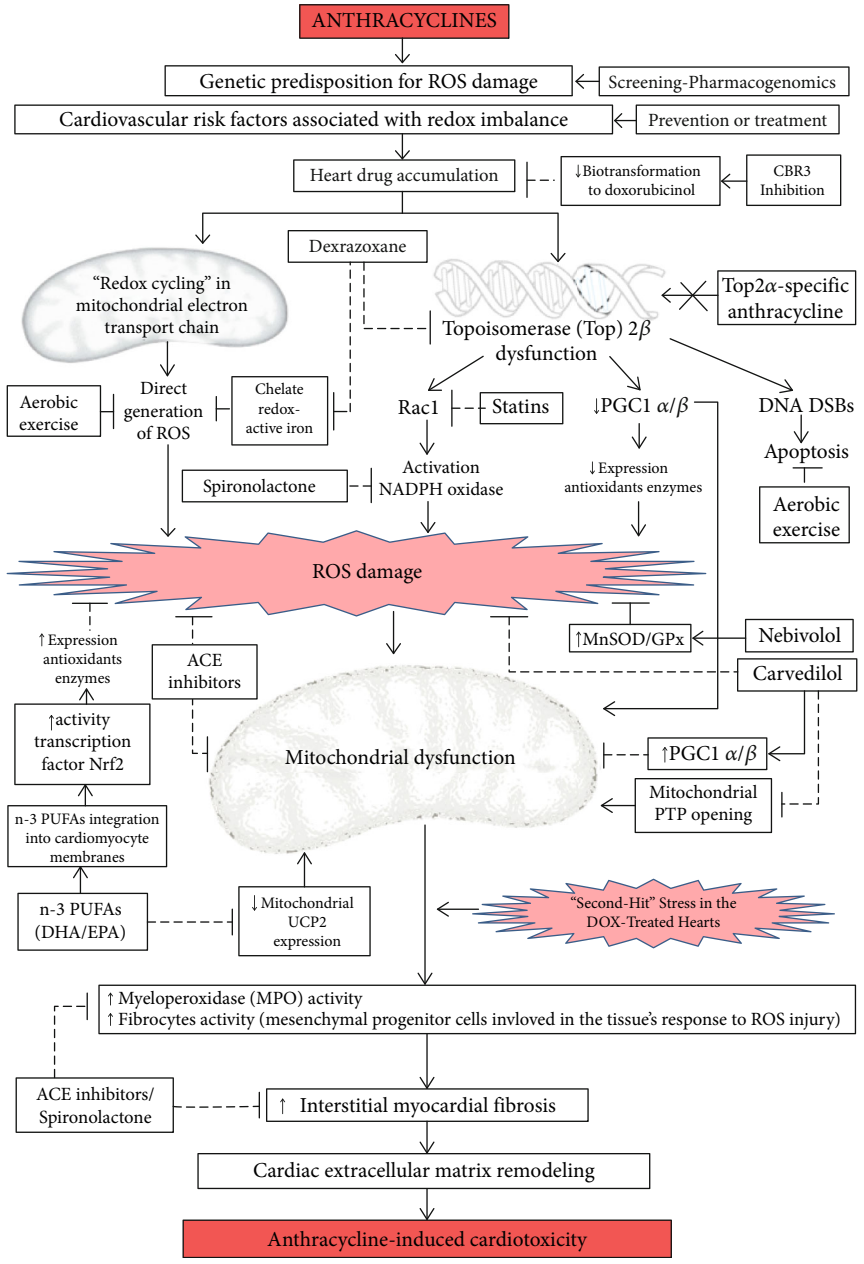

Figure 2: Potential cardioprotective therapies for anthracycline-induced cardiotoxicity. ACE: angiotensin-converting enzyme; CBR3: carbonyl reductase 3; DHA: docosahexaenoic acid; DNA DSBs: indicates deoxyribonucleic acid double-stranded breaks; EPA: eicosapentaenoic acid; GPx: glutathione peroxidase; MnSOD: Mn-superoxide dismutase; MPO: myeloperoxidase; n-3 PUFAs: n-3 polyunsaturated fatty acids; Nrf2: nuclear factor erythroid 2-related factor 2; PGC1 α/β: peroxisome proliferator-activated receptor-γ coactivator 1-α, and 1-β; PTP: permeability transition pores; Rac1: a subunit of NADPH oxidase; ROS: reactive oxygen species; Top2β: topoisomerase 2β; UCP2: mitochondrial uncoupling protein 2.

prevention strategy with antioxidants interventions. Also, the development of a genetic approach to identify some polymorphisms in genes related to anthracyclines biotransformation, antioxidant defences, or ROS generation could help to find patients with an increased risk of AIC.

Although in the future, it may be attractive to develop new preventive strategies for AIC, focused on targets such as topoisomerase 2β or the biotransformation of anthracyclines, the imperative need to have preventive interventions for AIC in the short term promotes continuing the evaluation of strategies that reduce oxidative stress through the use of drugs already available. The therapeutic interventions that have been clinically evaluated, such as ACE inhibitors, aldosterone antagonists, carvedilol, and nebivolol, contribute to decrease the severity of redox imbalance by different mechanisms and could also have a role in reducing the impact caused by "second hits" (Figure 2). New strategies involving non-ischemic cardiac preconditioning are being evaluated as preventative options for AIC due to their capacity of attenuating oxidative stress. Also, the role of dual strategies based

on combined interventions targeting different redox pathways for the primary prevention of AIC is being evaluated by new clinical trials.

This review also has some limitations. First, the evidence for most antioxidant interventions is still limited, and mostly all studies have small sample sizes. Second, although some interventions could theoretically prevent AIC through their antioxidant properties (such as carvedilol), most of the studies only evaluated clinical endpoints but not oxidative stress parameters to provide better mechanistic evidence.

Finally, despite the multiple new advances in knowledge of anthracycline-induced cardiotoxicity, oxidative stress remains one of the main therapeutic targets for cardioprotection. Therefore, further studies are needed with clinical interventions focused on the reduction of oxidative stress.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Protective Effects of Oroxylin A against Doxorubicin-Induced Cardiotoxicity via the Activation of Sirt1 in Mice

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Doxorubicin- (DOX-) related cardiac injury impairs the life quality of patients with cancer. This largely limited the clinical use of DOX. It is of great significance to find a novel strategy to reduce DOX-related cardiac injury. Oroxylin A (OA) has been identified to exert beneficial effects against inflammatory diseases and cancers. Here, we investigated whether OA could attenuate DOX-induced acute cardiotoxicity in mice. A single dose of DOX was used to induce acute cardiac injury in mice. To explore the protective effects, OA was administered to mice for ten days beginning from five days before DOX injection. The data in our study indicated that OA inhibited DOX-induced heart weight loss, reduction in cardiac function, and the elevation in myocardial injury markers. DOX injection resulted in increased oxidative damage, inflammation accumulation, and myocardial apoptosis in vivo and in vitro, and these pathological alterations were alleviated by treatment of OA. OA activated the sirtuin 1 (Sirt1) signaling pathway via the cAMP/protein kinase A, and its protective effects were blocked by Sirt1 deficiency. OA treatment did not affect the tumor-killing action of DOX in tumor-bearing mice. In conclusion, OA protected against DOX-related acute cardiac injury via the regulation of Sirt1.

1. Introduction

Doxorubicin (DOX), a quinone-containing anthracycline, is used to treat leukemia and malignant lymphomas. The use of DOX triggered toxic effects on the hearts and resulted in the cardiomyocyte loss and congestive heart failure, which limited the clinical use of DOX [1, 2]. DOX-induced characteristic change of acute cardiotoxicity included myofibrillar disruption, cardiomyocytes atrophy, and vacuolated preapoptotic cells, while cumulative chronic cardiotoxicity could lead to ventricular dilation [3]. Previous studies had identified the mammalian target of rapamycin (mTOR) as a key determinant of DOX-related cardiotoxicity [4–6]. However, there were no drugs that could effectively prevent the toxic effects of DOX. Accumulating evidences suggested that DOX-induced myocardial injury may be related to oxidative stress, calcium overload, mitochondrial damage, and cardiomyocyte apoptosis [7].

It has been reported that the metabolic products of DOX could transfer its unpaired electrons to oxygen, and thus, inducing the production of free radicals and cardiotoxicity [8]. In addition, the early activation of nuclear factor kappa-B (NF-κB) and subsequent inflammatory factors accumulation were also involved in DOX-related cardiac injury [9]. Oxidative stress caused the release of cytochrome c and the increased caspase-3 activity, promoting myocardial apoptosis [10]. Therefore, it is important to find a novel drug that constrains these pathological alterations for the treatment of DOX-related myocardial injury.

Oroxylin A, a natural flavonoid extracted from Scutellaria radix, has been reported to exert anticancer activities by inhibiting tumor invasion and metastasis [11]. The anticolon cancer effects of OA were associated with the inhibition of the inflammatory response [12, 13]. OA could also inhibit hypoxia-inducible factor-1 (HIF-1) α signaling pathway in mice [14]. In addition, OA treatment inhibited hydrogen peroxide-induced oxidative damage of PC12 cells [15] and attenuated oxidative stress caused by cigarette smoke via activating nuclear factor- (erythroid-derived 2-) like 2 (Nrf2) signaling pathway [16]. However, the effects of OA on DOX-induced acute cardiotoxicity and the related signaling mechanisms have not yet been reported. In the present study, we

show that OA protects the mice against DOX-induced acute cardiotoxicity by activating sirtuin 1 (Sirt1) signaling pathways. Our studies suggest that OA might have therapeutic utility in the treatment of DOX-induced myocardial injury.

2. Materials and Methods

2.1. Reagents. Oroxylin A (purity > 98% as determined by HPLC) was purchased from the National Institutes for Food and Drug Control (China). The specific Sirt1 inhibitors including EX527 (HY-15452) and nicotinamide (HY-B0150) were provided by MedChemExpress (Shanghai, China). DOX was provided by Sigma-Aldrich (St. Louis, MO, USA). The assay kits for Sirt1 activity (fluorometric, ab156065), NAD/NADH level (colorimetric, ab65348), protein kinase A (PKA, ab139435) activity, and cAMP level (competitive ELISA, ab138880) were provided by Abcam (Cambridge, UK).

2.2. Animals and Treatment. All animals' experimental protocols were approved by the Ethical Committee of Renmin Hospital of Wuhan University. The male C57BL/6 mice (age: 8-10 weeks, body weight: 23.5-27.5 g) were purchased from the animal experiment center of Wuhan University (Wuhan, China). All the mice were housed in the specific-pathogen-free mouse room of Renmin Hospital of Wuhan University under the standard conditions. Mice were randomly assigned into four groups as control+vehicle, control +OA, DOX+vehicle, and DOX+OA ($n = 10$ per group). The mice in our study were intraperitoneally injected with DOX at a dose of 20 mg/kg to establish the model of DOX-related cardiac injury [17]. To evaluate the effects of OA on DOX-induced acute cardiotoxicity, mice were orally given OA (40 mg/kg) or the same volume of CMC-Na solution for ten days beginning from five days before DOX injection. The dose of OA referred to a previous study [18]. Five days after DOX, the mice were anesthetized with isoflurane, and blood was collected via retro-orbital sinus for further detection. To confirm the role of Sirt1 in the protection of OA treatment, cardiac-restricted Sirt1 knockout (cKO) mice were used [19]. In brief, Sirt1 conditional floxed mice were bred with mice carrying the α-Mhc-MerCreMer transgene (Jackson Laboratory) to generate cardiac-restricted Sirt1 cKO mice. To delete Sirt1, Sirt1 cKO mice were intraperitoneally injected with tamoxifen (25 mg/kg/day) for 5 consecutive days.

In the tumor experiment, Lewis lung carcinoma (LLC) cells were subcutaneously implanted to isogenic mice. OA was administered on day 5 after tumor implantation, and this protective intervention lasted for ten days. DOX was injected on day 10. Five days after DOX, the mice were anesthetized with isoflurane, and blood and hearts were collected for further detection [20].

2.3. Hemodynamics. Left ventricle hemodynamics were detected as previously described [21]. Mice were anesthetized, and the apex of the left ventricle was exposed. After that, a micronanometer catheter (Millar 1.4F, SPR 835, Millar Instruments, TX, USA) was inserted into the left ventricle. This transducer was connected to a Power Laboratory system to detect and analyze the obtained data.

2.4. Detection of Myocardial Injury Markers. Five days after DOX, the mice were anesthetized with isoflurane, and blood was collected via retro-orbital sinus. The lactate dehydrogenase (LDH) detection kit (C0017) was provided by Beyotime Biotechnology (Beijing, China). And the creatine kinase myocardial bound (CK-MB) detection kit (E006-1-1) was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The homogenate of hearts and blood supernatants was used to measure the two myocardial injury markers.

2.5. Assessment of Biochemical Parameters. To detect myocardial oxidative injury, fresh heart samples were collected. The levels of myocardial 4-hydroxynonenal (4-HNE), nitrotyrosine, and glutathione (GSH) as well as the activities of glutathione peroxidase (Gpx) and superoxide dismutase (SOD) were detected following the manufacturer's instructions. 4-HNE ELISA Kit (E4645-100), nitrotyrosine ELISA Kit (K4158-100), and GSH Assay Kit (K264-100) were provided by BioVision (San Francisco, USA). Gpx BioAssay ELISA Kit (#356081) was also provided by BioVision. The activity of total SOD was detected using a kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.6. Cardiomyocyte Isolation and Contractile Assay. We used a Langendorff perfusion system to isolate adult cardiomyocytes from the hearts of DOX-treated mice according to a previous study [22]. Cardiomyocyte contractile function was detected using a SoftEdge MyoCam® system, which was obtained from IonOptix Corporation (MA, USA). Resting cell length, peak shortening, maximal velocity of shortening (+dL/dt), and maximal velocity of relengthening (-dL/dt) were detected.

2.7. Western Blot. After, the heart samples were collected and the total proteins were extracted using a BCA protein assay kit (Thermo Scientific, Rockford, USA). Nuclear protein was extracted using NE-PER™ Nuclear Extraction Reagents (Thermo Fisher Scientific). And then, the samples were separated by 10% SDS-PAGE and transferred to PVDF membranes [23, 24]. The membranes were then incubated with the primary antibodies against Nrf2 (Abcam, ab62352, 1 : 1000), heme oxygenase-1 (HO-1, Abcam, ab68477, 1 : 1000), NADPH quinone acceptor oxidoreductase 1 (NQO1, Abcam, ab28947, 1 : 1000), GAPDH (Abcam, ab8245, 1 : 1000), inhibitor-κBα (Abcam, ab32041, IκBα, 1 : 1000), P-IκBα (Abcam, ab38515, 1 : 1000), NF-κB (Abcam, ab16502, 1 : 1000), Histone H3 (Abcam, ab1791, 1 : 1000), Bcl-2 (Abcam, ab32124, 1 : 500), and Sirt1 (Abcam, ab189494, 1 : 1000) followed by the incubation of the secondary antibodies. These primary antibodies were obtained from Abcam (Cambridge, UK). These bands were detected by Bio-Rad imaging system and quantified by the Image Lab Software.

2.8. Quantitative Real-Time PCR. Total RNAs were isolated from heart tissues using the TRIzol reagent (Invitrogen Life Technologies, USA) [25]. cDNA synthesis was performed with the Bio-Rad iScript™ cDNA synthesis kit. The mRNA levels of genes were determined using the Transcriptor First Strand cDNA Synthesis kit (Roche, Germany). GAPDH was used as the reference gene.

2.9. Apoptotic Assay. The hearts were fixed with 4% paraformaldehyde for 24 hours. After that, the hearts were subjected to the standard procedures and then were sliced into sections. TUNEL staining was performed with a kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Myocardial apoptosis was also assayed by the detection of caspase3/7 activity and poly ADP-ribose polymerase (PARP) activity.

2.10. Cell Culture. H9c2 cell line was purchased from the Institute of Biochemistry Cell Biology (Shanghai, China), and the cells were maintained in DMEM supplemented with 10% fetal bovine serum [26]. The H9c2 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL for 24 h and pretreated with 40 μmol/l of OA for 24 h before challenged with DOX (5 μmol/l) for 24 h. The dose of OA was determined according to a previous study [27]. Cell viability was detected by a CCK-8 kit. Intracellular hydrogen peroxide was detected by an Intracellular Hydrogen Peroxide Detection Kit (BioVision, #K204-200), and superoxide was detected by a kit called Superoxide Anion Assay Kit (Sigma, CS1000). To verify the hypothesis that OA provided protection via activation of Sirt1, cells were subjected to EX527 (1 μmol/l) or nicotinamide (100 μmol/l) at 1 hour before DOX administration. NF-κB DNA binding activity and Nrf2 DNA binding activity were detected by the kits called TransAM® NFκB and TransAM® Nrf2 (Active motif, USA). To explore the mechanism by which OA treatment activated Sirt1, cells were incubated with H89 (a PKA inhibitor, 10 μmol/l), $2'5'$-dd-Ado (an adenylate cyclase inhibitor, 200 μmol/L) for 24 hours.

2.11. Detection of Cellular ROS. H9c2 myocytes were cultured in 96-well plates and pretreated with OA and DOX for 24 hours. 2,7-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Reactive oxygen species (ROS) were then detected by DCFH-DA. The cells were incubated with DCFH-DA (10 μmol/L) for 2 hours at 37°C, and immunofluorescence was detected by a fluorescence microplate reader as described previously [26].

2.12. Statistical Analysis. All the data were presented as the mean ± standard deviation (SD). We used unpaired Student's t-test to compare differences between the two groups. Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered as significant.

3. Results

3.1. OA Suppressed Cardiac Injury and Attenuated Cardiac Dysfunction in DOX-Induced Mice. Mice in the DOX group exhibited lower body weight (BW) and ratio of heart weight to tibial length (HW/TL) than those of the control group (Figures 1(a) and 1(b)). Compared to the DOX group, BW and HW/TL in the OA-treated group were significantly increased (Figures 1(a) and 1(b)). Next, we detected CK-MB and LDH, which are regarded as markers of cardiac injury. The increased plasma and cardiac CK-MB were significantly suppressed by OA treatment in DOX-treated mice (Figures 1(c) and 1(d)). The data in our study also suggested that DOX significantly increased the levels of LDH in the hearts and plasma, which were largely reduced by the treatment of OA (Figures 1(e) and 1(f)). Heart rate, ejection fraction (EF), maximum first derivative of ventricular pressure with respect to time (+dP/dt), -dP/dt, and left ventricle systolic pressure (LVSP) were significantly reduced in the DOX group. However, these alterations were largely attenuated by OA treatment (Figures 1(g)–1(k)). In addition, OA treatment suppressed the elevation of left ventricular end-diastolic pressure (LVEDP) in DOX-treated mice (Figure 1(l)).

3.2. OA Improved Contractile Function in Cardiomyocytes Isolated from DOX-Treated Mice. DOX injection did not affect the resting cell length of the isolated cardiomyocytes (Figure 2(a)). In response to DOX injection, cardiomyocytes isolated from DOX-treated mice showed decreased peak shortening, +dL/dt, and -dL/dt. And these pathological alterations were largely prevented by the treatment of OA (Figures 2(b)–2(d)).

3.3. OA Treatment Attenuated DOX-Induced Oxidative Damage in Cardiac Tissues. To investigate the effects of OA treatment on oxidative stress, we first detected the products of lipid peroxidation. In response to DOX injection, myocardial 4-HNE and nitrotyrosine levels were significantly increased. And these alterations were largely attenuated by OA treatment (Figures 3(a) and 3(b)). Administration of DOX decreased the content of GSH, Gpx, and total SOD activities in the hearts, and OA treatment prevented these pathological changes caused by DOX injection (Figures 3(c)–3(e)). Further detection revealed that the increased mRNA expression of gp91phox, NADPH oxidase 4, p47phox, and p67phox in DOX-treated mice was largely suppressed by OA treatment (Figures 3(f)–3(i)). The expression levels of Nrf2 and the downstream HO-1 and NQO1 were significantly decreased. In contrast, after OA treatment, these decreased protein expressions were significantly reversed in DOX-injected mice (Figure 3(j)).

3.4. OA Attenuated the Upregulation of Inflammatory Cytokines and Myocardial Apoptosis in DOX-Treated Mice. As shown in Figures 4(a)–4(c), DOX increased the mRNA levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β, which were remarkably inhibited by OA treatment (Figures 4(a)–4(c)). Unexpectedly, there was no difference between the four groups about the expression of

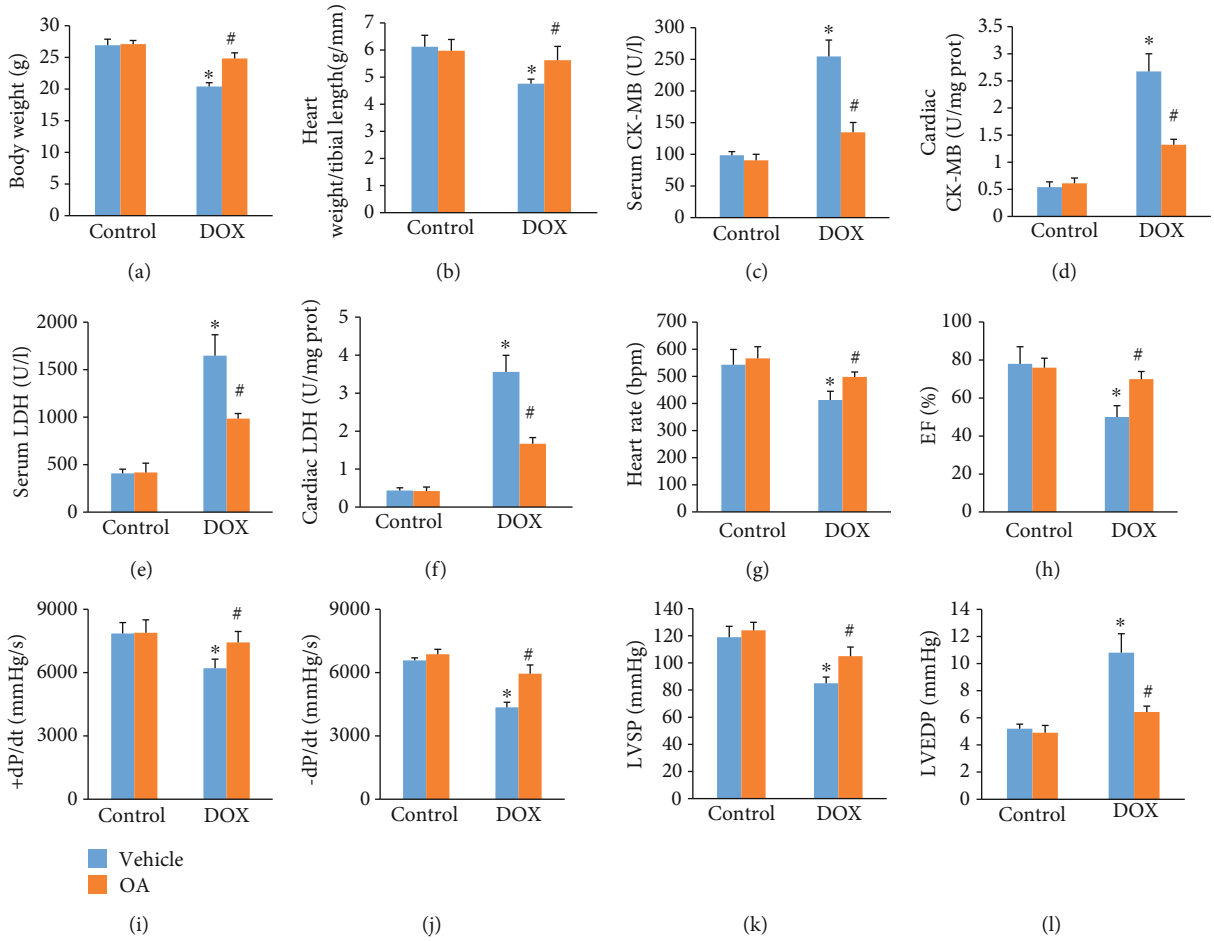

Figure 1: OA treatment attenuated cardiac dysfunction in DOX-treated mice. (a) Bodyweight in the indicated group ($n = 10$). (b) The ratio of heart weight to tibial length ($n = 10$). (c, d) Serum and cardiac CK-MB ($n = 6$). (e, f) Serum and cardiac LDH ($n = 6$). (g) Heart rate in the indicated groups ($n = 6$). (h) EF in the indicated groups ($n = 6$). (i, j) ±dP/dt in DOX-treated mice ($n = 6$). (k, l) LVSP and LVEDP in DOX-treated mice ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DOX group.

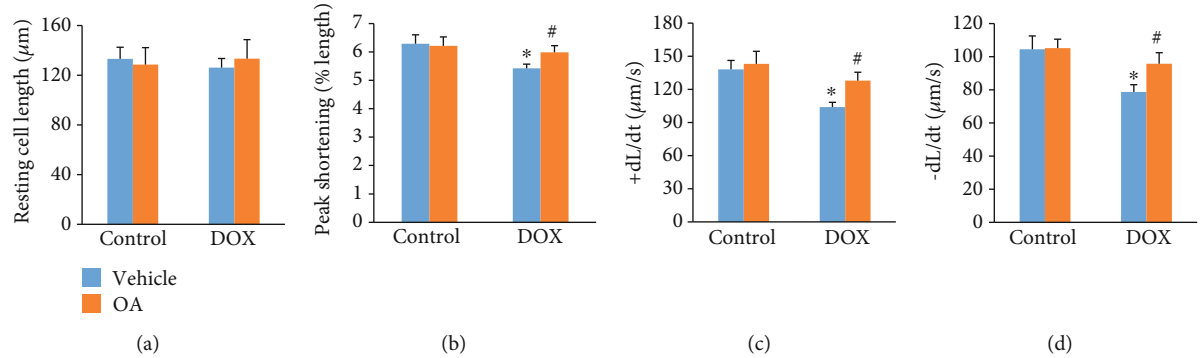

Figure 2: OA treatment improved contractile function in cardiomyocytes. (a) Resting cell length ($n = 6$). (b) Peak shortening ($n = 6$). (c, d) ±dL/dt ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $*P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DOX group.

monocyte chemotactic protein 1 (MCP-1) (Figure 4(d)). Inflammation induced the activation of matrix metalloproteinases (MMPs), which were closely involved in the pathogenesis of DOX-related cardiac injury [28]. Thus, we detected the mRNA levels of MMP2 and MMP-9 and found that the increased expression of MMP-2 and MMP-9 was suppressed by OA treatment (Figures 4(e) and 4(f)). NF-κB acted as a transcriptional factor and was responsible for the expression of several inflammatory cytokines including TNF-α and IL-6. The phosphorylation of IκBα protein played

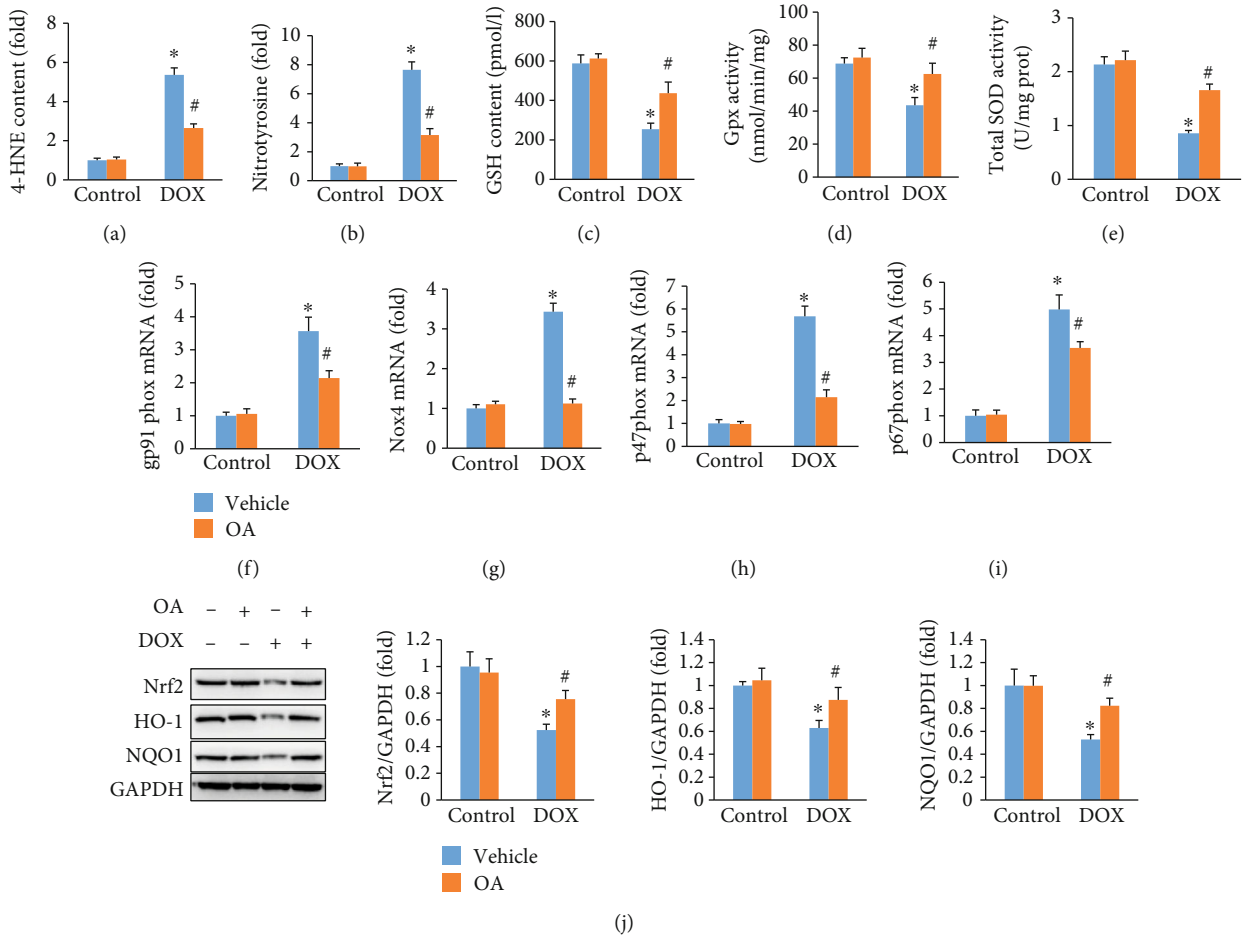

Figure 3: OA treatment prevented oxidative damage induced by DOX in cardiac tissue. (a, b) Myocardial 4-HNE and nitrotyrosine ($n = 6$). (c) GSH levels ($n = 6$). (c, d) Gpx and SOD activity ($n = 6$). (f–i) The mRNA levels of gp91phox, Nox4, p47phox, and p67phox ($n = 6$). (j) The protein expression of Nrf2 and downstream targets ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DOX group.

a key role in the activation of NF-κB [29]. Interestingly, DOX treatment significantly increased the phosphorylation of IκBα protein, and this alteration was blocked by OA (Figure 4(g)). In agreement with this finding, we also found that DOX increased the nuclear translocation of NF-κB, and this effect was attenuated by the treatment of OA (Figure 4(g)). Next, we assessed the antiapoptotic activity of OA. The decreased Bcl-2 protein expression caused by DOX injection was reversed by the treatment of OA (Figure 4(h)). Compared with the control group, DOX notably induced apoptosis in the hearts, as detected by TUNEL staining, caspase3/7 activity, and PARP activity. OA treatment attenuated these pathological alterations (Figures 4(i)–4(k)).

3.5. OA Treatment Inhibited DOX-Induced Cardiomyocytes Injury via Activating Sirt1. Previous studies suggested that Sirt1 could inhibit NF-κB [30]. Thus, we determined whether OA activated Sirt1 in vivo and in vitro. As shown in Figures 5(a) and 5(b), DOX decreased myocardial Sirt1 protein expression and activity, and these reductions were significantly normalized by OA treatment. In line with the findings in vivo, the decreased protein expression and activity of Sirt1 were also significantly restored in OA-treated H9c2 cells (Figures 5(c) and 5(d)). To confirm the role of Sirt1 in OA-mediated protection of DOX-related cardiac injury, we used the specific Sirt1 inhibitor EX527 and nicotinamide. We found that OA treatment significantly decreased the production of ROS and superoxide in DOX-treated cells, and these protection of OA against ROS and superoxide production were abolished by the use of EX527 or nicotinamide (Figures 5(e) and 5(f)). Next, we used a kit to detect Nrf2 DNA binding activity and found that OA enhanced Nrf2 transcriptional activity in response to DOX and lost this ability after Sirt1 inhibition by EX527 or nicotinamide (Figure 5(g)). The subsequent detection of the mRNA levels of HO-1 and NQO1 revealed that OA lost its effects on the expression of HO-1 and NQO1 after Sirt1 inhibition (Figures 5(h) and 5(i)). OA inhibited NF-κB transcriptional activity in response to DOX and lost this ability after Sirt1 inhibition by EX527 or nicotinamide (Figure 5(j)). EX527 or nicotinamide also abolished the inhibitory effects exhibited by OA treatment on the mRNA level of TNF-α in DOX-treated cells (Figure 5(k)). Cell viability was decreased in the DOX-treated group, increased after OA treatment, but declined again after Sirt1 inhibition by EX527 or nicotinamide (Figure 5(l)).

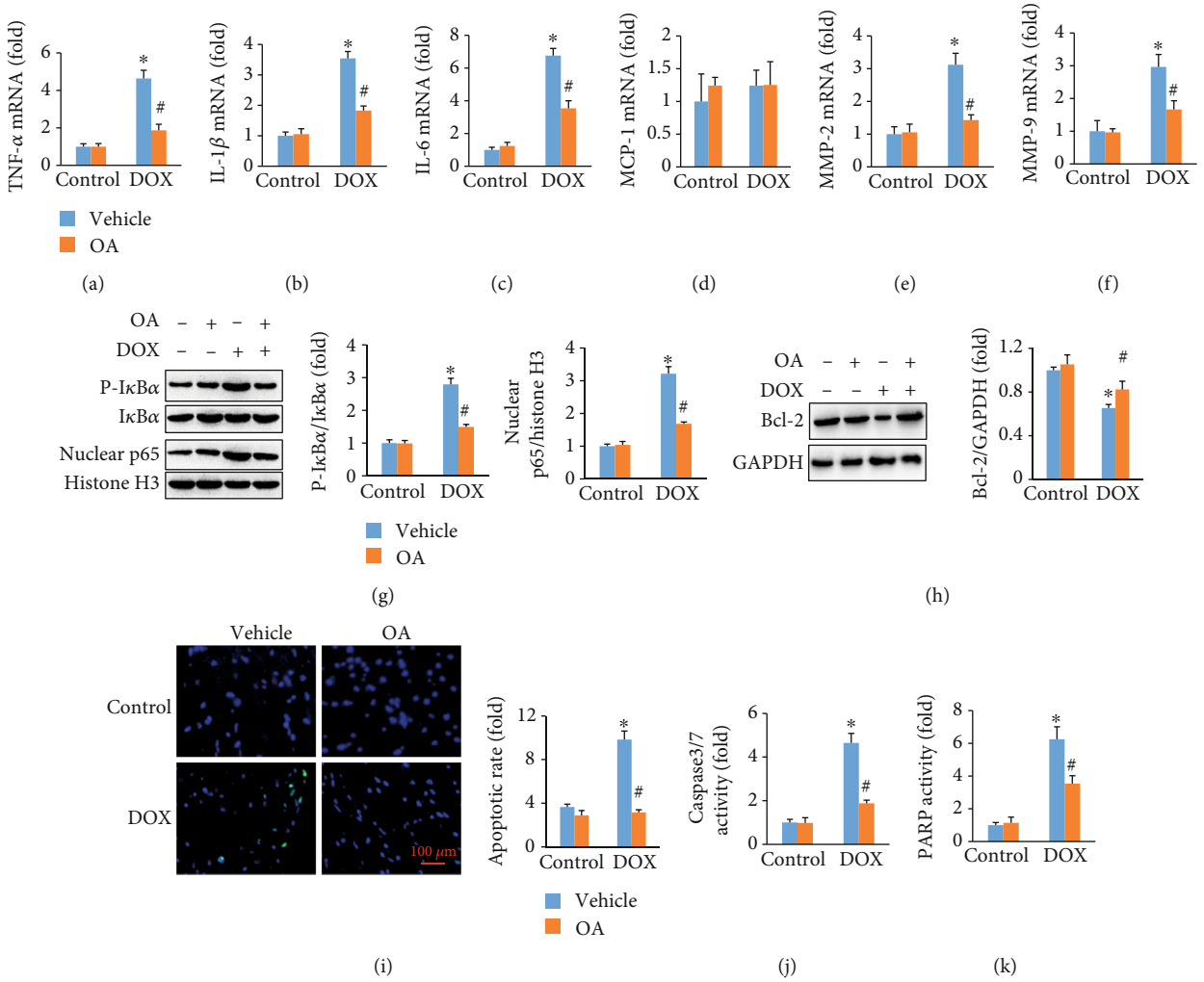

Figure 4: OA treatment attenuated inflammatory response and myocardial apoptosis in DOX-treated hearts. (a–d) Relative mRNA levels of inflammatory factors ($n = 6$). (e, f) The mRNA levels of MMP-2 and MMP-9 ($n = 6$). (g) The protein expression of p-IκBα and nuclear p65 ($n = 6$). (h) The protein expression of Bcl-2 ($n = 6$). (i) TUNEL staining and apoptotic rate ($n = 6$). (j, k) Caspase3/7 and PARP activities ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DOX group.

3.6. OA Lost the Protective Effects in Sirt1-Deficient Mice. Subsequently, we determined whether OA treatment lost its protective effects on DOX-related cardiac injury when Sirt1 signaling was blocked. To achieve this, we used mice with a cardiac-specific deletion of Sirt1 (Sirt1 cKO). Interestingly, OA treatment in the absence of Sirt1 did not attenuate DOX-induced cardiac injury. Instead, OA-treated Sirt1 cKO mice exhibited a similar phenotype as that in Sirt1 cKO mice in response to DOX injection, as reflected by cardiac CK-MB and LDH, cardiac function, 4-HNE, and nitrotyrosine (Figures 6(a)–6(e)). The inhibitory effect of OA nuclear translocation of NF-κB was also blocked by Sirt1 deficiency. The increased nuclear Nrf2 expression after OA treatment was also suppressed by Sirt1 deficiency (Figure 6(f)). The subsequent detection of the activities of caspase3/7 and PARP revealed that Sirt1 depletion also abolished the antiapoptotic effects of OA (Figures 6(g) and 6(h)).

3.7. OA Treatment Activated Sirt1 via cAMP/PKA in Mice. Sirt1 is a well-known NAD+-dependent deacetylase, and the NAD+ concentrations can affect Sirt1 activation. DOX decreased cardiac and cellular NAD+ levels, surprisingly, OA treatment could not affect NAD+ levels in DOX-treated hearts or cells (Figures 7(a) and 7(b)). Interestingly, we found OA treatment increased cAMP abundance in DOX-treated hearts and cells (Figures 7(c) and 7(d)). The decreased PKA activity in DOX-treated hearts and cells was restored by OA treatment (Figures 7(e) and 7(f)). Moreover, Sirt1 activation by OA treatment was blunted in cells with an adenylate cyclase inhibitor (2′5′-dd-Ado) or a PKA specific inhibitor (H89) treatment (Figure 7(g)). OA lost the protective effects on cell viability in DOX-treated cells with adenylate cyclase or PKA inhibition (Figure 7(h)).

3.8. OA Treatment Did Not Affect Tumor Growth or Tissue Concentrations of DOX. Next, we evaluated whether OA

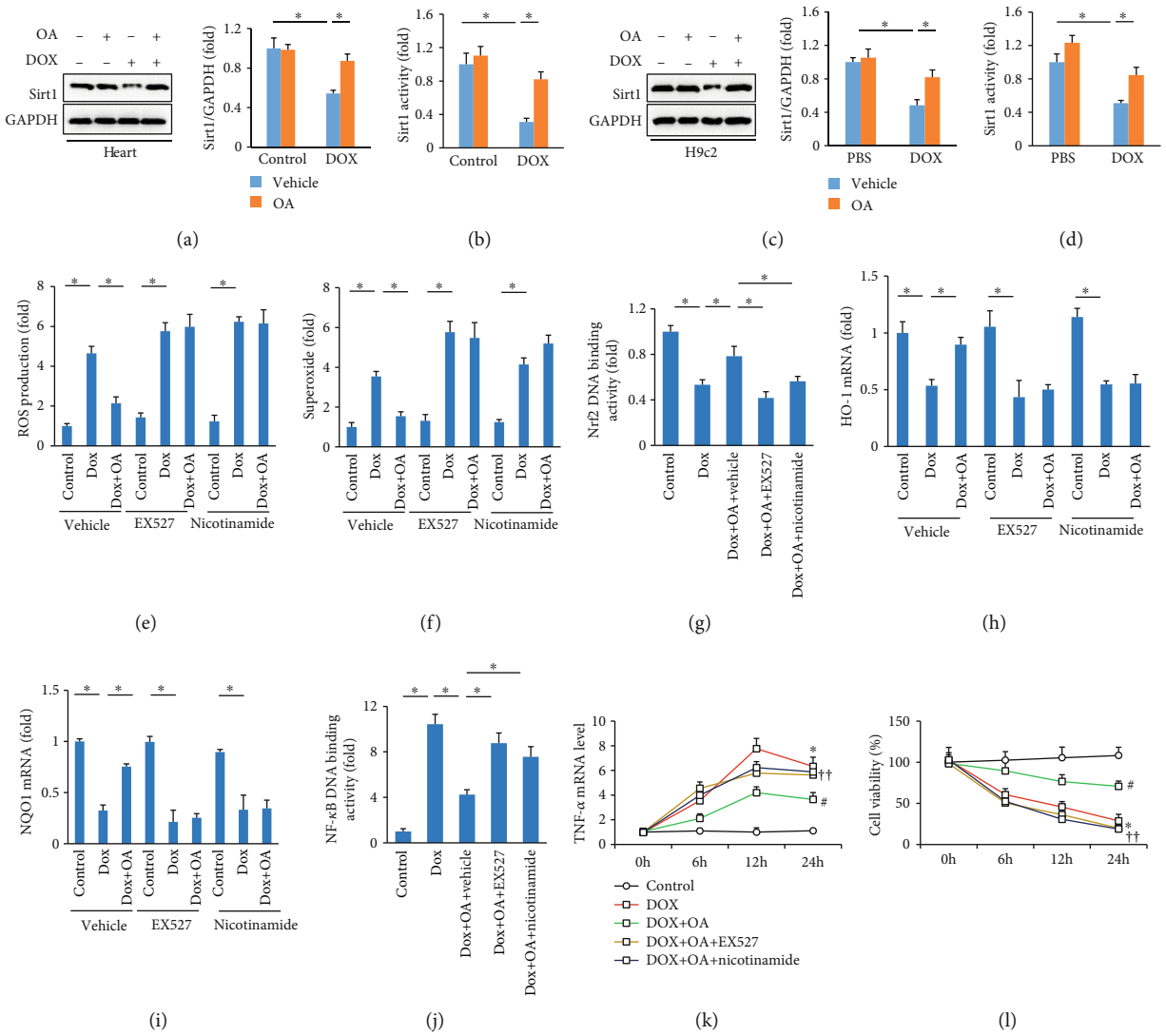

FIGURE 5: OA treatment exerted protection via Sirt1. (a, b) The Sirt1 protein expression and activity in vivo ($n = 6$). (c, d) The Sirt1 protein expression and activity in vitro ($n = 6$). (e, f) The production of ROS and superoxide ($n = 5$). (g) Nrf2 DNA binding activity ($n = 5$). (h, i) The mRNA levels of HO-1 and NQO1 ($n = 6$). (j) NF-κB binding activity ($n = 5$). (k) The mRNA level of TNF-α ($n = 5$). (l) Cell viability ($n = 5$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. For (a–i), $^{*}P < 0.05$ vs. matched control; for (j, k), $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DOX group, $^{\dagger}P < 0.05$ vs. DOX+OA group.

treatment affected tumor growth or tissue concentrations of DOX. Lewis lung carcinoma (LLC) cells were subcutaneously implanted to isogenic C57Bl6 mice. OA was administered on day 5 after tumor implantation, and this protective intervention lasted for ten days. DOX was injected on day 10 (Figure 8(a)). The data in our study indicated that OA treatment did not affect the growth of LLC xenotransplants (Figures 8(b) and 8(c)). Next, we detected DOX concentrations in serum, heart, and tumor tissue (Figures 8(d) and 8(f)). We found that there were no difference between DOX and DOX +OA groups, implying that the protective effects of OA were not attributed to reduce the availability of DOX.

4. Discussion

Here, we showed that OA treatment suppressed DOX-induced cardiotoxicity, as indicated by the improved cardiac function, reduced oxidative damage, inflammatory response, and myocardial apoptosis. Furthermore, we found that these protective effects of OA were mediated by the activation of Sirt1 in vivo and in vitro, and Sirt1 inhibition abolished OA treatment-mediated cardiac protection. In addition, we found that OA treatment did not affect tumor growth and compromise the effects of DOX. Collectively, our data define OA as a potential therapeutic drug for DOX-induced cardiotoxicity.

Some lines of evidence have suggested that oxidative stress was closely involved in the pathogenesis of DOX-induced myocardial damage [31, 32]. DOX-related toxicity was mainly caused by the free radicals during DOX metabolism, which impaired mitochondrial respiratory complex and promoted the production of superoxide [33]. Heart samples are much more sensitive to DOX-related redox imbalance for the lack of redox cycle-related enzymes and the high

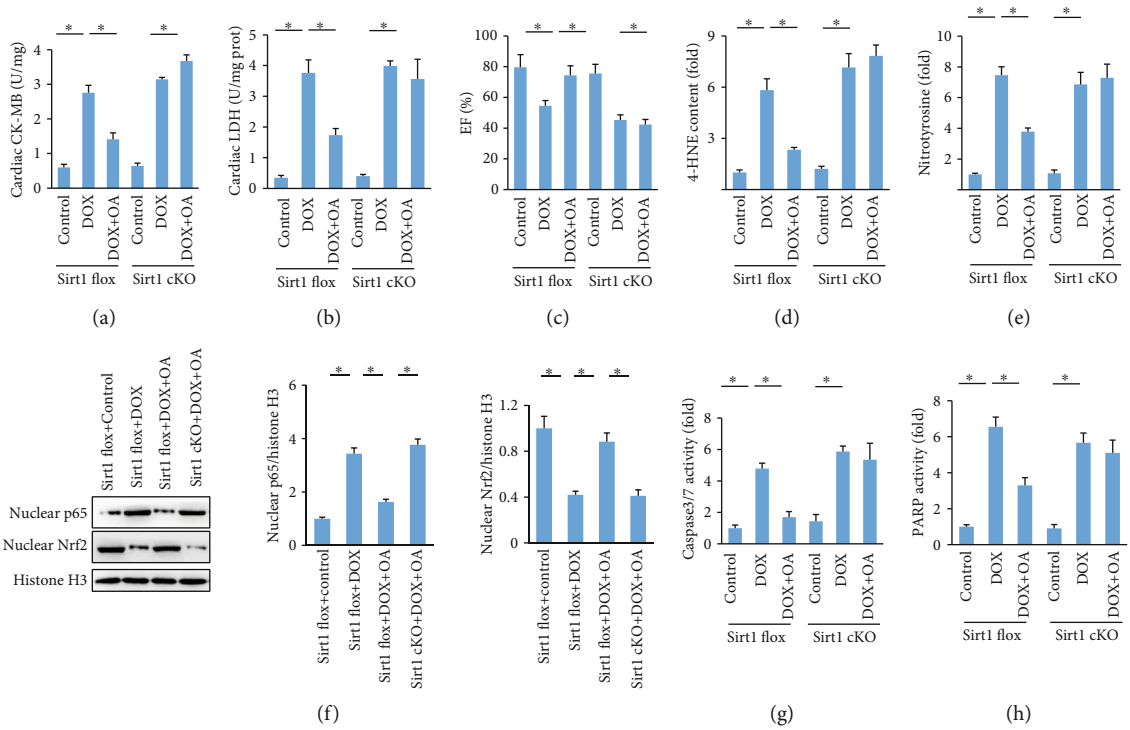

FIGURE 6: Sirt1 depletion abolished the protection provided by OA. (a, b) The cardiac CK-MB and LDH in DOX-treated mice ($n = 6$). (c) EF in the indicated groups ($n = 10$). (d, e) Myocardial 4-HNE and nitrotyrosine ($n = 6$). (f) The protein expression of nuclear Nrf2 and NF-κB ($n = 6$). (g, h) Caspase3/7 and PARP activities ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $^*P < 0.05$ vs. matched control.

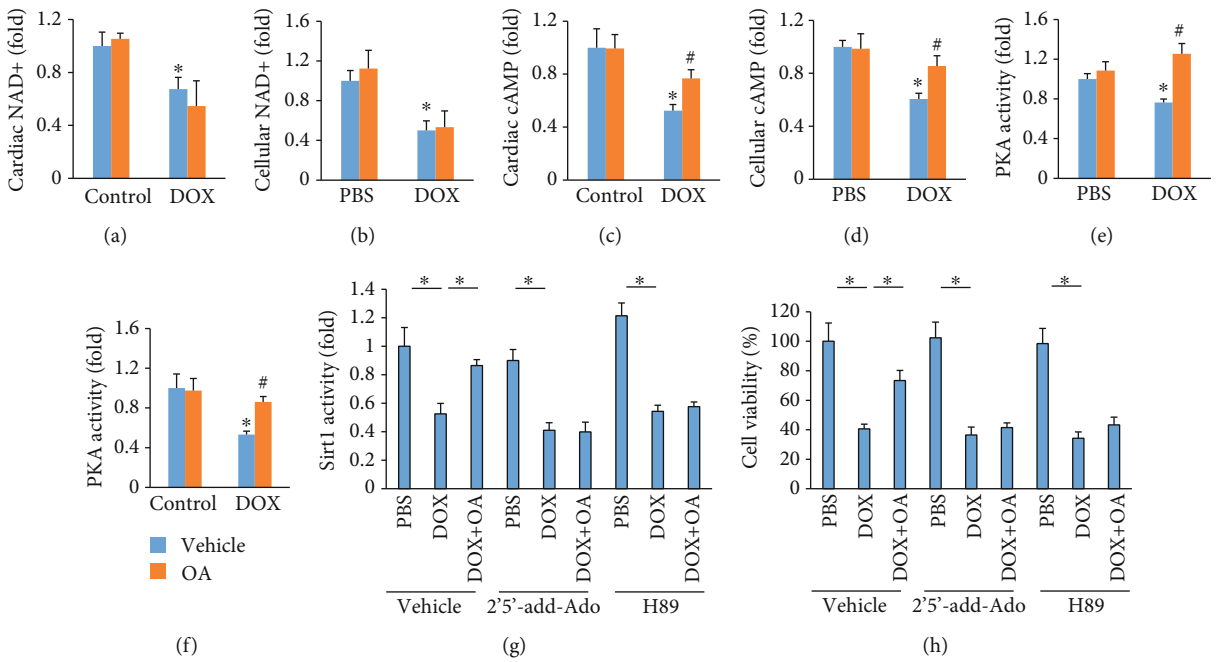

FIGURE 7: OA treatment activated Sirt1 via cAMP/PKA signaling axis. (a, b) Relative NAD+ levels ($n = 6$). (c, d) Relative cAMP levels ($n = 6$). (e) Relative PKA activity in cells ($n = 6$). (f) Relative PKA activity in the hearts ($n = 6$). (g) Relative Sirt1 activity in cells ($n = 6$). (h) Cell viability of cells ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. For (a–e), $^*P < 0.05$ vs. control/PBS group, $^{\#}P < 0.05$ vs. DOX group. For others, $^*P < 0.05$ versus the matched group.

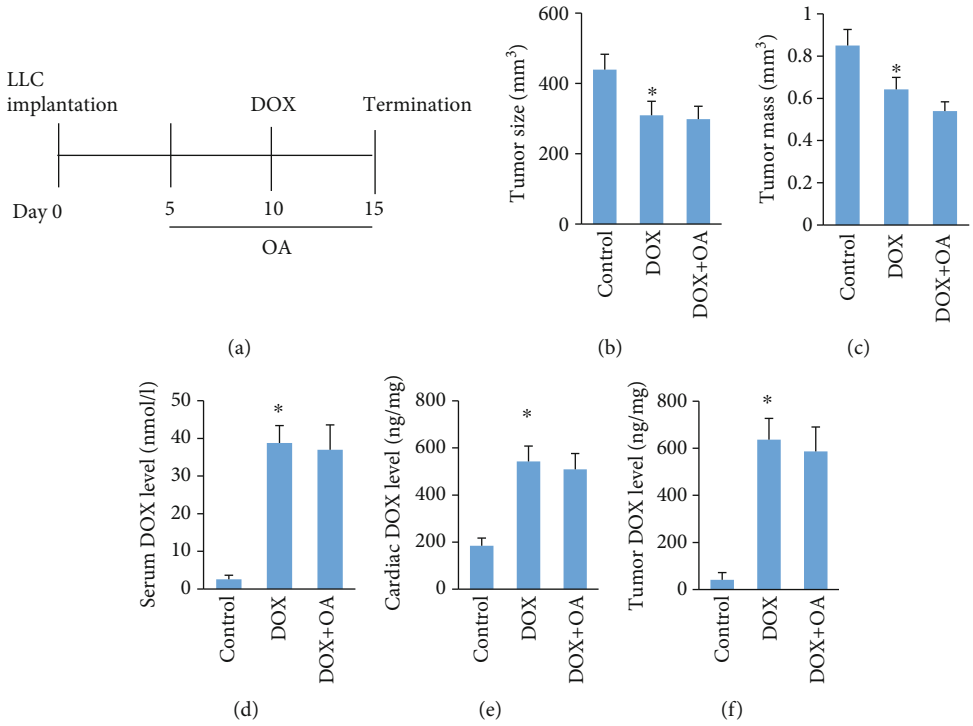

FIGURE 8: OA treatment did not increase tumor growth. (a) Schedule of the experiment. (b, c) Tumor size and tumor tissue mass ($n = 6$). (d–f) DOX concentrations in vivo in tissue ($n = 6$). Differences between multiple groups were determined by one-way ANOVA followed by Tukey's test. $^*P < 0.05$ vs. matched control.

volume of mitochondria [32]. Several lines highlighted the importance of modulating reactive oxidative damage in ameliorating the cardiotoxic effects caused by DOX. Nitric oxide synthase 3 deficiency could reduce the production of free radicals, thus, preventing the decline in cardiac function in DOX-treated mice [34]. Conversely, overexpression of SOD significantly decreased the oxidative damage and improved cardiac function in mice treated with DOX [35]. Previous studies found that OA could block free radical production in alcoholic liver disease [36]. In agreement with these findings, we also found that DOX impaired both SOD and Gpx activities, decreased GSH levels but increased lipid peroxidation contents, and these toxic effects of DOX were largely blocked by OA treatment. We also found that OA increased Nrf2 expression in DOX-treated mice. The attenuation of oxidative damage in OA-treated hearts might partly explain the protective effects of OA.

A number of studies suggested that the inflammatory response mediated the pathogenesis of DOX-induced heart dysfunction [37]. Upon stimuli, IκBα was phosphorylated and became degraded, and NF-κB translocated into the nucleus to trigger inflammatory cytokine synthesis [38]. Wang et al. first observed the activation of NF-κB in DOX-treated myocytes [39]. Here, we also found that treatment with OA attenuated the DOX-induced activation of NF-κB and upregulation of inflammatory factors. DOX-induced NF-κB activation was closely associated with myocardial apoptosis [40]. Here, we also found that DOX could induce myocardial apoptosis in vivo and impair cell viability in vitro. OA decreased myocardial apoptosis and improved cell viability in response to DOX. The attenuation of inflammatory response and myocardial apoptosis also contributed to the protective effects of OA.

Sirt1 has been suggested to play key roles in redox regulation, cell apoptosis, and inflammation [41]. Several lines of evidences suggested that Sirt1 was also involved in DOX-induced cardiac injury. A study found Sirt1 protein level was increased in response to DOX injection [42]. Inconsistent with this finding, the data from another lab indicated that DOX induced a significant decrease in Sirt1 expression. Here, we also found that DOX decreased Sirt1 protein level in vivo and in vitro. Of note, restoration of the expression of Sirt1 by OA treatment could improve cardiac function and attenuate DOX-related cardiac injury in mice. Moreover, Sirt1 depletion offsets the protective effects provided by OA treatment against DOX-induced cardiotoxicity. This finding suggested that OA exerted cardiac protection via Sirt1.

OA treatment did not alter the NAD+ level, suggesting that OA activated Sirt1 through a NAD+ independent manner. cAMP serves as an important second messenger to activate Sirt1 in NAD+ independent ways [43, 44]. cAMP/PKA induced the dissociation of Sirt1 with its endogenous inhibitor [44]. Moreover, it has been reported that PKA promoted the phosphorylation of Sirt1 to increase its enzymatic activity [43]. Here, we found that OA treatment increased cAMP levels and PKA activity in DOX-treated cells. Moreover, inhibition of adenylate cyclase and PKA abolished the activation of Sirt1 by OA, indicating that cAMP/PKA was required for the activation of Sirt1 by OA treatment.

To enhance the translational potential of our findings, we next confirmed that OA treatment did not compromise

therapeutic DOX levels or promote tumor growth. Actually, several studies have suggested that OA suppressed the development and growth of tumor [14]. Here, we found that OA treatment did affect tumor growth or tissue concentrations of DOX, implying that OA could not compromise therapeutic DOX levels.

In conclusion, mice treated with OA treatment revealed a suppressed myocardial toxicity, induced by DOX injection. OA may be considered as the new drug for the treatment of DOX-induced cardiotoxicity.

Data Availability

The data in our study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wen-Bin Zhang and Yong-Fa Zheng contributed equally this work.

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Research Article

Effect of Anticancer Quinones on Reactive Oxygen Production by Adult Rat Heart Myocytes

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This study investigated the effect of anthracycline antibiotics, mitomycin C, and menadione on oxygen consumption and hydrogen peroxide production by intact, beating, rat heart myocytes. Doxorubicin produced a dose-dependent increase in the rate of cyanide-resistant respiration by beating myocytes. The anthracycline analogs 4-demethoxydaunorubicin, 4′-epidoxorubicin, 4′-deoxydoxorubicin, and menogaril, as well as the anticancer quinones mitomycin C and menadione, also significantly increased oxygen consumption by cardiac myocytes. However, 5-iminodaunorubicin (which has a substituted quinone group) and mitoxantrone (which is not easily reduced by flavin dehydrogenases) had no effect on cardiac respiration. Both catalase (43%) and acetylated cytochrome c (19%) significantly decreased oxygen consumption that had been stimulated by doxorubicin; furthermore, extracellular hydrogen peroxide production was increased from undetectable control levels to 1.30 ± 0.02 nmol/min/10^7 myocytes ($n = 4$, $P < 0.01$) in the presence of 400 μM doxorubicin. These experiments suggest that the anthracycline antibiotics and other anticancer quinones stimulate cardiac oxygen radical production in intact heart myocytes; such a free radical cascade could contribute to the cardiac toxicity of these drugs.

1. Introduction

The anticancer quinones, including the anthracycline antibiotics and mitomycin C, are widely used for the treatment of hematopoietic malignancies and cancers of the breast and bladder [1]. Unfortunately, the utility of these drugs is hindered by cardiac toxicity that most often takes the form of a dose-related congestive cardiomyopathy [2–4]. Many different hypotheses have been suggested to explain the myocardial injury produced by antineoplastic quinones [5, 6]. These hypotheses include the observation that the formation of a doxorubicin-iron complex may enhance the formation of strong oxidants toxic to the heart [7]; cardiac toxicity could also occur through drug-induced effects on iron-binding proteins [8]. However, several lines of evidence support the possibility that stimulation of superoxide anion, hydrogen peroxide, and a chemical species with the characteristics of the hydroxyl radical in the heart after reduction of the quinone moiety by complex I of the mitochondrial electron transport chain [9], NADPH:cytochrome P-450 reductase associated with the sarcoplasmic reticular membrane, or cytoplasmic xanthine dehydrogenase [10] plays an important role in the mechanism of anthracycline cardiac toxicity [11, 12].

If reactive oxygen species are produced at these intracellular sites in intact heart cells [13], oxidative stress itself might explain the characteristic picture of mitochondrial swelling, sarcotubular vacuolization, and myofibrillar loss produced by anthracycline quinones [1]. Previous investigations utilizing neonatal rat heart cells in culture have demonstrated that treatment with the anthracycline analog daunorubicin decreases cellular reduced glutathione pools [14] and that inhibition of glutathione-mediated peroxide detoxification significantly enhances the cytotoxicity of

doxorubicin [15]. Hence, drug-induced oxygen radical formation appears to be at least one mechanism involved in the toxicity of anthracyclines for neonatal heart cells.

To confirm previous studies that used cardiac subcellular fractions from adult rats (but without disrupting intracellular architecture or utilizing cells that are actively dividing) [10], the experiments presented here were performed with intact, actively beating myocytes from adult rats. Our results indicate that the anthracycline and mitomycin C quinones are reduced by one electron in adult rat heart cells and can initiate an oxidation-reduction cycle in the presence of molecular oxygen that ultimately results in the formation of hydrogen peroxide.

2. Materials and Methods

2.1. Experimental Animals. Male 200-gram Sprague-Dawley rats were obtained from Simonsen Laboratories, Gilroy, CA and housed on hardwood bedding with access to feed and water *ad libitum*.

2.2. Materials. Doxorubicin hydrochloride and mitomycin C were purchased from commercial sources. Menogaril, 5-iminodaunorubicin, and mitoxantrone were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD. 4-demethoxydaunorubicin, 4′-epidoxorubicin, and 4′-deoxydoxorubicin were a gift of Farmitalia Carlo Erba, Milan, Italy. Menadione (2-methyl-1,4-naphthoquinone, sodium bisulfite salt), cytochrome c (type VI from horse heart), dimethyl sulfoxide (DMS0), diethylenetriaminepentaacetic acid (DTPA), hyaluronidase (type I-S), and bovine erythrocyte superoxide dismutase (SOD, 2900 units/mg) were from Sigma-Aldrich Chemical Co., St. Louis, MO. Prior to these experiments, cytochrome c was acetylated to eliminate the effects of potential exogenous oxidizing or reducing species present in our reaction mixtures [16]. Collagenase (type II) was purchased from Cooper Biomedical Inc., Freehold, NJ. Metrizamide (type AN 6300) of analytical grade was from Accurate Chemical and Scientific Corp., Westbury, NY. Catalase of analytical grade (65,000 units/mg) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, and was devoid of SOD activity when assayed by the method of McCord and Fridovich [17]. Only glass distilled, deionized water was used in these studies.

2.3. Preparation of Cardiac Myocytes. Experimental animals were euthanized by lethal inhalation of methoxyflurane. Ventricular tissue, typically from 6-8 rats, was rapidly removed and then washed and minced in an iced phosphate buffer at pH 7.4 containing 120 mM NaCl, 5.4 mM KCl, 1.5 mM Na_2HP0_4, 0.4 mM NaH_2P0_4, and 5 mM glucose. Myocytes were prepared as previously described [18] except that separation of intact from damaged cells was performed by metrizamide density centrifugation [19]. Cell viability in these studies was determined by the presence of rod-shaped morphology and exclusion of 0.1% trypan blue dye and ranged in typical experiments from 70-80%; myocyte yield was usually 0.5 to 1×10^6 viable cells per heart.

2.4. Measurement of Oxygen Consumption and Hydrogen Peroxide Formation. Oxygen consumption was determined using a YSI oxygen monitor with a Clarke-type electrode as previously described [20]. The final, 3 ml reaction mixture contained 125 mM potassium phosphate, 140 mM sodium chloride, and 10 mM glucose, pH 7.4 at 37°C. All reactants were bubbled with air for 30 min at 37°C before use; and most experiments were performed with 2×10^6 viable myocytes in the final 3 ml reaction mixture. Where utilized, myocytes were preincubated with 5 mM KCN for 10 min in the stirred, temperature-controlled glass oxygen electrode chamber open to the atmosphere prior to the initiation of the reaction with a chemotherapeutic agent. After insertion of the oxygen electrode, the linear rate of oxygen consumption was determined for 10 to 15 min thereafter. In some experiments, small volumes of specific reagents (typically 10 μl of catalase) were added to the reaction vessel through the access slot of the oxygen electrode plunger. The rate of oxygen consumption was based on a value of 597 nmol for the total dissolved oxygen content of the reaction vessel [21]. Hydrogen peroxide production was quantitated by the release of oxygen into the closed reaction chamber of the oxygen electrode as previously described [22], after the addition of 7500 units of catalase through the access slot of the plunger.

2.5. Statistical Methods. Data were analyzed with the 2-tailed t-test for independent means (not significant, $P > 0.05$ [23]).

3. Results

3.1. Effect of Anticancer Quinones on Oxygen Consumption by Rat Heart Myocytes. Oxygen consumption by intact, untreated cardiac myocytes isolated from adult rats was (mean ± S.E.) 5.35 ± 0.38 nmol/min/2×10^6 cells (Table 1). Cardiac respiration was reduced by 81% after the addition of KCN to inhibit mitochondrial cytochrome oxidase. Treatment with rotenone to inhibit mitochondrial electron flow beyond complex I of the electron transport chain decreased oxygen consumption by 72%. As demonstrated in Table 1, in the presence of myocytes, doxorubicin significantly increased the rate of both rotenone-resistant and cyanide-resistant respiration. In analogy to our previous experiments with cardiac submitochondrial particles [9] and cardiac sarcoplasmic reticulum [10], these studies suggest that the doxorubicin quinone may be reduced by mitochondrial complex I or other intracellular flavin dehydrogenases with subsequent electron transfer to molecular oxygen in the heart. The difference between our results with rotenone and KCN may reflect the inhibition of myocardial copper-zinc superoxide dismutase by cyanide. This could decrease the enzymatic breakdown of intracellular superoxide (ultimately to O_2 and H_2O) leading to an apparent increase in respiratory rate.

In the presence of cyanide, doxorubicin enhanced the respiratory rate of adult cardiac myocytes in a concentration-dependent manner (Figure 1); a significant increase in oxygen consumption occurred under our experimental conditions beginning with a doxorubicin concentration of 10 μM, (1.4 ± 0.01 nmol/min/2×10^6 cells, $P < 0.05$, $n = 3$). At the highest doxorubicin concentration tested (400 μM), drug-

Table 1: Requirements for quinone-enhanced oxygen consumption in rat heart myocytes. Oxygen consumption was determined polarographically in a 3 ml reaction system at 37°C that contained 125 mM potassium phosphate buffer, pH 7.4, 140 mM NaCl, 10 mM glucose, and 2×10^6 viable cardiac myocytes.

Reaction system	Oxygen consumption (nmol O_2/min/2×10^6 cells)
Control	5.35 ± 0.38[a]
+KCN (5 mM)	1.02 ± 0.08[b]
+Rotenone (10 μM)	1.52 ± 0.02[b]
Doxorubicin (400 μM)	
+Rotenone (10 μM)	2.20 ± 0.14[c]
Doxorubicin (400 μM)	
+KCN (5 mM)	5.74 ± 0.28[c]
Doxorubicin (400 μM)	
+KCN (5 mM)	
-Cells	ND[d]

[a]Mean ± S.E. of 3 to 5 experiments. [b]Significantly different from the control, at $P < 0.01$. [c]Significantly different from samples containing mitochondrial inhibitors alone, at $P < 0.01$. [d]N.D. is not detectable.

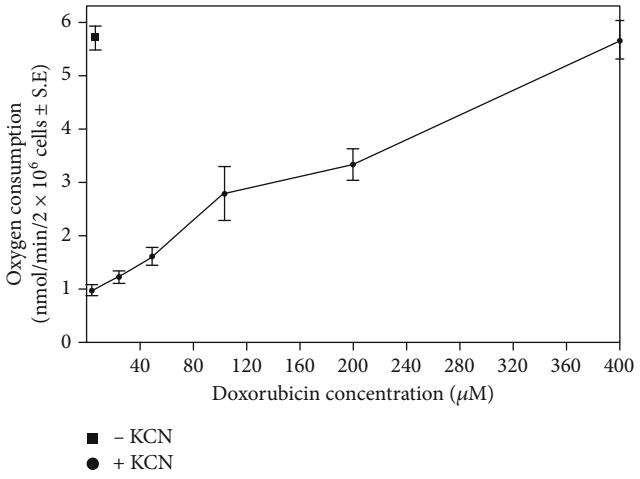

Figure 1: Effect of doxorubicin concentration on the rate of cyanide-resistant respiration in adult rat heart myocytes. Data represent the mean ± S.E. of 3 to 6 experiments for every concentration of doxorubicin tested; $P < 0.05$ for each drug level compared to the untreated control.

stimulated oxygen consumption was equivalent to that produced intrinsically by mitochondrial respiration. Furthermore, as shown in Figure 2, drug-stimulated oxygen consumption varied with the myocyte concentration examined. No oxygen was consumed in the absence of cells, and the difference in respiratory rate between drug-treated and control cells increased with the number of myocytes studied (Figure 2).

We also investigated the effect of other anticancer quinones (including anthracycline antibiotics, mitomycin C, mitoxantrone, and menadione) on cyanide-resistant respiration by rat heart myocytes. As demonstrated in Table 2, essentially all the anthracyclines tested significantly increased cardiac oxygen consumption. Only 5-iminodaunorubicin,

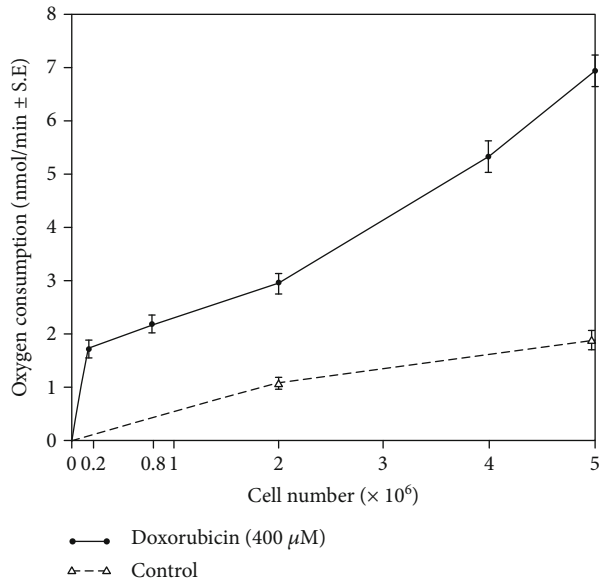

Figure 2: Effect of myocyte concentration on doxorubicin-stimulated, cyanide-resistant (5 mM) oxygen consumption. The results shown represent the mean ± S.E. of 3 experiments for each myocyte concentration tested.

which has a substituted quinone group that substantially limits oxidation-reduction cycling [24] was incapable of stimulating myocyte oxygen consumption under our experimental conditions. Of the nonanthracyclines examined, both mitomycin C and menadione (2-methyl-1,4 naphthoquinone) stimulated cyanide-resistant respiration significantly, whereas mitoxantrone, at equimolar concentration, proved ineffective (Table 2). These results are consistent with previous investigations suggesting that the mitoxantrone quinone does not produce an active redox cycle in the heart mitochondria or sarcoplasmic reticulum [25]. On the other hand, mitomycin C significantly enhances oxygen consumption by cardiac NADPH:cytochrome P-450 reductase but not by the mitochondrial electron transport chain [26]. Although the effect of menadione on myocyte oxygen consumption has not been examined previously, reduction of molecular oxygen by this compound after treatment of hepatocytes has been described [27].

3.2. Effect of Oxygen Radical Scavengers on Doxorubicin-Stimulated Myocyte Oxygen Consumption. To further examine the mechanism(s) underlying the increase in myocyte oxygen consumption produced by doxorubicin, we investigated the effect of various oxygen radical modifying agents on drug-related respiration. Both catalase and acetylated cytochrome c, scavengers of hydrogen peroxide and superoxide anion, respectively, significantly decreased doxorubicin-enhanced, cyanide-resistant myocyte oxygen consumption (Table 3). Heat-inactivated catalase was without effect. These results are of interest in that neither protein would be expected to enter viable myocytes. Thus, it seems likely that at least some extracellular superoxide anion and H_2O_2 were present following doxorubicin exposure in these studies. The addition of DTPA, an efficient iron chelator that does

Table 2: Effect of anticancer quinones on cyanide-resistant oxygen consumption by cardiac myocytes. Oxygen consumption was measured with a Clark-type electrode in a 3 ml reaction system at 37°C that contained 125 mM potassium phosphate buffer, pH 7.4, 140 mM NaCl, 10 mM glucose, 5 mM KCN, and 2×10^6 viable cardiac myocytes, with or without drugs.

Drug	Oxygen consumption (nmol O_2/min/2×10^6 cells)
None	1.02 ± 0.08[a]
4-Demethoxydaunorubicin	
50 μM	1.82 ± 0.15[b]
400 μM	2.22 ± 0.02[b]
4′-Epidoxorubicin (400 μM)	3.48 ± 0.34[b]
Menogaril (400 μM)	2.57 ± 0.06[b]
4′-Deoxydoxorubicin (400 μM)	2.67 ± 0.53[b]
5-Iminodaunorubicin (400 μM)	1.24 ± 0.24
Mitomycin C	
50 μM	1.08 ± 0.06
400 μM	1.55 ± 0.06[b]
Mitoxantrone (400 μM)	1.39 ± 0.11
Menadione	
10 μM	1.90 ± 0.30[b]
50 μM	4.80 ± 1.20[b]
400 μM	9.37 ± 1.41[b]

[a]Mean ± S.E. of 3 to 5 experiments. [b]Significantly different from the control, at $P < 0.05$.

Table 3: Effect of oxygen radical modifiers on doxorubicin-enhanced, cyanide-resistant oxygen consumption by rat cardiac myocytes. Oxygen consumption was measured with a Clark-type electrode in a 3 ml reaction system at 37°C exactly as described in Table 2.

Reaction system	Oxygen consumption (percent of control)
Control	100[a]
+Catalase (2500 units/ml)	102 ± 8[b]
+Heat-inactivated catalase (2500 units/ml)[c]	134 ± 24
+Acetylated cytochrome c (50 μM)	93 ± 3
+Dicumarol (50 μM)	125 ± 25
Doxorubicin (400 μM)	100[d]
+Catalase (2500 units/ml)	57 ± 1[e]
+Heat-inactivated catalase (2500 units/ml)	103 ± 5
+Acetylated cytochrome c (50 μM)	81 ± 5[e]
+Dicumarol (50 μM)	97 ± 9
+DTPA (100 μM)	99 ± 6
+DMSO (100 mM)	100 ± 3

[a]Control rate of oxygen consumption was 1.00 ± 0.05 nmol/min/2×10^6 myocytes, $n = 4$. [b]Mean ± S.E. of 3 to 5 experiments. [c]Catalase was inactivated by autoclaving for 60 min. [d]Doxorubicin-stimulated, cyanide-resistant oxygen consumption was 6.4 ± 0.6 nmol/min/2×10^6 cells, $n = 3$. [e]Significantly different from samples containing doxorubicin alone, $P < 0.05$.

not penetrate mammalian cell membranes [28], also did not decrease drug-stimulated oxygen consumption. Since DTPA chelates iron in a form that is unavailable for oxidation-reduction reactions, our findings do not support formation of an extracellular doxorubicin-iron complex as the species responsible for reduction of oxygen in these experiments [29]. Drug-related oxygen consumption was, furthermore, not diminished by DMSO, a potent scavenger of the hydroxyl radical; this was expected because DMSO does not detoxify hydrogen peroxide or superoxide anion under aqueous conditions and because the reaction of DMSO with the hydroxyl radical does not yield molecular oxygen [22].

Finally, although previous investigations have demonstrated that treatment of neonatal cardiac myocytes in culture with the anthracycline daunorubicin significantly increases intracellular DT-diaphorase activity [30], addition of the potent DT-diaphorase inhibitor dicumarol did not change the rate of doxorubicin-stimulated myocyte respiration (Table 3). Additional experiments using 50 μM doxorubicin also failed to reveal any effect of dicumarol on drug-stimulated respiration (data not shown). If DT-diaphorase played an important role in cardiac detoxification by catalyzing the two-electron reduction of the doxorubicin quinone, enzyme inhibition by dicumarol should have increased the rate of oxygen consumption by favoring semiquinone over hydroquinone formation.

3.3. Effect of Doxorubicin on Hydrogen Peroxide Production by Heart Myocytes. As demonstrated in Figure 3, addition of catalase to cyanide-treated myocytes in the presence of doxorubicin released oxygen, indicating that hydrogen peroxide had been formed in these studies. In multiple experiments, identical to the one shown in Figure 3, the rate of hydrogen peroxide formation was 1.30 ± 0.02 nmol/min/10^7 myocytes ($n = 4$) in the presence of doxorubicin (400 μM). Release of H_2O_2 was not detectable in the absence of the drug ($n = 4$, $P < 0.01$). It is important to point out that our studies only measured H_2O_2 that leaves the cell and is accessible to exogenous catalase. Thus, these results represent a lower limit estimate of the rate of drug-stimulated H_2O_2 formation. Furthermore, because of this methodological limitation, no precise quantitation of the stoichiometry between myocyte oxygen consumption and H_2O_2 production is possible under our experimental conditions.

4. Discussion

In these experiments, we have demonstrated that a wide range of anthracycline antibiotics, as well as mitomycin C and menadione, are capable of stimulating oxygen consumption by heart myocytes isolated from adult rats. Furthermore, drug-related oxygen consumption is accompanied by the formation of hydrogen peroxide. Hence, our studies confirm that reactive oxygen metabolism by anticancer quinones previously studied with cardiac subcellular fractions or purified enzyme preparations occur in the intact cell. Since doxorubicin significantly increased myocyte oxygen consumption in the presence of rotenone (which is not known to inhibit antioxidant enzyme activity) as well as cyanide and led to the

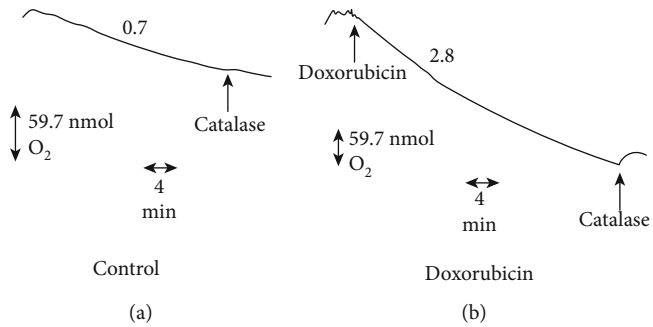

Figure 3: Effect of doxorubicin on cyanide-resistant oxygen consumption and hydrogen peroxide production by rat heart myocytes. Numbers above each experiment indicate oxygen consumption in nmol/min/10^6 cells. Catalase (arrow, 7500 units) was added through the access slot of the oxygen electrode plunger. (a) The control rate of rat heart myocytes in the presence of 5 mM KCN. (b) The doxorubicin concentration was 400 μM.

presence of detectable extracellular levels of H_2O_2, our investigations suggest that reactive oxygen formation after treatment of cardiac myocytes with doxorubicin exceeds cellular pathways for both enzymatic and nonenzymatic detoxification of oxygen radical metabolites. Hence, it seems possible that drug-related oxygen radical formation *in vivo* could be involved in the cardiac toxicity of the anthracycline quinones.

It has been recognized for several years that the risk of clinical congestive heart failure is increased in patients who are treated with mitomycin C after having previously received doxorubicin [31, 32]. Furthermore, previous studies have demonstrated that mitomycin C increases oxygen consumption and superoxide production by NADPH:cytochrome P-450 reductase from the heart sarcoplasmic reticulum; however, mitomycin C is not actively reduced by the mitochondrial electron transport chain [26]. At equimolar concentrations, in this study, mitomycin C and doxorubicin increased myocyte oxygen consumption by 52% and 56.3%, respectively (Tables 1 and 2). Hence, these experiments suggest that mitomycin C may undergo more limited free radical metabolism than the anthracyclines in intact myocytes. Because of the abundance of mitochondria in the heart, and because of the clinical observation that mitomycin C by itself is seldom a cardiotoxin, it may be speculated that reduction of the anthracycline quinone by the mitochondria plays a predominant role in generating drug-induced free radicals in intact heart cells.

We also found in these experiments that oxygen consumption was not decreased by a potent iron-chelating agent. This finding does not eliminate the possibility that iron may play an important role in the myocardial toxicity of anticancer quinones. Our experiments suggest only that an extracellular doxorubicin-iron complex was unlikely to be the cause of the oxygen radical production that we observed. The role of an intracellular drug-iron complex or of free or protein-bound iron in facilitating the production of the hydroxyl radical or other reactive species has not been examined in these experiments. However, because of the potent oxidizing power of the hydroxyl radical, and studies suggesting that this species is of importance to anthracycline toxicity [33]; further definition of the role of intracellular iron in anthracycline heart toxicity remains of interest.

5. Conclusions

In summary, the experiments reported here strongly suggest that adult rat heart myocytes metabolize anticancer quinones to species capable of reducing molecular oxygen. It is noteworthy, furthermore, that generation of reactive oxygen species occurred at anthracycline concentrations that are formed intracellularly following exposure of intact heart cells to this class of drugs [34]. In view of the presence of an extracellular flux of H_2O_2 following exposure of rat heart myocytes to doxorubicin, and the recent demonstration of the adverse consequences of a doxorubicin-related free radical cascade on cardiac fibroblasts [15], these experiments suggest, finally, that multiple components of the cardiac matrix, as well as myocyte membranes, may be at risk from anthracycline-induced oxidative stress.

Abbreviations

DMSO: Dimethyl sulfoxide
DTPA: Diethylenetriamine-pentaacetic acid
SOD: Superoxide dismutase
KCN: Potassium cyanide.

Data Availability

The data used to support the findings of this study are included with the article.

Conflicts of Interest

There are no conflicts of interest associated with this paper.

Acknowledgments

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Review Article

Possible Susceptibility Genes for Intervention against Chemotherapy-Induced Cardiotoxicity

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Recent therapeutic advances have significantly improved the short- and long-term survival rates in patients with heart disease and cancer. Survival in cancer patients may, however, be accompanied by disadvantages, namely, increased rates of cardiovascular events. Chemotherapy-related cardiac dysfunction is an important side effect of anticancer therapy. While advances in cancer treatment have increased patient survival, treatments are associated with cardiovascular complications, including heart failure (HF), arrhythmias, cardiac ischemia, valve disease, pericarditis, and fibrosis of the pericardium and myocardium. The molecular mechanisms of cardiotoxicity caused by cancer treatment have not yet been elucidated, and they may be both varied and complex. By identifying the functional genetic variations responsible for this toxicity, we may be able to improve our understanding of the potential mechanisms and pathways of treatment, paving the way for the development of new therapies to target these toxicities. Data from studies on genetic defects and pharmacological interventions have suggested that many molecules, primarily those regulating oxidative stress, inflammation, autophagy, apoptosis, and metabolism, contribute to the pathogenesis of cardiotoxicity induced by cancer treatment. Here, we review the progress of genetic research in illuminating the molecular mechanisms of cancer treatment-mediated cardiotoxicity and provide insights for the research and development of new therapies to treat or even prevent cardiotoxicity in patients undergoing cancer treatment. The current evidence is not clear about the role of pharmacogenomic screening of susceptible genes. Further studies need to done in chemotherapy-induced cardiotoxicity.

1. Introduction

Cancer therapeutics have seen tremendous progress in recent years [1, 2] and have revolutionized the treatment strategies and outcomes of some types of cancer [3]. These novel therapeutic strategies target specific molecular entities implicated in disease pathogenesis. Advances in cancer treatment have improved the survival rates of cancer patients, but they have also increased morbidity and mortality due to side effects [4, 5], in particular, cardiovascular complications, including hypertension, arrhythmias, left ventricular (LV) dysfunction, and HF, which can manifest many years after the completion of chemotherapy [6]. For example, regardless of the infusion rate [7], maximum cumulative doses [8], and alternative drugs [9] to reduce heart injury, the incidence of cardiotoxicity caused by anthracyclines is 9% to 18% [10, 11]. Within 2 years of HF, patients have a mortality rate of 60%, an extremely poor prognosis [12]. Further, the incidence of

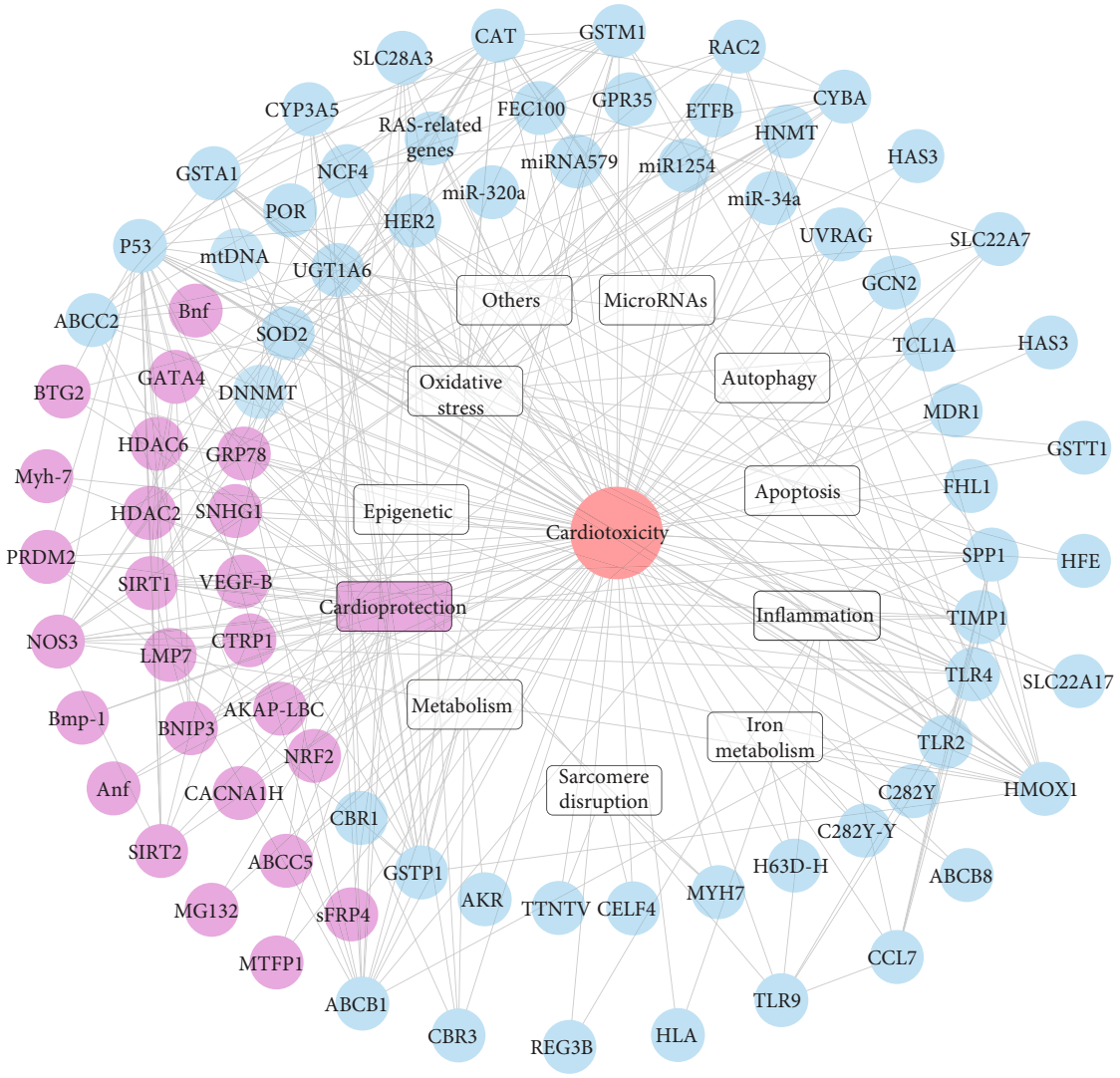

Figure 1: Graph of the protein network comprising combinations based on genetic studies that indicates the protective targets in the chemotherapy-induced cardiotoxicity.

myocarditis with checkpoint inhibitors can be as high as 13.9% [13]. As a result, cancer patients often suffer from a variety of cardiotoxicities induced by treatment, which can result in substantial adverse impact on their emotional, economic, and social well-being [14, 15]. Unfortunately, the mechanisms underlying chemotherapy-induced cardiotoxicity remain poorly understood.

Although clinical and demographic factors may increase the susceptibility of some individuals to the risk and severity of toxicity, individual differences in toxicity manifestations are considerable, exacerbating these toxicities. Genetics, therefore, could provide insights into the mechanism for toxicity induced by chemotherapy. The identification of genetic biomarkers able to predict whether a patient is at risk of developing cardiac dysfunction induced by chemotherapy will minimize cardiotoxicities during cancer treatment, through the administration of cardioprotective drugs or the use of optimized cancer therapies. Data from studies on genetic defects and pharmacological interventions have suggested that many molecules, primarily those regulating oxidative stress, inflammation, autophagy, apoptosis, and metabolism, contribute to the pathogenesis of cardiotoxicity induced by chemotherapy. In this article, we review the progress made in genetic research to elucidate the molecular mechanisms of chemotherapy-induced cardiotoxicity. Furthermore, a network of functionally related proteins from a STRING database [16] (Figure 1) was established to determine whether these targets play a role in the prediction of or protection against chemotherapy-induced cardiotoxicity. We propose a variety of cardioprotective mechanisms and provide insights for the development of therapies to reduce, or even cure, the cardiotoxicity induced by chemotherapy in future studies.

2. Susceptibility Genes in Chemotherapy-Induced Cardiotoxicity

Genes positively correlated with cardiotoxicity have been found to contain alleles that change the encoding of protein expression, leading to the development of disease [17, 18]. Genetic markers that predict whether patients will develop

cardiotoxicity from chemotherapy would allow for the careful monitoring of patients, the administration of cardioprotective drugs, and the early initiation of treatment after cardiotoxicity [19–21]. This review provides an overview of all the genetic variants that have been found to influence susceptibility to cardiotoxicity (Table 1 and Figure 2). The identified gene variants are discussed in view of the latest theories regarding the complex pathological mechanisms responsible for this adverse drug reaction.

2.1. Oxidative Stress. Chemotherapy produces reactive oxygen species (ROS) via multiple pathways, including hydroxyl radicals (-OH), superoxide radicals (O^{2-}), and hydrogen peroxide (H_2O_2). Excessive ROS generation is the most widely theorized mechanism for mediating chemotherapy-induced cardiotoxicity [22–24]. H_2O_2 and O^{2-} may generate the toxic -OH and cause myocardial injury [25]. The heart is particularly vulnerable to oxidative stress because of the low levels of enzymes that neutralize these substances found in cardiac tissue [26, 27]. ROS interacts with DNA, proteins, and lipid membranes to destroy them.

Chemotherapy produces excessive free radicals by exploiting cellular oxidoreductases, including nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase, resulting in cardiotoxicity [28–30]. The NADPH oxidase (NOX) multienzyme complex uses NADPH or NADH as an electron donor to promote a 1-electron reduction of oxygen. This enzyme has been studied in the endothelium and macrophages, and was recently confirmed as a possible primary source of ROS in the myocardium [31]. Genotypic variations of alpha-1 class glutathione S-transferase (GSTA1, rs3957357) and NOX p22phox (CYBA, rs4673) are predictors of event-free survival. The influence of single-nucleotide polymorphisms (SNPs) on toxicity was assessed in 658 rituximab-CHOP- (R-CHOP-) 21 courses [32]. Overall, the SNPs influencing CYBA rs4673 and GSTA1 rs3957357 may predict patient prognosis after R-CHOP-21 treatment. In addition, a variant of the NOX subunit NCF4 (rs1883112) may prevent hematological and nonhematological toxicity [33, 34]. Another study investigated genotype participants and conducted a follow-up study for the occurrence and development of HF [35]. The SNPs were selected from 82 genes potentially associated with cardiotoxicity. Among 1,697 patients, 55 had acute anthracycline-induced cardiotoxicity (ACT) and 54 had chronic ACT. This study detected 5 genes that were related to polymorphisms in NOX and doxorubicin (DOX) efflux transporters, while chronic ACT was found to be related to NCF4 (rs1883112). Additionally, acute ACT was found to be related to the p22phox subunit (rs4673) and the RAC2 subunit (rs13058338). Consistent with these results, mice with insufficient NOX activity were resistant to chronic DOX therapy [35–37].

Meanwhile, another previous study investigated 2,950 patients who had undergone hematopoietic cell transplantation (HCT) from 1988 to 2007 [38]. Genotyping was performed on 77 cases of HCT germline DNA and 178 cases of control. The results of multivariate analysis showed that the incidence of congestive heart failure (CHF) was higher in patients with pre-HCT chest radiation and with gene variants coding for the NOX subunit RAC2 (rs13058338), HFE (rs1799945), or the DOX efflux transporter ATP-binding cassette subfamily C member 2 (ABCC2, rs8187710) [35, 39]. In addition, the polymorphisms of NOX subunits and transporters ABCC1, ABCC2, and SLC28A3 were genotyped in patients with aggressive CD20 B-cell lymphoma [40, 41]. The RAC2 subunit genotypes were found to have statistical significance in the multivariate logistic regression analysis. In summary, RAC2 and CYBA genotypes appear to be related to ACT [34, 42], which demonstrates that NOX is associated with ACT.

ABCC1, also known as multidrug resistance-associated protein 1 (MRP1), is expressed in the heart and is involved in detoxifying and protecting against the toxic actions of xenoorganisms [43, 44]. One study investigated the correlation between left ventricular (LV) function and SNPs in the ABCC1 gene in children treated with anthracyclines [45]. The data of acute lymphoblastic leukemia in children were analyzed, and echocardiography and genotyping of 9 polymorphisms of the ABCC1 gene were performed. The results revealed that the combination of ABCC1 rs3743527TT and rs3743527tt-rs246221tc/TT is associated with lower LV fractional shortening (FS), suggesting that genetic variations in the ABCC1 gene may impact LV dysfunction induced by anthracycline. Moreover, the synonymous encoding variant rs7853758 in the SLC28A3 gene was significantly related to ACT [46–48]. The risk and protection variants of other genes have been described, including SLC28A1 and several kinds of ATP-binding cassette transporters (ABCB1, ABCB4, and ABCC1). The novel relevance of the Top2b (topoisomerase-IIb) SNPs was verified [49], which suggested an association between the SNPs of RAC2, NCF4, and SLC28A3, and 23 SNPs associated with ACT [50]. Another study examined the relationship between 36 candidate polymorphisms of MAP (methotrexate, adriamycin, and cisplatin) pathway genes and grade 3-4 chemotherapy toxicity [48]. Blood samples were taken from patients who had completed MAP chemotherapy. All patients were manually genotyped to identify five polymorphisms, while the remaining 31 polymorphisms were genotyped using Illumina 610-Quad microarray. The results suggested that the toxicity of methotrexate was enhanced in the MTHFR, ABCB1, and ABCC2 variants [48, 51, 52].

The P450 oxidoreductase (POR) gene encodes a flavin protein that transfers electrons from NADPH to various kinds of proteins, including the cytochrome P450 enzymes [53]. Anthracyclines and other quinone compounds are transformed by microsomes into hemiquinone radical form through an electron reduction reaction catalyzed by POR. This biological activation step stabilizes the drug's cross-linking to DNA and is thought to greatly enhance its cytotoxicity [54]. This study detected 60 gene-encoding proteins participating in drug metabolism and efflux, with the POR gene and daunorubicin (DNR) showing the strongest cardiotoxic effects in patients with acute myeloid leukemia (AML) [55]. In this cohort of patients with AML, the estimated variation in the POR gene after DNR treatment accounted for

Table 1: Susceptibility genes in chemotherapy-induced cardiotoxicity.

Study	Drug used	Type of cancer examined	Gene	SNP ID/location of pathogenic mutation	Targets	Cardiac toxicity	References
Rossi et al. (2009)	Doxorubicin	Large B-cell lymphoma	CYBA GSTA1 NCF4	rs4673 rs3957357 rs1883112	NAD(P)H oxidase, p40phox	EF decreased, echocardiography abnormalities, electrocardiogram abnormalities	[32]
Wojnowski et al. (2005)	Doxorubicin	Non-Hodgkin's lymphoma	NCF4 His72Tyr 7508T3A Gly671Val Val1188Glu Cys1515Tyr	rs1883112 rs4673 rs1305833 rs8187694 rs8187710	NAD(P)H oxidase, p22phox	Arrhythmia, myocarditis-pericarditis, acute HF	[35]
Armenian et al. (2013)	Anthracyclines	Hematopoietic cell transplantation	RAC2 HFE	rs8187710 rs13058338 rs1799945	NAD(P)H oxidase	CHF, depressed EF or SF	[38]
Reichwagen et al. (2015)	Anthracyclines	CD20+ B-cell lymphomas	RAC2 CYBA	rs13058338 rs4673	NADPH oxidase	Arrhythmia, reduced EF, ischemia	[40]
Sági et al. (2018)	Anthracyclines	ALL, OSC	CYP3A5 SLC28A3 ABCC2 NQO1 SLC22A6	rs4646450 rs7853758 rs3740066 rs1043470 rs6591722	ROS	LV function, SF, EF	[41]
Semsei et al. (2012)	Anthracyclines	ALL	ABCC1	rs3743527	ROS	LV dysfunction, reduced LVFS	[45]
Visscher et al. (2013)	Anthracyclines	Childhood cancer	UGT1A6 SLC28A3	rs17863783 rs7853758 rs885004	No report	SF < 26%	[46]
Visscher et al. (2012)	Anthracyclines	Childhood cancer	SLC28A3	rs7853758	No report	CHF, SF < 26%	[47]
Windsor et al. (2012)	Methotrexate	Malignant bone tumor	ABCC2 GSTP1	No report	ROS	Cardiac dysfunction, EF decreased	[48]
Hertz et al. (2016)	Doxorubicin	Breast cancer	ABCB1 CBR3	No report	Metabolism	EF < 55%	[50]
Lubieniecka et al. (2013)	Anthracyclines	AML	POR	rs2868177 rs13240755	ROS	LVEF decreased	[55]
Huang et al. (2017)	Daunorubicin	ALL	CYP3A5 (POR)	No report	Cytochrome P450 family 3	Cardiac dysfunction	[56]
Vivenza et al. (2013)	Anthracyclines	Breast cancer	GSTM1	No report	Oxidative/electrophilic species	Congestive HF, LVEF	[65]
Rajić et al. (2009)	Anthracyclines	ALL	CAT GSTT1 GSTM1	rs10836235	ROS, SOD	Cardiac damage	[71]
Ruiz-Pinto et al. (2018)	Anthracyclines	Breast cancer	ETFB	rs79338777	Mitochondrial dysfunction	Myocardial injury, LVEF decreased	[74]

Table 1: Continued.

Study	Drug used	Type of cancer examined	Gene	SNP ID/location of pathogenic mutation	Targets	Cardiac toxicity	References
Shizukuda et al. (2005)	Doxorubicin	No report	p53	No report	ROS, Cu/Zn, SOD	Cardiac injury, LV systolic dysfunction	[77]
Wang et al. (2014)	Anthracyclines	Children's oncology	HAS3 gene	rs2232228	ROS	LV dysfunction, EF < 40%, and FS < 28%	[79]
Visscher et al. (2015)	Anthracyclines	Childhood cancer	SLC22A17 SLC22A7	rs4982753 rs4149178	ROS, SOD	LV dysfunction	[83]
An et al. (2017)	Doxorubicin	Intermittent fasting	UVRAG	No report	LC3 II and p62 protein	Cardiac dysfunction	[101]
Wang et al. (2018)	Doxorubicin	No report	GCN2	No report	Bcl-2, Bax, ATF4, UCP2	LV dysfunction	[103]
McCaffrey et al. (2013)	Doxorubicin	Breast cancer	TCL1A MDR1	rs11849538	PI3K, AKT, cIAP2, IAP-C, MIHC	Congestive HF, EF < 40%, LV dysfunction	[106]
Todorova et al. (2017)	Doxorubicin	Breast cancer	HLA	rs9264942 rs2523619 rs10484554	Inflammation, autoimmune disorders	Cardiac dysfunction, LVEF decline	[111]
Mori et al. (2010)	Doxorubicin	No report	Spp1 Fhl1 Timp1 Ccl7 Reg3b	No report	Degeneration of myocardium and inflammation	Cardiac injury	[112]
Pop-Moldovan et al. (2017)	Doxorubicin	Hematological malignancies	TLR2 TLR4	No report	TLR	Diastolic dysfunction, LVEF decreased	[118]
Li et al. (2018)	Doxorubicin	Mammary tumor	TLR9	No report	PI3Kγ	Myocardial dysfunction	[120]
Todorova et al. (2017)	Doxorubicin	Breast cancer	MicroRNA	No report	IL-17, TNF-α, NF-κB	Cardiac dysfunction, LVEF declined	[129]
Zhao et al. (2014)	Bevacizumab	Colorectal cancer	miRNA1254	No report	CRP, MMPs	CHF	[132]
Yin et al. (2016)	Doxorubicin	No report	miR-320a	No report	VEGF	Cardiac dysfunction	[135]
Zhu et al. (2017)	Doxorubicin	DLBCL	miR-34a	No report	Caspase-3, Bcl-2	Cardiac dysfunction	[136]
Cascales et al. (2012)	Doxorubicin	Hematological	C282Y-Y H63D-H	No report	Iron metabolism disorder	HF, LVEF decrease	[142]
Lipshultz et al. (2013)	Doxorubicin	ALL	C282Y	No report	Iron metabolism disorder	Cardiac dysfunction, LVEF, cTnT, NT-proBNP	[144]
Ichikawa et al. (2014)	Doxorubicin	No report	ABCB8	No report	Mitochondrial iron	Cardiomyopathy	[146]
Fang et al. (2019)	Doxorubicin	No report	Hmox1	No report	Mitochondrial iron	Cardiomyopathy	[151]
Blanco et al. (2008)	Anthracyclines	Childhood cancer	CBR3 V244M	No report	Metabolism	CHF	[155]
Reinbolt et al. (2016)	Adriamycin, cytoxan	Breast cancer	CBR1 CBR3	No report	Metabolism	EF < 50% and >15%	[156]
Salanci et al. (2012)	Anthracyclines	No report	CBR3 GSTP1	No report	Metabolism	Cardiac dysfunction, LVEFs < 50%	[157]

Table 1: Continued.

Study	Drug used	Type of cancer examined	Gene	SNP ID/location of pathogenic mutation	Targets	Cardiac toxicity	References
Blanco et al. (2012)	Anthracyclines	Childhood cancer	CBRs	No report	Metabolism	Cardiomyopathy, EF < 40%, SF < 28%	[158]
Lubieniecka et al. (2012)	Anthracyclines	AML	AKR CBR	No report	Metabolism	LVEF% drop	[162]
Wasielewski et al. (2014)	Anthracyclines	Adult and childhood cancer	MYH7	No report	Sarcomere disruption	Dilated cardiomyopathy	[166]
Wang et al. (2016)	Anthracyclines	Children oncology	CELF4	rs1786814	Sarcomere disruption	Cardiomyopathy	[171]
Garcia-Pavia et al. (2019)	Anthracyclines	Multiple cancers	TTNtv	No report	Sarcomere disruption	Dilated cardiomyopathy	[179]
Ferreira et al. (2017)	Doxorubicin	No report	DNA methylation	No report	Epigenetic	Decreased mtDNA levels	[183]
Ferreira et al. (2019)	Doxorubicin	No report	DNMT1	No report	Epigenetic	Upregulation of mtDNA transcripts	[185]
Beauclair et al. (2007)	Trastuzumab	Breast cancer	Her2	No report	No report	LVEF decreased	[186]
Stanton et al. (2015)	Trastuzumab	Breast cancer	Ile 655 Val Pro 1170 Ala	rs1058808 rs1136201	No report	CHF, LVEF < 50%	[188]
Peña et al. (2015)	Trastuzumab	Breast cancer	HER2 655 A>G	rs1136201	No report	CHF, LVEF < 50%	[189]
Roca et al. (2013)	Trastuzumab	Breast cancer	HER2 Ile655Val	No report	MAPK and PI3 K/Akt	CHF, LVEF < 50%	[190]
Ruiz-Pinto et al. (2017)	Anthracyclines	Pediatric cancer	GPR35	rs12468485	No report	LV dysfunction, SF < 26%	[196]
Sachidanandam et al. (2012)	Doxorubicin	Childhood cancer	HNMT	rs17583889	No report	SF < 26%	[199]
Salata et al. (2013)	Chemotherapy Radiotherapy	Breast cancer	RAS-related genes	No report	AT1 receptor	Cardiac remodeling	[203]
Schneider et al. (2017)	Anthracyclines	Breast cancer	SNP	rs28714259	No report	CHF, LVEF < 50%, acute coronary syndrome, supraventricular tachycardia, myocardial dysfunction	[205]
Kitagawa et al. (2012)	Epirubicin Cyclophosphamide 5-Fluorouracil	Breast cancer	FEC100	No report	No report	Arrhythmias, QTc interval prolongation	[208]

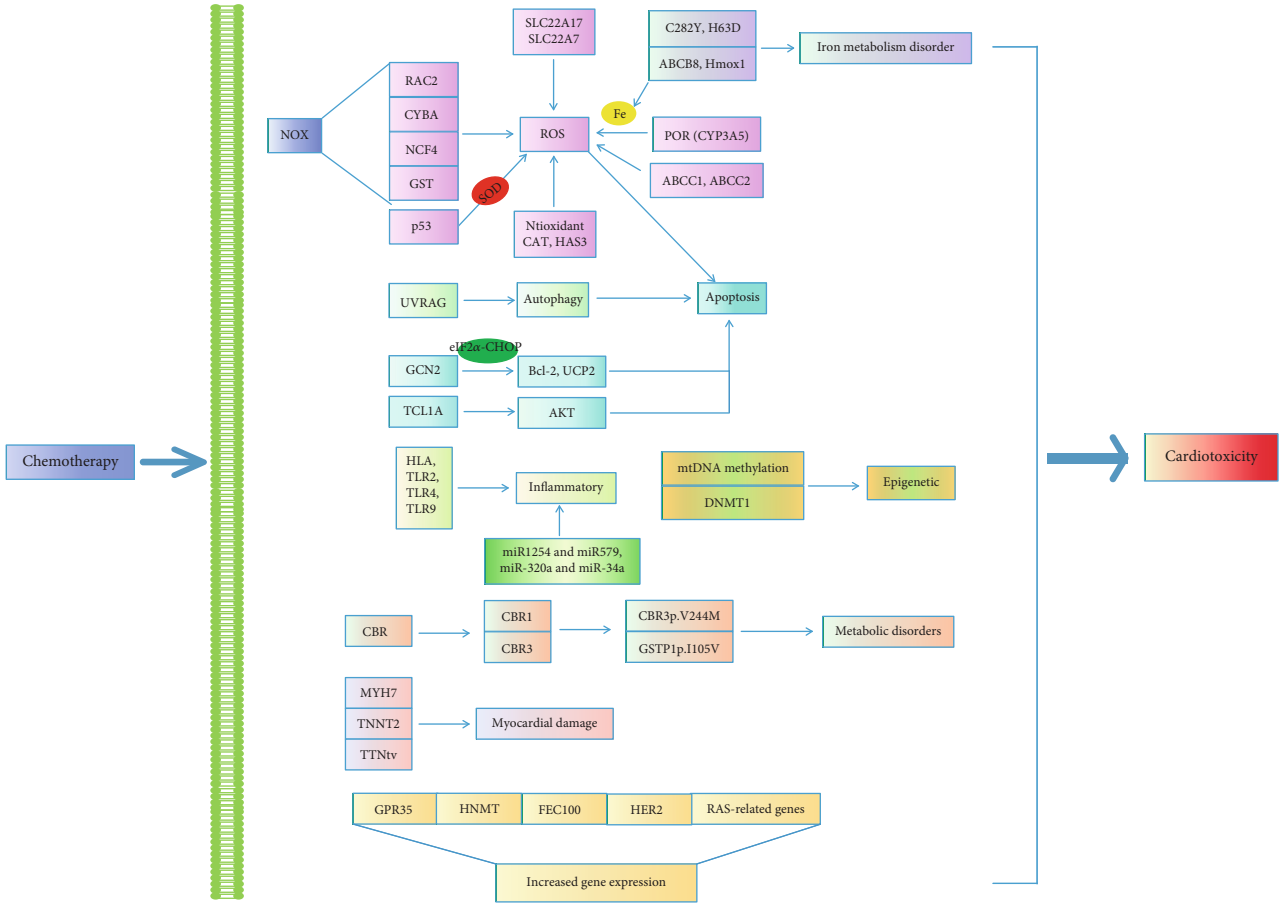

Figure 2: Mechanism of cardiotoxicity induced by susceptibility genes in chemotherapy. ROS: reactive oxygen species; NOX: nicotinamide adenine dinucleotide phosphate oxidase; POR: P450 oxidoreductase; GST: glutathione S-transferase; CYP3A5: cytochrome P450 family 3 subfamily A member 5; CAT: catalase; HAS3: hyaluronan synthase 3; SOD: superoxide dismutase; UVRAG: ultraviolet irradiation resistance-associated gene; GCN2: general control nonderepressible 2; eIF2α: eukaryotic initiation factor 2α; UCP2: uncoupling protein 2; Bcl-2: B-cell lymphoma-2; TCL1A: T cell leukemia/lymphoma 1A; HLA: human leukocyte antigen; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TLR9: Toll-like receptor 9; Hmox1: heme oxygenase-1; CBR: carbonyl reductase; CBR1: carbonyl reductase 1; CBR3: carbonyl reductase 3; TTNtv: titin-truncating variants; DNMT1: DNA methyltransferase 1; GPR35: G protein-coupled receptor 35; HNMT: histamine n-ethyltransferase; RAS-related genes: renin-angiotensin system-related genes.

approximately 11.6% of the LVEF-decreased patients and 13.2% of the LVEF-decreased patients with a cumulative dose. In post hoc analysis, this association was driven by a linear interaction of 3 SNPs (rs2868177, rs13240755, and rs4732513) with a cumulative dose of DNR. Another study examined the relationship between cytochrome P450 family 3 subfamily A member 5 (CYP3A5) genetic polymorphism and the DNR plasma concentration in patients with AML [56]. The study included 36 children who had been recently diagnosed with acute lymphoblastic leukemia (ALL). Polymerase chain reaction- (PCR-) derived sequencing was used to detect the CYP3A5 $*$ 3 genotype, and then PCR was used to detect the mRNA expression of CYP3A5. The enzyme activity of CYP3A was detected using a midazolam probe, and the DNR concentration was determined via high-performance liquid chromatography. The expression levels of CYP3A5 mRNA in children with different genotypes were different, while the activity of the CYP3A5 enzyme in the CYP3A5 $*$ 1 allele was higher than that in the CYP3A5 $*$ 3 allele. The polymorphism of the CYP3A5 $*$ 3 gene is closely related to CYP3A enzyme activity, the mRNA expression of CYP3A5, and the DNR plasma drug concentration, and results in different adverse drug reactions [56–58].

The evidence is increasingly indicating that drug metabolizing enzymes, such as the members of the glutathione S-transferase (GST) family, have great effect for characterizing the response of patients to chemotherapeutic drugs [59, 60]. The corresponding genes, such as GSTM1, glutathione s-transferase Pi (GSTP1), and GSTT1, encode the phase II detoxifying proteins that are involved in conjugating substrates that are toxic to cancer cells, including the type of chemotherapy used in the treatment of breast cancer [61–63]. However, the key participant in the pathophysiology of CHF is the renin-angiotensin-aldosterone system (RAAS) [64]. This study determined whether polymorphisms in the RAAS and GST II detoxification enzyme families might be useful predictors of LVEF dynamics and CHF risk [65]. The association between the gene polymorphisms and cardiotoxicity development was investigated in 48 early breast cancer patients undergoing anthracycline-assisted

chemotherapy. The following polymorphisms were analyzed: p.Met235Thr and p.Thr174Met in angiotensinogen (AGT), Ins/Del in angiotensin-converting enzyme (ACE), A1166C in angiotensin II type 1 receptor (AGTR1A), and p.Ile105Val in GSTP1 in c.-344t>c aldosterone synthase (CYP11B2). In addition, GSTM1 can be used as a biomarker with a higher risk of cardiotoxicity, as demonstrated previously in patient cohorts [62, 66, 67].

The cardiotoxicity of anthracyclines is thought to be caused by cardiomyocyte damage mediated by ROS, which is produced by the mitochondrial respiratory chain and the non-enzymatic iron pathways. A high oxidative metabolic rate and weak antioxidant defense make cardiomyocytes especially sensitive to free radical damage [68–70]. Catalase (CAT), GSTT1, GSTM1, and superoxide dismutase II (SOD2) play important roles in ROS metabolism. Rajić et al. demonstrated that deactivating the variants of CAT (rs1001179 and rs10836235), SOD2 (rs4880), GSTM1, and GSTT1 may increase cardiotoxicity risk [71]. This hypothesis was investigated in a long-term survival cohort of 76 children with ALL. Compared to genetic polymorphisms, cardiac injury was assessed as a property variable [72]. The results suggested a significant association between CAT (rs10836235) and cardiac damage after exposure to anthracyclines. The most important gene was electron transfer flavoprotein beta subunit (ETFB, rs79338777), which participated in mitochondrial b oxidation and adenosine triphosphate (ATP) production, and whose association was replicated in a group of independent cancer patients treated with anthracyclines [73, 74].

An additional study investigated whether targeted damage to the p53 gene could enhance the cardiotoxicity induced by DOX [75, 76] by randomly assigning wild-type (WT) mice and p53 knockout (p53 KO) mice to saline or DOX by intraperitoneal injection. The continuous imaging of animals using high-frequency two-dimensional echocardiography and the LV systolic function measurements assessed by FS indicated weight loss in the WT mice as early as 4 days and 2 weeks after DOX injection. On the contrary, LVFS remained unchanged after DOX injection in the p53 KO mice. After DOX treatment, the apoptosis of cardiomyocytes measured using TUNEL and the ligase reaction were found to increase significantly, whereas the level of glutathione and Cu/Zn SOD did not change in the p53 KO mice, but not in the WT mice. Therefore, the p53 gene in p53-mediated signaling may play an important role in the cardiotoxicity induced by DOX, and may regulate ROS induced by DOX [77].

Hyaluronan (HA) generated by hyaluronan synthase 3 (HAS3) is a common ingredient and has a positive effect on a variety of diseases [78]. Furthermore, HA is known to decrease heart damage caused by ROS in cardiovascular disease. This study examined host sensitivity to anthracycline-associated cardiomyopathy using a cardiovascular SNP array to analyze common SNPs in 2,100 genes associated with cardiovascular disease [79]. The study identified a common SNP (rs2232228) in the HAS3 gene that modifies the risk of anthracycline-induced cardiomyopathy. Compared to the GG genotype, the rs2232228 AA genotype increased the risk of cardiomyopathy by 8.9 times [38].

SLC22A17 was first identified in the brain as an orphan transporter of unknown endogenous substrates, expressed in a variety of tissues, including the heart [80]. SLC22A17 transports naturally occurring nucleotides, preferentially selects guanine analogs and several nucleoside-based drugs, and has a considerable substrate overlap with concentrated nucleoside transporters [81, 82]. This study verified novel variants related to ACT and evaluated them in a risk prediction model. Two cohorts for the treatment of childhood cancer were genotyped for 4,578 SNPs in the drug ADME (absorption, distribution, metabolism, and elimination) and toxicity genes [83]. An important association between SLC22A7 (rs4149178) and SLC22A17 (rs4982753) was found, and evidence was also found for some genes associated with ROS [84]. Two new variants in SLC22A17 and SLC22A7 were associated with cardiotoxicity induced by anthracyclines, thereby improving risk stratification in patients.

2.2. Autophagy. Autophagy in its normal state is essential for maintaining homeostasis [85, 86]; however, disorders of autophagy in cardiomyocytes have been linked to a variety of cardiovascular diseases [87–89]. Autophagy is associated with cardiomyopathy induced by DOX [90–95], and the ultraviolet irradiation resistance-associated gene (UVRAG), an autophagy-related protein, can adjust autophagosome formation [96], maturation [97], and autophagosomal lysosomal reformation (ALR) [98]. Studies on UVRAG-deficient mice found that the autophagy flux was impaired and autophagosomes were accumulated in the heart, suggesting that UVRAG may regulate the maturation of autophagosomes [99, 100]. An et al. evaluated the effect of UVRAG-mediated autophagy in cardiotoxicity induced by DOX [101]. The deficiency of UVRAG will aggravate the cardiotoxicity induced by DOX, which is manifested by an enhancement of cytoplasmic vacuoles, an increased collagen accumulation, increased serum levels of lactate dehydrogenase (LDH) and myocardial creatine kinase (CK), increased ROS levels, increased apoptosis, and reduced cardiac function. The autophagy flux was impaired in cardiotoxicity induced by DOX, while a deficiency of UVRAG exacerbated autophagy flux impairment in cardiotoxicity induced by DOX. In summary, these data suggest that UVRAG deficiency in part aggravates cardiotoxicity by exacerbating DOX-induced autophagy impairment.

2.3. Apoptosis. General control nonderepressible 2 (GCN2) is a eukaryotic initiation factor 2α (eIF2α) kinase that damages ventricular adaptation to pressure overload by influencing myocardial apoptosis [102]. After DOX treatment, systolic dysfunction, apoptosis, and ROS were found to be reduced in $Gcn2^{-/-}$ mice. GCN2 deficiency attenuated eIF2 phosphorylation, induced its downstream targets, activated transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP), and retained B-cell lymphoma-2 (Bcl-2) and mitochondrial uncoupling protein 2 (UCP2). In addition, this study found that the knockdown of GCN2 weakened DOX-induced ROS, while the overexpression of GCN2 intensified it, and reduced Bcl-2 and UCP2 through the eIF2α-CHOP pathway [103–105]. Furthermore, another study found that

oxidative byproducts accumulated in the plasma of patients treated with DOX [106]. At the RNA level, compared with women who received chemotherapy but maintained normal EF, the 260 transcripts of women with low EF changed after chemotherapy, with a difference of >2 times. Notably, the transcription of T cell leukemia/lymphoma 1A (TCL1A) decreased by 4.8 times in women with chemotherapy-induced low EF. TCL1A, also known as an AKT helper activator, is one of the primary presurvival factors of cardiac myocytes. In addition, patients with low EFs had a twofold reduction in ABCB1 transcription encoding multidrug resistant protein 1 (MDR1), which may lead to higher cardiac drug levels [107, 108]. Hence, cancer treatment-induced cardiomyopathy may result in genetic susceptibility or decreased TCL1A levels, decreased AKT activity, and augmented sensitivity to DOX apoptosis.

2.4. Inflammation. Previous studies have found that individual susceptibility to low doses of DOX treatment is related to the differential expression of genes involved in the inflammatory response [109], which correlates with increasing reports on the important function of human leukocyte antigen (HLA) to the hypersensitivity of complex polymorphism to drug toxicity [110]. A study analyzing DNA from breast cancer patients treated with DOX and its role in the DOX-related cardiotoxicity risk identified 18 SNPs of 9 genes in the HLA region that may be associated with DOX cardiotoxicity [109, 111]. This result suggested that increased susceptibility to DOX-induced cardiotoxicity is associated with the dysregulation of autoimmune and inflammatory disease-related genes [111]. In addition, Mori et al. treated rats with three typical cardiotoxic compounds, namely, isoproterenol, DOX, and carbofuran, which resulted in cardiac lesions in rats [112]. This study was followed by microarray analysis and histopathological examination. Using statistical and cluster analysis, 36 probe groups were extracted from the upregulation of three cardiotoxic compounds. The analysis showed that these genes were involved in the myocardial degeneration and inflammation observed in histopathological analysis. Among the selected genes, Timp1, Spp1, Ccl7, Fhl1, and Reg3b showed a sustained upregulation of high expression levels in all three compounds at both time points [113–115].

Toll-like receptors (TLRS), including TLR4, TLR2, and TLR9, allow cardiomyocytes to respond to endogenous or exogenous stimuli, and may alter their pathophysiological response [116, 117]. One study investigated the potential role of TLR2 and TLR4 gene expression as early biomarkers of cardiomyopathy induced by DOX [118]. In this study, blood collection, RNA isolation, cDNA reverse transcription, quantitative reverse transcription PCR (qRT-PCR), and relative expression quantification were performed on samples from 25 patients with DOX-treated hematologic malignancies via qRT-PCR. The results showed that TLR4 and TLR2 expression was higher in patients with diastolic dysfunction and DOX treatment [118, 119]. In addition, DOX was found to participate in PI3Kγ downstream signaling of TLR9, which converged to autophagy inhibition and maladaptive metabolic remodeling, ultimately leading to cardiomyocyte death and systolic dysfunction. One study treated chronic DOX in mice expressing inactive PI3Kγ or receiving selective PI3Kγ inhibitors [120]. Cardiac function was assessed by echocardiography, and DOX-mediated signaling was evaluated in the heart tissue and cardiomyocytes. The dual cardioprotective and anticancer effects of PI3Kγ inhibition were evaluated in mice tumor models. The results showed that PI3Kγ kinase dead (KD) mice exhibited preserved cardiac function after a long-term low dose of DOX therapy and were protected by DOX-induced cardiotoxicity. The effect of PI3Kγ inhibition was found to have a causal relationship with enhanced autophagy processing in the DOX-damaged mitochondria. In terms of its mechanism, PI3Kγ was triggered downstream of TLR9 in DOX-treated mice hearts by mitochondrial DNA released by damaged organelles and contained in the autolysosomes [121, 122].

2.5. MicroRNAs (miRNAs). MicroRNAs (miRNAs) are universally expressed small noncoding RNAs, which adjust gene expression at the posttranscriptional level [123]. The importance of miRNAs in a wide range of human diseases suggests their potential as biomarkers for clinical use [124]. Numerous studies have shown that miRNA expression profiles are associated with cardiovascular diseases, including fibrosis, hypertrophy, arrhythmia, and HF, and can have powerful and unexpected effects [125–128]. One study obtained information about microRNA in cancer patients treated with DOX to determine whether these patients developed cardiac abnormalities after chemotherapy [129]. Plasma from 20 breast cancer patients who had undergone DOX treatment were analyzed using quantitative RT-PCR and qPCR. The circulating microRNA profiles of patients with cardiotoxicity induced by DOX were then compared with those without cardiotoxicity induced by DOX. The results indicated that 32 microRNAs were severely misregulated in patients with cardiac dysfunction, the analysis of which suggested that they were associated with inflammation [130, 131].

Another study determined whether specific miRNA levels were discharged into the circulation due to cardiotoxicity induced by bevacizumab [132]. After miRNA array analysis using isolated RNA, this study selected 19 candidate miRNAs from the array for a validation study of 90 controls and 88 patients with cardiotoxicity induced by bevacizumab. Compared to the control group, the circulating levels of the 5 miRNAs were significantly increased in patients with cardiotoxicity induced by bevacizumab. To verify these findings, the study compared selected miRNAs in plasma from 66 patients with acute myocardial infarction (AMI) with cardiotoxicity induced by bevacizumab. The results confirmed a specific rise in the expression of two miRNAs, miR1254 and miR579, in patients with cardiotoxicity induced by bevacizumab, with miR1254 showing the strongest association with the clinical diagnosis of bevacizumab-induced cardiotoxicity [132–134].

Furthermore, some studies have suggested that miR-320a [135] and miR-34a [134] play important roles in chemotherapy-induced cardiotoxicity. After DOX treatment, miR-320a was found to increase in the cardiomyocytes, and participated in DOX-induced cardiotoxicity due to its direct

targeting of VEGF-A [135]. Therefore, the overexpression of miR-320a enhanced cardiac apoptosis and caused vessel abnormalities in the heart tissue and cardiac dysfunction in mice. miR-34a had been shown to be upregulated in the myocardium and plasma of DOX-treated rats and in the H9C2 cells of rat myocardium treated with DOX [136]. In terms of its mechanism, miR-34a contributed to DOX-induced cardiotoxicity by targeting the Sirt1/p66shc pathway [136]. It was also shown that miR-34b/c was upregulated in the myocardial cell line HL-1 treated with DOX [137]. This study showed that the itchy E3 ubiquitin protein ligase (ITCH) was a direct target of miR-34b/c, and that miR-34b/c reduced HL-1 viability, promoted NF-κB expression, and increased proinflammatory cytokines through ITCH downregulation [137]. Overall, these studies demonstrated that DOX treatment is associated with miRNA signaling, which may potentially predict cardiac dysfunction in breast cancer patients [138]. Thus, these data provide a basis for future studies to identify biomarkers for cardiotoxicity induced by DOX.

2.6. Iron Metabolism. Hereditary hemochromatosis (HH) is an inherited iron metabolism disorder that leads to tissue damage associated with excess levels of iron. Homozygotes of the C282Y mutation are present in 52-100% of HH patients [139]. Non-cancer-related idiopathic cardiomyopathy and early pathological LV remodeling were found to be higher in patients [140] than in healthy controls [141]. This study retrospectively assessed 97 consecutive necropsies for HFE genotypes, cardiac iron, and cardiac events from patients with solid and hematologic tumors [142]. The iron concentrations in the heart and liver were tested using atomic absorption spectrometry, and the HFE gene mutations related to HH were analyzed. Haplotypes 282C/63D and 282Y/63H of HFE mutations were found to be related to higher cardiac iron deposition [143]. Other studies also confirmed a link between HH associated with the mutation frequency of the HDE gene and its association with DOX-related cardiotoxicity in children at high risk of ALL [144]. C282Y and H63D were analyzed in the peripheral blood, while serum cardiac troponin-T (cTnT) and N-terminal pro-brain natriuretic peptide (NT-proBNP), biomarkers for heart injury and cardiomyopathy, were measured during treatment. The results suggested that the heterozygous C282Y genotype was related with multiple increases in the concentration of cTnT. LV structure and function were evaluated by echocardiography. The results showed that LVFS and end-systolic and -diastolic posterior wall thickness were abnormal in children with both alleles. In short, DOX-induced associated cardiotoxicity is associated with C282Y HFE carriers [141, 145].

DOX-dependent cardiotoxicity is presumed to occur through ROS production and cellular iron accumulation. One study found that DOX treatment produced cardiotoxicity through preferential iron accumulation in mitochondria [146]. In cardiomyocytes, DOX became concentrated in the mitochondria and enhanced mitochondrial iron and cellular ROS levels. ABCB8 is a mitochondrial protein that promotes iron output both *in vitro* and in the heart of transgenic mice, such that its overexpression was found to reduce the content of mitochondrial iron and cellular ROS, and provided protection against DOX-induced cardiomyopathy [147, 148]. The mitochondrial levels of iron were significantly higher in patients with DOX-induced cardiomyopathy than in patients with other types of cardiomyopathy or normal heart function. These results suggested that the cardiotoxic effects of DOX were caused by an accumulation of mitochondrial iron, such that reducing the mitochondrial iron levels could prevent DOX-induced cardiomyopathy.

Ferroptosis is a new form of regulatory cell death, characterized by the iron-dependent accumulation of lipid peroxides to lethal levels, which is different from apoptosis, necrosis, and autophagy morphobically, biochemically, and genetically [149, 150]. In typical apoptotic or necrotic mice, DOX-induced cardiomyocytes exhibited characteristic ferroptotic cell death. RNA sequencing results showed that heme oxygenase-1 (Hmox1) was markedly upregulated in the DOX-treated mouse heart [151]. By administering DOX to the mice, heme degradation caused by the Nrf2-mediated upregulation of Hmox1 and cardiomyopathy caused by rapid and systematic accumulation of nonheme iron were induced, but were not observed, in Nrf2-deficient mice. Since ferroptosis is driven by damage to lipid membranes, excess free iron was found to accumulate in the mitochondria, which led to lipid peroxidation in the membrane. MitoTEMPO, a mitochondria-targeted antioxidant, can rescue DOX cardiomyopathy and supports oxidative mitochondrial damage, which is the main mechanism of heart damage caused by ferroptosis.

2.7. Metabolism. Carbonyl reductase (CBR) catalyzes the metabolism of anthracyclines, and SNPs in CBR affect metabolic efficiency. CBRs catalyze the reduction of anthracyclines into the cardiotoxic alcohol metabolites, especially carbonyl reductase 1 (CBR1) and carbonyl reductase 3 (CBR3), whose polymorphism affects the synthesis of these metabolites [152–154]. Blanco et al. and Reinbolt et al. investigated whether the SNPs in CBR1 (1096GA) and CBR3 (V244M) altered the risk of anthracycline-associated cardiomyopathy in cancer patients [155, 156]. They found that the CBR genotype was related to an increased risk of cardiomyopathy. Another study evaluated the relationship between changes in functional cardiac parameters after treatment with anthracyclines and the polymorphism of CBR3 and GSTP1 [157]. This study included 70 patients with normal cardiac function who received anthracyclines to assess cardiac function using gated blood pool scintigraphy and echocardiography. A TaqMan probe was used to genotype the polymorphisms of 70 patients, which were verified via DNA sequencing. In terms of the CBR3p.V244M polymorphism, the systolic and diastolic parameters from GG to AA all showed a worsening trend [158]. Meanwhile, G allele carriers with the GSTP1p.I105V polymorphism were common, and PFR was significantly reduced compared to patients with the AA genotype. Therefore, the variation of CBR3 and GSTP1 may be related to changes in short-term functional cardiac parameters after chemotherapy [159, 160].

Previous studies have also suggested that 13 of the naturally existing nonsynonymous SNPs in aldo-keto reductases

(AKR) and CBR decrease the metabolic rate of anthracyclines *in vitro* [161]. This study investigated these SNPs individually and jointly for their correlation with cardiotoxicity in patients with DNR induced by AML [162]. Five of the 13 SNPs showing an *in vitro* action on anthracycline drug metabolism were tested in 185 AML patients. The results indicated the *in vitro* role of nonsynonymous SNPs in the reductase genes in the metabolism of anthracycline [163]. Another study validated the evidence of a link between SNPs and cardiotoxicity in ABCB1 in breast cancer patients treated with anthracyclines [50]. An echocardiography was used to analyze 166 breast cancer patients treated with DOX, with 19 cases of abnormal systolic function and 147 control cases. After applying the appropriate statistical correction, four high-priority SNPs were detected in the main analysis, while 23 other SNPs were screened using uncorrected secondary analysis. Two SNPs, including ABCB1 and CBR3, which are associated with cardiotoxicity, were identified as a result.

2.8. Sarcomere Disruption. Although anthracyclines have been successfully used to treat cancer, their use is limited by their cardiotoxic side effects [164]. There are several known risk factors for anthracycline-associated cardiomyopathy (AACM) [165]; however, the absence of these known risk factors lead to the development of AACM. One study investigated whether genetic susceptibility to dilated cardiomyopathy (DCM) is a risk factor for AACM [166]. A hospital-based and two hospital registries for cancer patients treated with systemic cancer were reviewed, with an emphasis on AACM. Mutations in genetically related cardiomyopathy in selected AACM family patients were analyzed and their presymptomatic cardiology was evaluated. The study analyzed 5 AACM families with DCM and 1 AACM family member with potential early signs of mild DCM. As a result, pathogenic MYH7 mutations were identified in the two families. Moreover, in the DCM family with AACM, mutations in MYH7 c.1633G>A and c.2863G>A were identified. Therefore, it can be hypothesized that genetic susceptibility to DCM may be a potential risk factor for AACM [166, 167].

The SNP rs1786814 on the CELF4 gene is an important cut-off for the interaction between genes and the environment [168–170]. Genome-wide association studies were used to investigate the potential mechanistic implications of verified SNPs. Multivariate analysis showed that cardiomyopathy was rare and dose independent in patients with the A allele. However, in patients exposed to anthracyclines, compared to those with the GA/AA genotype, the rs1786814 GG genotype had a 10.2-fold increased cardiomyopathy risk. The CUG-BP and ETR-3-like factor proteins control the developmental regulatory splicing of TNNT2, and this gene encodes cTnT. More than one cTnT variant may cause a transient mitotic myofilament response to calcium, resulting in a reduction in contractile force. Analysis showed that the rs1786814 GG genotype was correlated with more than one TNNT2 splicing variant. In summary, this study suggests that the CELF4 (rs1786814) polymorphism modifies the dose-dependent association between anthracyclines and cardiomyopathy, possibly through pathways involving abnormal splicing of TNNT2 variants [171–173].

Titin-truncating variants (TTNtv) are observably conspicuous in DCM, occurring in 15% of outpatients and 25% of end-stage patients [174–177], but are rarely found in childhood-onset DCM [178]. Meanwhile, this study found TTNtv in 8.1% of adults and 5.0% of children with cancer treatment-induced cardiomyopathy (CCM). Garcia-Pavia et al. studied patients from three cohorts, retrospectively enrolling patients with multiple cancers, breast cancer, and AML, and sequenced their cardiomyopathy genes, including nine prespecified genes [179]. This study compared the incidence of rare mutations between the CCM cohort and the cancer genome atlas (TCGA) participants, healthy volunteers, and reference populations with matched lineages. The prevailing CCM genotype was simulated in anthracycline-treated mice based on the genotype assessment of clinical characteristics and results. Of the nine priority genes, CCM patients had more rare protein-altered variants than their peers. TTNtv was found to be dominant, occurring in 7.5% of patients with CCM. Compared to patients without TTNtv, patients with CCM TTNtv experienced more HF, atrial fibrillation, and impaired myocardial recovery. This finding is consistent with data showing that TTNtv mice treated with anthracyclines and isolated TTNtv cardiomyocytes showed persistent systolic dysfunction, which varied from that of the wild type [179, 180].

2.9. Epigenetics. Since mitochondrial dysfunction can dramatically reprogram the epigenome [181, 182], cardiotoxicity may also be induced by the epigenetic changes associated with mitochondrial dysfunction. For verification, the study used rats injected with DOX or saline for 8 weeks [183]. Gene expression, global DNA methylation, and the acetylation status of proteome lysine were assessed by qPCR, ELISA, and Western blot, respectively, in saline- or DOX-treated rat cardiac tissue. This study showed that DOX treatment reduced global mtDNA methylation in the heart, which was accompanied by obvious changes in the expression of multiple functional genes. DOX disrupted the cardiac mitochondrial biogenesis, which was demonstrated by the reduced ratio of mitochondrial DNA versus genomic DNA and the decreased transcription levels of several mitochondrial genes [184]. Furthermore, the transcription of genes involved in the lipid metabolism and epigenetic regulation was also affected. Western blot analysis showed that the protein acetylation patterns in DOX-treated rat heart mitochondrial fractions were different from the control. These results indicated that the interaction between epigenetic alterations and mitochondrial dysfunction may be the main determinant of DOX-induced cardiotoxicity. In addition, Ferreira et al. investigated the correlation between nanomolar DOX concentration and epigenetic-related mitochondrial adaptation [185]. H9C2 cardiomyocytes were cultured with DOX for 24 hours and then recovered in nontoxic medium for 9 days. It was found that nanomolar DOX pretreatment led to the upregulation of mitochondrial DNA transcripts, with the decrease of DNA methyltransferase 1 (DNMT1) and the global methylation levels. This result suggested that nanomolar DOX preconditioning induction may be based on epigenetic mitochondrial adaptation.

2.10. Others

2.10.1. HER2 (erbB-2, neu). HER2 (erbB-2, neu) is a transmembrane protein with tyrosine kinase activity but no definite physiological ligands. Milano et al. found that HER2 gene polymorphism coding for the HER2 (Ile655Val) transmembrane domain may be a predictor of cardiac toxicity [186, 187]. A case-control study tested 11 ErbB2 single-gene SNPs that led to changes in the amino acid sequence of the HER2-neu protein related to cardiotoxicity in trastuzumab therapy [188]. Only the two ErbB2 SNPS (Ile 655 Val and Pro 1170 Ala) were discovered to be mutated by single-gene SNP analysis. The HER2/neu Pro 1170 Ala polymorphism could be used to identify an increased risk of cardiotoxicity in patients receiving trastuzumab. Another study used TaqMan allele identification to genotype the HER2 655 A>G (rs1136201) genetic variation [189]. The result showed that the polymorphism of HER2 655 A>G was significantly correlated with cardiotoxicity, and supported the role of HER2 655 A>G polymorphism as a genetic marker of cardiotoxicity in trastuzumab-induced HER2-positive breast cancer patients. Roca et al. investigated the predictive value of HER2, FCGRIIA, and FCGRIIIA gene polymorphisms on cardiotoxicity [190]. A total of 132 patients with HER2-positive breast cancer were analyzed, and the results showed that the HER2-I655V genotype was significantly associated with cardiotoxicity, whereas the FCGR2A-131 H/H genotype was markedly associated with shorter event-free survival (EFS). These results may contribute to improved efficacy and reduced toxicity, leading to the selection of HER2 blockers in adjuvant therapy. Another study examined the effects of a HER2 gene polymorphism (Ile655Val) on the pharmacodynamics of trastuzumab-induced cardiotoxicity, suggesting that the presence of the Val allele may be a risk factor for cardiotoxicity induced by trastuzumab in breast cancer patients [191, 192].

2.10.2. G Protein-Coupled Receptor 35 (GPR35). The G protein-coupled receptor 35 (GPR35) is the family of G protein-coupled receptors, a membrane protein that mediates a wide range of physiological processes [193]. The *in vitro* functional analysis of cardiomyocytes suggested that the overexpression of GPR35 decreased cell viability and promoted morphological changes [194, 195]. Ruiz-Pinto et al. studied the variation association on the Illumina HumanExome BeadChip array in 83 cancer patients treated with anthracyclines [191]. A gene-based analysis identified a novel and significant association between GPR35 and chronic ACT. This study found the greatest contribution to this association in rs12468485, where the T allele was associated with lower anthracycline doses and an increased risk of chronic ACT for more severe symptomatic cardiac presentation. Using exome array data, the results indicated that GPR35 was a novel susceptibility gene associated with the induction of ACT in cancer patients during treatment [196, 197].

2.10.3. Histamine N-Ethyltransferase (HNMT). The exact relevance between histamine n-ethyltransferase (HNMT) and cardiotoxicity is currently unknown. However, it has been proposed that antihistamines may be able to reverse multidrug resistance in breast cancer cells [198]. Recent research has shown that many SNPs play a role in ACT in children. One study investigated two adult ACT sisters who had developed ACT after administration with relatively low doses of DOX [199]. One of the sisters carried the HNMT variant genotype (rs17583889), while the other was heterozygous, suggesting that these genotypes had similar effects in ACT adults. Although further studies are needed, these gene types may play important roles for the clinical application of adriamycin liposomes.

2.10.4. Renin-Angiotensin System- (RAS-) Related Genes. In the heart, variations in certain renin-angiotensin system (RAS) components are frequently observed in the conditions leading to HF progression, such as ACE and angiotensin II type 1 receptor (AT1) [200–202]. One study investigated whether the renin-angiotensin-related gene could be altered using chemotherapy and radiation in a rat model [203]. Female rats were divided into three groups: the control group, the radiation (IR) group, and the chemotherapy+radiation (TC+IR) group. Left ventricular analysis was performed five months after treatment, and changes in the mRNA levels of several RAS-related genes were assessed by RT-PCR, such as angiotensinogen, renin, ACE, AT1, and vascular endothelial growth factor (VEGF), which may be involved in ACE. Compared with the control group, only decreased levels of ACE and VEGF were observed in renin, TC+IR, and IR, while increased levels of AT1 mRNA were observed in the TC+IR group and IR groups. In summary, both chemotherapy and radiotherapy may result in significant changes to the expression of some RAS-related genes [203, 204].

2.10.5. Others. A genome-wide association study (GWAS) was conducted on 3,431 patients from a randomized phase III study-adjuvant breast cancer trial (E5103) to identify the SNP genotypes associated with an increased risk of CHF after treatment with anthracyclines [205]. The study attempted to validate the drug candidates in two separate phase III adjuvant trials, E1199 and BEATRICE. When CHF was assessed by a cardiologist, 11 SNPs were found, 9 of which were independent chromosomal regions associated with increased risk. A study of the two most important SNPs in E1199 showed that the SNP rs28714259 was associated with an increased risk of CHF at a critical level. Subsequently, rs28714259 was tested in BEATRICE and was found to be significantly correlated with LVEF reduction. Therefore, the SNP rs28714259 represents a validated SNP associated with anthracycline-induced CHF in breast cancer clinical trials [205, 206].

A susceptibility to the chemotherapeutic drug-induced prolongation of QT interval is thought to be associated with SNPs or genetic mutations, some of which are present in the potassium channel gene [207]. Using electrocardiograms, the QTc intervals and arrhythmia characteristics were assessed in early breast cancer patients undergoing FEC100 chemotherapy. In the treated patients, a total of 131 ECG records were obtained, and the QTc interval was measured in 127 records. After each treatment, a marked trend in

QTc interval prolongation was observed, lasting for four chemotherapy cycles. In the first to the fourth chemotherapy cycle, the median length of QTc interval prolongation was 13, 11, 18, and 14 ms, respectively. In the first and fourth weeks before and after treatment, the QTc intervals were significantly different, and a supraventricular premature beat was found in 3 of the 131 cycles in 2 of the 34 patients. Therefore, this study confirmed that FEC100 is associated with significantly longer QTc intervals in early breast cancer patients [208].

CHF: congestive heart failure; LVEF: left ventricular ejection fraction; SF: shortening fraction; DLBCL: diffuse large B-cell lymphoma; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; OSC: osteosarcoma; NADPH: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; NOX: nicotinamide adenine dinucleotide phosphate oxidase; POR: P450 oxidoreductase; GST: glutathione S-transferase; CYP3A5: cytochrome P450 family 3 subfamily A member 5; CAT: catalase; HAS3: hyaluronan synthase 3; SOD: superoxide dismutase; UVRAG: ultraviolet irradiation resistance-associated gene; GCN2: general control nonderepressible 2; eIF2α: eukaryotic initiation factor 2α; UCP2: uncoupling protein 2; Bcl-2: B-cell lymphoma-2; TCL1A: T cell leukemia/lymphoma 1A; HLA: human leukocyte antigen; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TLR9: Toll-like receptor 9; Hmox1: heme oxygenase-1; CBR: carbonyl reductase; CBR1: carbonyl reductase 1; CBR3: carbonyl reductase 3; TTNtv: titin-truncating variants; GPR35: G protein-coupled receptor 35; HNMT: histamine n-ethyltransferase; RAS-related genes: renin-angiotensin system-related genes.

3. Protective Genes in Cancer Treatment-Induced Cardiotoxicity

Genes are known to play important roles in various human cancers, as well as in the pathogenesis of heart development and cardiovascular disease, due to their involvement in adjusting heart function, cardiac hypertrophy, and HF [209]. The following provides a summary of various cardiac protective mechanisms and insights into the development of new drugs and personalized therapies to decrease, or even eliminate, the toxic effects of chemotherapy on the heart (Table 2 and Figure 3).

3.1. Oxidative Stress. Anthracycline-induced cardiotoxicity has been associated with polymorphisms in genes encoding for NOX complex subunits, namely, ABCC1 and ABCC2, among survivors of various cancers [35, 38, 45]. Krajinovic et al. analyzed 251 children with ALL using echocardiography to determine the impact of the metabolic and functional pathway polymorphism of DOX on cardiotoxicity [210]. The results of association analysis indicated a regulatory role of the variants A-1629 T (an ATP-binding cassette transporter) and G894T (the NOS3 endothelial nitric oxide synthase gene). The ABCC5 tt-1629 genotype had an average reduction in EF and SF of 8–12%, while the NOS3 TT894 genotype exerted a protective role on EF and FS in the patients [210, 211], especially in those who were not administered dexrazoxane.

Another study investigated the mechanisms and targets for DOX-induced cardiotoxicity [212]. Both *in vitro* models of cells and *in vivo* models of mice were established, the results of which indicated that DOX could significantly reduce the activity of H9C2 cells, increase the levels of LDH and CK, and induce histopathological and electrocardiac changes in mice, thereby inducing myocardial oxidative damage. An mRNA microarray assay was used to select miR-140-5p as the target miRNA responsible for a significant increase in DOX-induced cardiotoxicity. A double-luciferase reporter gene assay suggested that miR-140-5p was able to directly target Nrf2 and Sirt2, thereby increasing DOX-induced oxidative damage to the myocardium. Furthermore, the intracellular ROS levels were found to prominently increase or decrease after miR-140-5p mimic or inhibitor transfection, with changes in the expression levels of Nrf2 and Sirt2 [213–216]. In addition, DOX-induced oxidative damage to the myocardium was found to be alleviated in mice treated with a miR-140-5p antagomir. Therefore, miR-140-5p/Sirt2 and miR-140-5p/Nrf2 may become new targets for the treatment of DOX-induced cardiotoxicity.

3.2. Endoplasmic Reticulum (ER) Stress. It has been shown that DOX causes endoplasmic reticulum (ER) dilation in both human and mouse hearts [217, 218], suggesting that ER dysfunction is related to DOX-induced cardiotoxicity, and that the inhibition of ER stress is a feasible method to improve DOX-induced cardiotoxicity [219]. One study found that DOX caused the ER in the hearts of mice to expand, suggesting that DOX may affect ER function. DOX activated the ER transmembrane stress sensor in cultured cardiomyocytes and mouse hearts and activated transcription factor 6 (ATF6) [220]. However, DOX inhibited the expression of ATF6 downstream genes, including the X-box binding protein 1 (XBP1). Reduced levels of XBP1 resulted in an inability to induce the expression of ER chaperone glucose regulatory protein (GRP) 78, which plays a major role in the adaptive response to ER stress. Moreover, DOX activated caspase-12, an apoptotic molecule located in the ER membrane, resulting in cardiac dysfunction. In brief, DOX can activate the apoptosis response caused by ER stress, further increasing ER stress in the mouse heart. However, the overexpression of heart-specific GRP78 or the administration of the chemical ER partner alleviates the cardiac dysfunction caused by DOX.

CACNA1H was found to be related to DOX-induced cardiac toxicity, while the CACNA1H-specific inhibitor ABT-639 significantly reduced DOX-induced cardiac damage and dysfunction, and relieved ER stress and the apoptosis of cardiac myocytes [221, 222]. One study assessed DOX-induced heart damage and changes in CACNA1H expression, and investigated the effects of ER stress and apoptosis on DOX-induced heart damage in mice [222]. To determine the effect of CACNA1H in this process, this study assessed the DOX-induced changes in heart injury and ER stress after treatment with a CACNA1H-specific inhibitor, ABT-639. Lastly, the ER stress inhibitor UR906 was used to determine

Table 2: Protective genes in chemotherapy-induced cardiotoxicity.

Study	Drug used	Type of cancer examined	Gene	ΔExpression	Targets	Cardiac toxicity	References
Krajinovic et al. (2016)	Doxorubicin	ALL	ABCC5 NOS3	No report	ROS	Lower LVEF; reduction of EF and SF	[210]
Zhao et al. (2018)	Doxorubicin	No report	miR-140-5p	Downregulated	ROS	ECG abnormality; histopathological changes of heart	[212]
Fu et al. (2016)	Doxorubicin	No report	GRP78	Upregulated	ER stress	Decreased the LVFS and LVEF	[220]
Hu et al. (2019)	Doxorubicin	No report	CACNA1H	Downregulated	ER stress	Myocardial dysfunction, myocardial apoptosis	[222]
Aung et al. (2017)	Doxorubicin	No report	Mtfp1	Downregulated	ROS, apoptosis	Severe cardiomyopathy	[233]
Kobayashi et al. (2006)	Doxorubicin	No report	GATA4	Upregulated	LC3-II, Bcl-2	Cardiomyocyte death	[240]
Tong et al. (2015)	Doxorubicin	No report	BTG2	Upregulated	Apoptosis, miR-21	Depressed LV function, decreased heart indices	[244]
Chen et al. (2018)	Doxorubicin	No report	CTR P1	Upregulated	PKB/AKT	Impaired cardiac function	[250]
Caso et al. (2017)	Doxorubicin	No report	AKAP-Lbc	Downregulated	Protein kinase D1, Bcl-2, Bax	CytC release and mitochondrial dysfunction	[262]
Chen et al. (2019)	Doxorubicin	No report	SNHG1	Upregulated	miR-195/Bcl-2 axis	Impairment of heart function	[266]
Wells et al. (2017)	Anthracycline	Non-Hodgkin's lymphoma and breast cancer	PRDM2	Upregulated	DNA repair, metabolism, cardiac remodeling	LV function, LVEF	[275]
Zheng et al. (2020)	Doxorubicin	No report	Bnip3	Downregulated	Pyroptosis	Declined in LVEF and FS, increased LDH and CK-MB	[279]
Singla et al. (2019)	Doxorubicin	No report	ES-Exos	Upregulated	Pyroptosis	Cardiac dysfunction	[282]
Dimitrakis et al. (2012)	Doxorubicin	No report	MURF-1	Upregulated	UPS	HF	[287]
Sishi et al. (2013)	Doxorubicin	No report	E3 ligase	Upregulated	UPP	Myocardium dysfunction	[289]
Zhao et al. (2015)	Doxorubicin	No report	β1i, β2i and β5i	Upregulated	UPS	Cardiac dysfunction	[290]
Chen et al. (2015)	Adriamycin	No report	MG132	Upregulated	Cx43, ZO-1, 20S proteasome	HF	[295]
Spur et al. (2016)	Doxorubicin	No report	LMP7	Downregulated	b5 standard proteasome	HF	[296]
Song et al. (2018)	Doxorubicin	No report	α-Tubulin acetylation	Downregulated	HDAC6	Acute cardiomyopathy	[302]
Hanf et al. (2019)	Doxorubicin	No report	Histone 3 acetylation	Downregulated	SIRT1 and HDAC2	Cardiomyopathy	[303]
Piotrowska et al. (2017)	Doxorubicin	No report	Anf, Bnf, Bmp-1, Myh-7	Upregulated	HDACs	Cardiac remodeling	[307]
Bhuvanalakshmi et al. (2017)	Doxorubicin	No report	sFRP4	Upregulated	HDACs	Cardiac injury	[308]
Räsänen et al. (2016)	Doxorubicin	No report	VEGF-B	Upregulated	Apoptosis	Decreased LV mass, left ventricular wall and septum thickness, diastolic and systolic volume, and stroke volume; decreased LVFS and LVEF	[313]

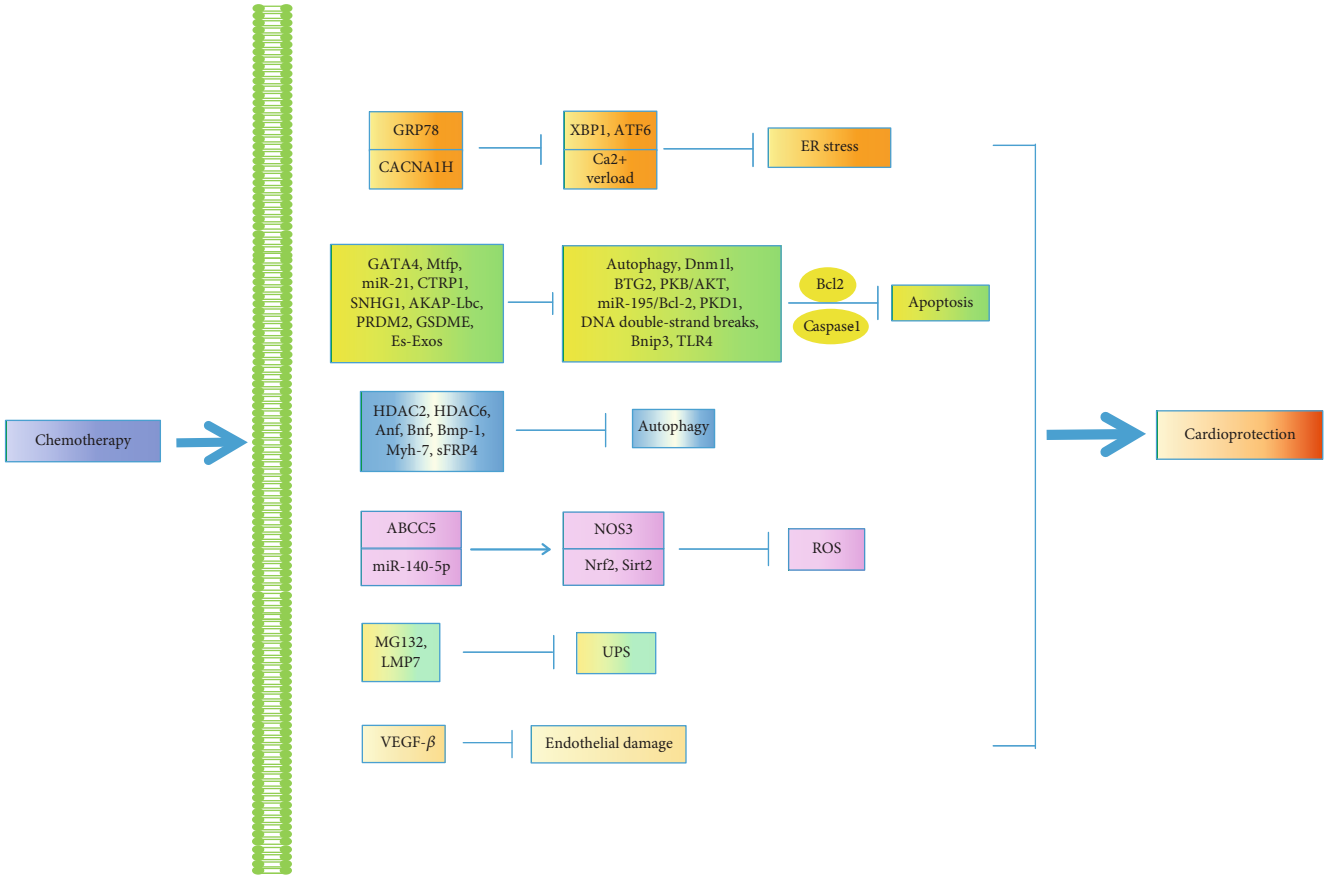

Figure 3: The mechanism by which genes protect against cardiotoxicity in chemotherapy. ROS: reactive oxygen species; ER stress: endoplasmic reticulum stress; ATF6: transcription factor 6; XBP1: X-box binding protein 1; GRP78 glucose regulatory protein; Mtfp1: mitochondrial fission protein 1; Dnm1l: dynamin 1-like; BTG2: B-cell translocation gene 2; CTRP1: C1q/TNF-related protein 1; PKB/AKT: protein kinase B phosphorylation; Bcl-2: B-cell lymphoma-2; AKAP: A-kinase anchoring protein; SNHG1: small nucleolar RNA host gene 1; PRDM2: PR domain-containing 2 with ZNF domain; GSDME: gasdermin D; Bnip3: Bcl-2/adenovirus E1B 19 kDa interaction protein 3; ES-Exos: embryonic stem cell-derived exosomes; TLR4: Toll-like receptor 4; UPS: ubiquitin-proteasome system; HDAC2: histone deacetylase; VEGF-β: vascular endothelial growth factor-β.

the effect of ER stress on DOX-induced cardiac toxicity in H9C2 cells. The results showed that DOX treatment resulted in cardiac injury, decreased cardiac function, increased myocardial cell apoptosis, and a significant increase in CACNA1H expression in the heart tissue. The CACNA1H inhibitor ABT-639 was found to partially protect cardiac function and reduce apoptosis in mice [223]. These results suggest that CACNA1H may reduce DOX-induced cardiotoxicity by decreasing the severity of ER stress, since ABT-639 significantly altered the expression of ER stress-related proteins, including PERK, P-PERK, ATF6, CHOP, ATF4, and GRP78. Therefore, the inhibition of CACNA1H may significantly reduce DOX-induced ER stress, cardiac toxicity, and apoptosis.

3.3. Apoptosis. Apoptosis plays an important role in cardiovascular disease. It is associated with the loss of cardiomyocytes in several kinds of heart diseases, including myocardial infarction, myocardial hypertrophy, HF, and cardiotoxicity [224–226]. Recent studies have shown that the inhibition of cardiomyocyte apoptosis can significantly reduce DOX-induced cardiac dysfunction [227–230]. Therefore, the discovery of novel genes that alleviate the apoptosis of cardiomyocytes is essential for the treatment of DOX-induced cardiotoxicity. Currently, a new mitochondrial inner membrane protein, mitochondrial fission protein 1 (Mtfp1), has been authenticated [231] and is considered to be indispensable for maintaining mitochondrial membrane integrality; it has, therefore, been associated with mitochondrial fission regulation [232]. One study reported on the role of Mtfp1 in mitochondrial division and on the induction of apoptosis in DOX-induced cardiotoxicity [233]. The knockdown of Mtfp1 can prevent mitochondrial fission in cardiomyocytes, subsequently decreasing DOX-induced apoptosis by preventing the accumulation of mitochondrial-type dynamin 1-like (Dnm1l). Conversely, when Mtfp1 is overexpressed, DOX can lead to large amounts of cardiomyocytes undergoing mitochondrial apoptosis. These results indicate that the knockdown of Mtfp1 can minimize myocardial cell loss in DOX-induced cardiotoxicity. Therefore, Mtfp1 expression regulation is a potential new treatment for cardiotoxicity induced by chemotherapy [233, 234].

The transcription factor GATA4 has been shown to influence the expression of various cardiac-related genes

[235, 236]. Previous studies have shown that DOX could downregulate GATA4 transcription in myocardial cells [237, 238]. The GATA4 level protection by the 1-adrenergic agonist phenylephrine or GATA4 overexpression by the adenovirus-mediated gene transfer protected myocardial apoptosis induced by DOX [237–239]. The protective effect of GATA4 against DOX-induced cardiotoxicity is mediated at least in part by its ability to upregulate the expression of the Bcl-2 gene [240], which is a survival factor that inhibits apoptosis and autophagy. Kobayashi et al. investigated the ability of GATA4 to suppress autophagy and act as the underlying mechanism of protection against DOX-induced toxicity in cardiomyocytes [94]. DOX treatment decreased the GATA4 protein levels, leaving cardiomyocytes vulnerable to DOX-induced toxicity. Indeed, autophagy activated by GATA4 gene silencing was found to increase the toxicity of DOX, while the overexpression of GATA4 restrained the autophagy induced by DOX, thereby decreasing cardiomyocyte apoptosis. This mechanism indicates that GATA4 may upregulate Bcl-2 gene expression and inhibit the activation of autophagy-related genes induced by DOX, thus the antiapoptosis and antiautophagy roles of GATA4. These findings suggest that the activation of autophagy mediated DOX-induced cardiotoxicity, while the preservation of GATA4 inhibited autophagy by regulating the Bcl-2 and autophagy-related gene expression, thereby suppressing cardiotoxicity induced by DOX [94, 241, 242].

mRNA-21 (miR-21) plays an important role in adjusting apoptosis [243]. Although miR-21 is involved in cardiovascular disease, little is known about its biological function in response to cardiotoxicity induced by DOX. One study reported on the effects of DOX on cardiac function and miR-21 expression in mouse heart tissue and H9C2 cardiac myocytes [244]. The results suggested that the cardiac function of mice with chronic DOX injury was worse than that of mice with acute DOX injury; DOX treatment prominently enhanced the expression of miR-21 in mice cardiac tissues and H9C2 cardiomyocytes. The overexpression of miR-21 weakened apoptosis in cardiomyocytes induced by DOX and decreased the levels of miR-21 expression attenuated by the DOX-induced apoptosis of cardiomyocytes. The results of functional gain and loss experiments suggested that the B-cell translocation gene 2 (BTG2) was a target of miR-21, with BTG2 expression being prominently reduced in DOX-treated cardiomyocytes. In this study, miR-21 was found to protect mice myocardial and H9C2 cells from cardiotoxicity induced by DOX by targeting BTG [245, 246].

C1q/TNF-related protein 1 (CTRP1) is a highly conserved family of proteins [247] expressed in the heart [248, 249]. Chen et al. studied the expression of CTRP1 in the heart using an *in vivo* gene delivery system [250]. Two weeks after the gene was delivered, an intraperitoneal injection of DOX was administered to the mice to induce cardiac injury. In the DOX-treated mice, the levels of CTRP1 were reduced. The overexpression of CTRP1 then decreased cardiac troponin I, recovered cardiac function, and weakened cardiac cell apoptosis. CTRP1 expression also ameliorated cell viability and decreased the release of LDH. In contrast, DOX led to a reduction in protein kinase B phosphorylation (PKB/AKT) [251], but this was recovered by CTRP1 overexpression. The inhibition of AKT can counteract the inhibitory roles of CTRP1 on myocardial cell apoptosis [252]. In AKT-deficient mice, CTRP1 lost its ability to provide protection against cardiac damage caused by DOX. However, transfusion with recombinant CTRP1 could reverse preestablished cardiac damage caused by DOX therapy. Overall, CTRP1 provided protection against cardiotoxicity induced by DOX by activating the AKT signal pathway [250, 253]. Therefore, CTRP1 has therapeutic potential against cardiotoxicity induced by DOX.

A-kinase anchoring proteins (AKAPs) have been proposed to coordinate and synchronize the activity of a variety of signal transducers to regulate key cellular processes in the heart [254, 255]. AKAP-Lbc is a protein primarily expressed in the cardiac tissue that coordinates the activation of the hypertrophic transduction pathway downstream of α1-Ars [256–258]. In *in vivo* experiments, AKAP-Lbc has been shown to promote compensatory hypertrophy and cardiomyocyte protection in stress-overloaded hearts [259–261]. The stimulation of myocardial cells by the α1-adrenergic receptor (AR) agonist phenylephrine (PE) was found to prominently inhibit DOX-induced apoptosis [262]. Importantly, this result suggests that AKAP-Lbc is crucial for sending protection signals downstream of α1-Ars [263]. This study also found that the inhibition of AKAP-Lbc expression in the ventricular myocytes infected with lentivirus RNA may reduce PE's ability to reduce DOX-induced apoptosis [238]. AKAP-Lbc-mediated cardiomyocytes activate the expression of antiapoptotic protein Bcl-2 and suppress the transport of proapoptotic protein Bax to the mitochondria [239, 240]. In summary, AKAP-Lbc can provide cardiomyocytes with protection against DOX-induced toxicity.

Long noncoding RNA (lncRNA), a group of RNA molecules with lengths greater than 200 nucleotides, has limited protein-coding potential and has recently been identified as a key factor in many diseases, including cardiovascular disease [264]. lncRNA small nucleolar RNA host gene 1 (SNHG1) on human chromosome 11 has been found to be abnormally expressed in a variety of human cancers [265]. Chen et al. investigated whether DOX toxicity in AC16 cardiomyocytes *in vitro* can be adjusted by lncRNA SNHG1, with the aim of identifying potential mechanisms [266]. This study found that DOX treatment resulted in severe damage in AC16 cells by reducing cell viability and increasing cell apoptosis, while the overexpression of SNHG1 reduced apoptosis in DOX-treated AC16 cells. In addition, this study found that SNHG1 could counteract the inhibitory role of miR-195 on Bcl-2, while miR-195 restoration blocked the beneficial action of SNHG1 against DOX toxicity in AC16 cells [267]. In short, this study provided convincing evidence that SNHG1 partially protects cardiomyocytes from DOX-induced toxicity by modulating the miR-195/Bcl-2 axis [266, 267].

PR domain-containing 2 with ZNF domain (PRDM2) is crucial for the BRCA1-dependent repair of DNA double-strand breaks [268]. Damage to this mechanism increases DOX cardiotoxicity in mice [269]. In addition, PRDM2 is a heme oxygenase-1 transcriptional regulator [270], which, in addition to preventing oxidative stress [271, 272], has also

been shown to promote the repair of DOX-induced DNA double-strand breaks [273] and decrease cardiomyocyte apoptosis [274]. One study examined the genetic factors that influence changes in cardiac LV function following chemotherapy with anthracyclines [275]. GWAS was conducted in this study which identified LV function changes in 385 cases of anthracyclines using BioVU after exposure to anthracyclines. The DNA samples were subsequently linked to an unidentified electronic medical record data. In a prospective clinical trial, 181 patients exposed to anthracyclines were independently replicated for variants. This study used path analysis to evaluate the combined roles of various kinds of genetic variations. These results were among the 11 candidate genes found in GWAS and located in SNP rs7542939 near PRDM2. Pathways associated with cell metabolism, DNA repair, and cardiac remodeling were identified. Therefore, using genome-wide associations, this study confirmed a susceptibility site near PRDM2 [275, 276].

Pyroptosis is a novel form of programmed cell death characterized by the swelling of cells, the blowing of large bubbles in plasma, and cytolysis, which results in the release of the cell contents and proinflammatory molecules [277, 278]. A study investigated the role of gasdermin D-(GSDME-) mediated pyroptosis in DOX-induced cardiac injury to assess the effect of BH3 protein Bcl-2/adenovirus E1B 19 kDa interaction protein 3 (Bnip3) in regulating of DOX-induced pyroptosis [279]. *In vitro* and *in vivo* cardiotoxicity models induced by DOX were established by DOX treatment. Cell transfection was used to regulate the expression of GSDME, caspase-3, and Bnip3. The release of LDH was determined using the LDH-cytotoxicity assay. Western blotting was used to measure protein level expression, flow cytometry analysis was used to determine cell death, echocardiography was used to detect heart function, and HE staining was used to observe the pathological features of the cardiac tissue. The results showed that GSDME-mediated pyroptosis was associated with DOX-induced cardiotoxicity *in vivo*. Furthermore, DOX induced the activation of caspase-3 and ultimately activated GSDME-dependent pyroptosis, which was inhibited by the silencing or inhibition of caspase-3. Other studies have shown that GSDME inhibition can inhibit the DOX-induced pyroptosis of cardiomyocytes *in vitro*. Lastly, DOX increased the expression of Bnip3, where Bnip3 silencing inhibited DOX-induced myocardial apoptosis [280, 281]. As such, this study revealed a novel pathway, the Bnip3-caspase-3-GSDME pathway, by which myocardial pyroptosis is regulated after DOX therapy.

Another study investigated whether embryonic stem cell-derived exosomes (ES-Exos) in DOX-induced cardiotoxicity attenuated inflammation-induced pyroptosis, inflammatory cell signal transduction, proinflammatory M1 macrophages, and poor cardiac remodeling [282]. To this end, the study transplanted ES-Exos and compared them with ES cells (ESCs) to detect pyroptosis, inflammation, cell signaling, adverse cardiac remodeling, and their effects on DOX-induced cardiac dysfunction. The results showed that DOX treatment significantly increased the expression of inflammasome markers (TLR4 and NLRP3), pyroptotic markers (caspase-1, IL1-β, and IL-18), cellular signaling proteins (MyD88, p-P38, and p-JNK), proinflammatory M1 macrophages, and TNF-α cytokines. ES-Exos or ESCs inhibited this increased expression of pyroptosis, inflammation, and cell signaling proteins. In addition, ES-Exos or ESCs increased M2 macrophages and anti-inflammatory cytokine IL-10, significantly inhibited cytoplasmic vacuoles and hypertrophy, and improved cardiac function [283, 284].

3.4. Proteasome Activity. DOX enhanced ubiquitin-proteasome system- (UPS-) mediated proteolysis in the heart, indicating that UPS hyperfunction may be an important mechanism of DOX-induced acute cardiotoxicity [285–287]. The O-linked attachment of monosaccharide-N-acetylglucosamine (O-GlcNAc) is a highly dynamic and ubiquitous protein modification [288]. Protein O-GlcNAcylation has rapidly become a key regulator of several important biological processes, including proteasomal degradation and apoptosis. However, proteasome inhibition has been found to be very effective in inhibiting cell proliferation in the treatment of cancer and for preventing restenosis [289]. These findings also suggest that the use of DOX with antitumor proteasome inhibitors may reduce the toxicity of DOX. Moreover, the overexpression of immunoproteasome-catalyzed subunits was found to markedly attenuate DOX-induced myocyte apoptosis and other UPS gene expression [290], while its knockdown significantly increased DOX-induced myocyte apoptosis [291].

UPS has been reported to be involved in Cx43 degradation [292]. The proteasome inhibitor MG132 has been found to suppress the internalization and degradation of Cx43 [293, 294]. This study investigated the roles of the MG132 proteasome inhibitor on Cx43, Zo-1, and 20S proteasome, and ubiquitin expression levels in adriamycin-induced HF rats [295]. MG132 was found to reduce adriamycin-induced injury in HF. Moreover, MG132 suppressed the expression of 20S proteasome and ubiquitin, while upregulating Cx43 and ZO-1. These findings indicate that inhibiting UPS upregulates Cx43 expression and suggest that proteasome inhibitors may be used against Cx43 degradation, thus preventing CX43-mediated arrhythmia in HF.

In another study, the role of UPS as a key monitoring pathway for maintaining cell viability and counteracting the toxicity of DOX treatment was also reported [296]. In addition to DOX treatment, the inhibition of proteasome activity is another reasonable strategy for the treatment of multiple myeloma (MM). As such, the mechanism by which small molecular compounds with clinically relevant proteasome subunit specificity affect DOX cytotoxicity was investigated. The activity of the b5 standard proteasome subunits was found to be critical in limiting off-target cytotoxicity in primary cardiomyocytes during DOX therapy. LMP7 inhibition in primary cardiomyocytes or the genetic ablation of LMP7 in cardiac tissue did not affect the development of DOX cardiotoxicity. These results suggest that immunoproteasome-specific inhibitors with known antitumor activity against MM cells may be beneficial in reducing cardiomyocyte death, compared with the compound carfilzomib [297], which targeted both the b5 standard proteasome and the LMP7 immunoproteasome subunit.

3.5. Histone Deacetylase (HDAC) Inhibitors. Histone deacetylases (HDACs) are widely expressed enzymes that can catalyze the removal of acetyl groups from histones, resulting in reduced DNA accessibility and gene silencing [298]. Although the exact mechanism of HDAC inhibitors in chemotherapy-induced cardiotoxicity is unclear, HDAC inhibitors are known to have a variety of effects [299–301]. Song et al. showed that HDAC6 was upregulated in DOX-treated cardiomyocytes *in vitro* and in an *in vivo* mice model, resulting in the deacetylation of α-tubulin [302]. Therefore, the genetic or pharmacological inhibition action of HDAC6 in mice has a cardioprotective effect on DOX by restoring the autophagic flux. In another study, Hanf et al. proved that DOX treatment affected the expression level of HDAC (SIRT1 and HDAC2) [303]. Nevertheless, pterostilbene, a natural analog of resveratrol and an antioxidant, was found to reduce cardiotoxicity induced by DOX both *in vitro* and *in vivo* [304]. This effect was attributed to the increased deacetylation activity of SIRT1, indicating its cardioprotective effect on DOX. In summary, HDAC inhibitors have a cardioprotective effect on DOX [305]. In Piotrowska et al.'s study, it was found that DOX, in a generally considered "safe" dose, caused adverse myocardial changes as soon as 2 weeks after continuous infusion in a mature chronic DOX infusion mouse model [306, 307]. The study also found that the low doses of DOX led to specific changes in several of the HDAC transcription profiles, which are epigenetic regulators of heart remodeling. These results indicated a potential cardioprotective therapy by modulating HDAC (Hdac2, Hdac4, Hdac6, and Hdac7) expression or activity during DOX treatment. Another study used various combinations of DNA methyltransferase and HDAC inhibitors, including DC301, DC302, and DC303 [308]. Induced by DC301 and DC302, Wharton's jelly mesenchymal stem cells (WJMSCs) differentiated into myocardial structures with Wnt antagonists, sFRP3 and sFRP4, and Dickkopf 1 (Dkk1) and Dkk3 upregulated. Cardiac progenitor cells were injected in vivo in a DOX-induced cardiotoxic mouse model. Bisulfite sequencing was used to examine the promoter methylation status of the cardiac transcription factor Nkx2.5 and the Wnt antagonist secreted frizzled-related protein 4 (sFRP4) after cardiac differentiation and revealed that sFRP4 was activated by promoter CpG island demethylation during cardiogenesis. The MSC-derived cardiac progenitors not only successfully transplanted to the site of DOX-induced cardiac injury in mice but also formed functional cardiomyocytes and recovered cardiac function [309–311]. These studies revealed the connection between Wnt inhibition and epigenetic modification to activate cardiac differentiation, which could strengthen the efficacy of stem cells in the treatment of cardiac injury.

3.6. Others. Vascular endothelial growth factor-β (VEGF-β), which promotes coronary angiogenesis and physiological cardiac hypertrophy, has potential for protection against DOX-induced cardiotoxicity [312]. In one study, doses at simulated clinical concentrations were administered to adenoviral vectors or control vectors expressing VEGF-β in normal mice 1 week prior to DOX treatment [313]. VEGF-β treatment suppressed DOX-induced heart atrophy, protected the sparse capillaries in the heart, and ameliorated the endothelial function of DOX-treated mice. VEGF-β also increased the volume of the LV without compromising cardiac function and decreased the expression of genes related to cardiovascular disease [314–316]. Importantly, VEGF-β did not affect tumor growth. As such, the inhibition of DOX-induced endothelial injury and the prevention of chemotherapy-related cardiotoxicity provide new therapeutic directions.

ALL: acute lymphoblastic leukemia; LV: left ventricular; LVFS: left ventricular fractional shortening; LVEF: left ventricular ejection fraction; HF: heart failure; UPP: ubiquitin-proteasome pathway; UPS: ubiquitin-proteasome system; LDH: lactic dehydrogenase; CK-MB: creatine kinase-MB; ROS: reactive oxygen species; ER: endoplasmic reticulum; ATF6: transcription factor 6; XBP1: X-box binding protein 1; GRP78 glucose regulatory protein; Mtfp1: mitochondrial fission protein 1; Dnm1l: dynamin 1-like; BTG2: B-cell translocation gene 2; CTRP1: C1q/TNF-related protein 1; PKB/AKT: protein kinase B phosphorylation; Bcl-2: B-cell lymphoma-2; AKAP: A-kinase anchoring protein; SNHG1: small nucleolar RNA host gene 1; PRDM2: PR domain-containing 2 with ZNF domain; GSDME: gasdermin D; Bnip3: Bcl-2/adenovirus E1B 19 kDa interaction protein 3; ES-Exos: embryonic stem cell-derived exosomes; TLR4: Toll-like receptor 4; HDAC: histone deacetylase; HDAC2: histone deacetylase 2; sFRP4: secreted frizzled-related protein 4; VEGF-β: vascular endothelial growth factor-β.

4. Discussion

This review provides an integrated overview of all the genetic variations that have been found to affect susceptibility to cardiotoxicity induced by chemotherapy. Genetics provides an insight into the development of toxicity associated with these cancer treatments, and by identifying the functional genetic variants related to these toxicities, we can improve our understanding of the potential mechanisms and pathways, thus paving the way for the development of novel therapies for these toxicities [317]. In addition, genetic markers with underlying predictive power could be used to identify patients who would benefit from careful monitoring and the prescription of cardioprotective drugs. Once chemotherapy-induced cardiotoxicity occurs, the use of appropriate therapeutic measures can alleviate this toxicity [18, 318, 319]. Meanwhile, clinicians can select specific treatments for patients according to the genotype studied and compare the differences in drug efficacy, toxicity, and side effects among patients with different genotypes [320]. Gene polymorphisms are closely related to individual differences in the effect of drugs. The research results are applied to rational drug use, thereby providing guidance for clinical drug therapy of tumors.

The majority of genes studied were related to biochemical pathways of chemotherapy-induced cardiotoxicity. For these genes, animal and mechanism studies have shown that their alleles changed the expression or activity levels of the encoded protein, thereby promoting the occurrence and development of disease. Cardiac toxicity results from

oxidative stress, autophagy, apoptosis, inflammation, DNA damage, metabolism, and sarcoplasmic reticulum, among others. To date, several potential cellular and molecular mechanisms involving several genes for cardiotoxicity have been identified. Accordingly, the main susceptibility genes related to cardiotoxicity after chemotherapy are CYBA, GSTA1, NCF4, RAC2, ABCC1, ABCC2, CAT, UVRAG, GCN2, TCL1A, TLRS, C282Y, Hmox1, CBRs, MYH7, TNNT2, and TTNtv.

ROS is considered the primary mediator of chemotherapy-induced cardiotoxicity. Mitochondria are abundant in cardiomyocytes and are the main source of ROS. Changes in gene expression (CYBA, GSTA1, NCF4, RAC2, ABCC1, ABCC2, and CAT) lead to mitochondrial dysfunction, which results in increased ROS production and, ultimately, muscle cell damage. The turnover of damaged mitochondria via autophagy is essential to maintain the structure and function of cardiomyocytes [321], and UVRAG deficiency exacerbates DOX-induced cardiotoxicity. Moreover, a decreased ratio of Bcl-2/Bax can lead to the formation of pores in the mitochondria and the activation of the apoptotic pathway [322, 323]. GCN2 deficiency confers resistance to DOX-induced cardiomyocyte apoptosis by increasing the ratio of Bcl-2 and Bax. Moreover, an accumulation of iron (C282Y and Hmox1) in the mitochondria has recently been shown to cause chemotherapy cardiotoxicity, primarily by promoting ROS generation. Meanwhile, DOX-induced cardiac injury was found to be morphologically characterized by inflammation [324]. The genes TCL1A, TLR4, TLR2, and TLR9 appear to be strongly related with the inflammation and repair processes that occur following myocardial injury.

This study has some limitations which deserve discussion. Firstly, we found a total of 64 articles associated with chemotherapy-induced cardiotoxicity. Most of the studies were single case and animal studies and there were inconsistencies in the results reported between the studies. Secondly, the majority of the included studies had a small sample size. To ensure that the research results more effectively influence the development of personalized medicines, future studies should use large populations. Finally, the participants had different backgrounds. Multicenter research on patients from other regions, particularly Asia, Australia, Africa, Oceania, and South America, should be performed. Furthermore, an objective definition of cardiotoxicity and the frequency of events for each genotype should be considered. We also selectively discussed the role of genes included in the literature. It should be noted that the genes discussed in this review do not mean that they are superior to the other genes identified. Therefore, high-quality studies are needed to determine the susceptibility genes in chemotherapy-induced cardiotoxicity, thus providing guidance for clinical drug therapy of tumors.

5. Conclusion

In recent times, with improved treatment regimens, cancer patients have a better chance of survival. Unfortunately, they are at risk of developing long-term cardiotoxicity because of their anticancer therapies. However, there is a serious lack of reliable and sensitive biomarkers for the clinical evaluation of chemotherapy-induced cardiotoxicity. Based on genetic analyses, the combination of chemotherapy-induced cardiotoxicity and treatment targeting molecular targets of specific genes may prevent or mitigate the cardiotoxicity induced by chemotherapy in patients. In the context of inevitable cardiotoxicity, the effective and safe treatment of different types of cancer is important and deserves further study. This review reveals a number of potential therapeutic targets and provides a viable hypothesis for the development of new gene-targeted drugs for the treatment of chemotherapy-induced cardiotoxicity. But more high-quality studies are needed to determine the susceptibility genes in chemotherapy-induced cardiotoxicity, thus providing guidance for clinical drug therapy of tumors.

Conflicts of Interest

All authors claim that there is no conflict of interests about the publication of this review.

Authors' Contributions

Yanwei Xing, Saumya Das, and Hongcai Shang designed the idea for drafting this review. Xinyu Yang and Guoping Li collected the documents and wrote the paper. Tao Yang, Manke Guan, Na An, and Fan Yang contributed to the discussion. Qianqian Dai, Changming Zhong, Changyong Luo, and Yonghong Gao revised and edited the review. All authors commented on the manuscript. Xinyu Yang and Guoping Li contributed equally to this work.

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