Original Article
MiR-378 modulates energy imbalance and apoptosis of mitochondria induced by doxorubicin

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Abstract: Doxorubicin (DOX) is an effective anticancer drug, however its clinical application is limited due to its cardiotoxicity. Therefore, understanding the mechanisms of cardiotoxicity induced by DOX is essential. We found that the level of miR-378 was decreased in the hearts of DOX-treated rats. Increasing the expression of miR-378 resulted in a decrease of lactate dehydrogenase (LDH) upon DOX treatment *in vitro* by targeting lactate dehydrogenase A (LDHA). Furthermore, bioinformatics analysis indicated that cyclophilin A (PPIA), a regulator of apoptosis, is also a direct target gene of miR-378. We confirmed this by Western blot. Our results also showed that the overexpression of miR-378 inhibited the hyperactivation of ER stress signaling induced by DOX. In addition, MiR-378 overexpression was found to protect cardiomyocytes from DOX-induced energy imbalance and apoptosis of mitochondria. These results may allow for a therapeutic approach that overcomes the cardiotoxicity of DOX-based treatments for cancer.

Keywords: miR-378, apoptosis, energy imbalance

Introduction

Some highly effective anticancer drugs, such as doxorubicin, have been widely used in the treatment of multiple types of cancers, including breast cancer, liver cancer, and gastric cancer, and they have led to important gains in survival [1]. However, the usage of doxorubicin can result in serious cardiotoxicity, which can lead to congestive heart failure, thus restricting its clinical application. The molecular signaling of DOX-induced cardiotoxicity has been a controversial topic in recent years.

Mitochondria are the main target organs in the pathogenesis of DOX, which can lead to mitochondrial dysfunction and energy imbalance [2]. As a glycolytic enzyme, LDH catalyzes lactic acid to pyruvate. Evidence demonstrates the importance of LDHA, a subunit of LDH, in energy imbalance [3].

The heart has evolved a complex mechanism of apoptotic control and has highly expressed anti-apoptotic proteins. Endoplasmic reticulum (ER) stress-induced cell apoptosis is a newly discovered signal transduction pathway [4]. Functions of the ER include maintaining metabolism, lipoprotein secretion, and Ca^{2+} homeostasis [5]. Our previous work showed that ER stress was activated in rats with DOX-induced cardiomyopathy [6]. ER stress could regulate the ratio of Bax/Bcl-2 to influence the function of mitochondria and lead to apoptosis. As a highly abundant protein, PPIA accounts for up to 0.6% of the total cytosolic protein content [7]. PPIA is involved in a growing number of biological processes, including protein folding and cell apoptosis [8]. Similar to the ER stress chaperone GRP78, the protein expression of PPIA is regulated in ER stress [9]. The changes and regulations of myocardial energy metabolism in DOX-induced cardiotoxicity are poorly understood. Specifically, the regulation of ER stress and energy metabolism in DOX-induced cardiotoxicity have rarely been reported.

Research has shown that miR-378 transfection significantly enhances cell viability and inhibits cardiomyocyte apoptosis [10]. miR-378 is en-

riched in cardiac tissue during the postnasal period of cardiac growth but down-regulated in patients with heart failure, suggesting that it may participate in the disease progression of cardiomyopathy [11, 12].

In our present study, we aimed to understand the biological functions of miR-378 in cardiotoxicity. Our results demonstrated that miR-378 was able to decrease the cardiotoxicity in response to DOX stimulation *in vitro*. Furthermore, we found that miR-378 can regulate the expression of PPIA and LDHA. This will provide new insights into understanding the pathogenesis of DOX-induced cardiotoxicity.

Material and methods

Animal experiment

Animal care and study protocols were approved by the Animal Care and Use Committee of Inner Mongolia University for Nationalities. Male 200-220 g SD rats were purchased from Basic Medical School of Jilin University Animal Center (Changchun, China). Rats were kept with free water and food *ab libitum* and housed in an air-conditioned room on a 12-h light/12-h dark cycle. SD rats were randomly divided into 2 groups (n = 10/each group): the DOX group, which was intraperitoneally injected with doxorubicin (2 mg/kg) once per week for 4 weeks, and the control (untreated) group, which were given the same amount of 0.9% NaCl solution. After 4 weeks, the rats were killed, the heart was exposed and removed, and H&E staining was performed with the upper 1/3 of the cardiac tissue.

Isolation, culture, and treatment of neonatal mouse cardiomyocytes

Primary cultures of neonatal mice cardiomyocytes were obtained as described previously [13]. The cardiomyocytes were maintained in DMEM supplemented with 10% FBS (fetal bovine serum), 1% penicillin, and streptomycin under an atmosphere of 95% air and 5% CO_2 at 37°C. The medium was replaced every 2 days and cells were cultured again or subjected to experimental procedures at an amount to achieve 70-80% confluence. Cytotoxicity was induced by DOX at this concentration [14].

Lentiviral transfection of the target cells

The recombinant adenoviruses used for the overexpression of miR-378 were generated using the BLOCKiT adenoviral RNAi expression system (Invitrogen, Carlsbad, CA). A certain amount of the viral liquid (the number of viruses vs. cells was 100:1) was collected and added into the prepared medium using pipettes. Polybrene was then added into the medium (6 g/ml) to improve the efficiency of infection. The original culture medium was aspirated from all of the cultures. New culture medium with viral fluid and new culture medium without viral fluid were added to lentivirus transfection cells and control cells, respectively. After 24 h, normal culture medium was replaced by lentiviral medium and continuously cultured in the incubator with 5% CO_2 at 37°C. After infection by the lentivirus for 24 h, cells were replenished with complete medium. After treatment with drugs for 24 h, cells were collected and used for subsequent experiments.

Lactate dehydrogenase (LDH) release assay

There were 1.5×10^5 cardiomyocytes per well that were placed in 24-well cell culture plates overnight for incubation. The supernatants were collected and added into black culture plates with 96 wells (200 μL per well). Release level of LDH was measured using an LDH cytotoxicity assay kit (Beyotime, China) as per the manufacturer's instructions. Absorbance at a wavelength of 450 nm in each well was determined using an enzyme linked immunoassay instrument.

Echocardiographic assessment of cardiac function

GE Vivid E9 color doppler imaging high-frequency ultrasound probe ML6-15 with a frequency of 13 MHz and image depth of 3.5 cm was used for transthoracic echocardiography. After anesthetizing the rats with 10% chloral hydrate (0.2 g/kg) by intraperitoneal injection, M-type ultrasound was used to measure left ventricular posterior wall thickness at end diastole (LVPWd), end-diastolic interventricular septum thickness (IVSd), and fractional shortening (FS). Data from three consecutive cardiac cycles were used to calculate the average value.
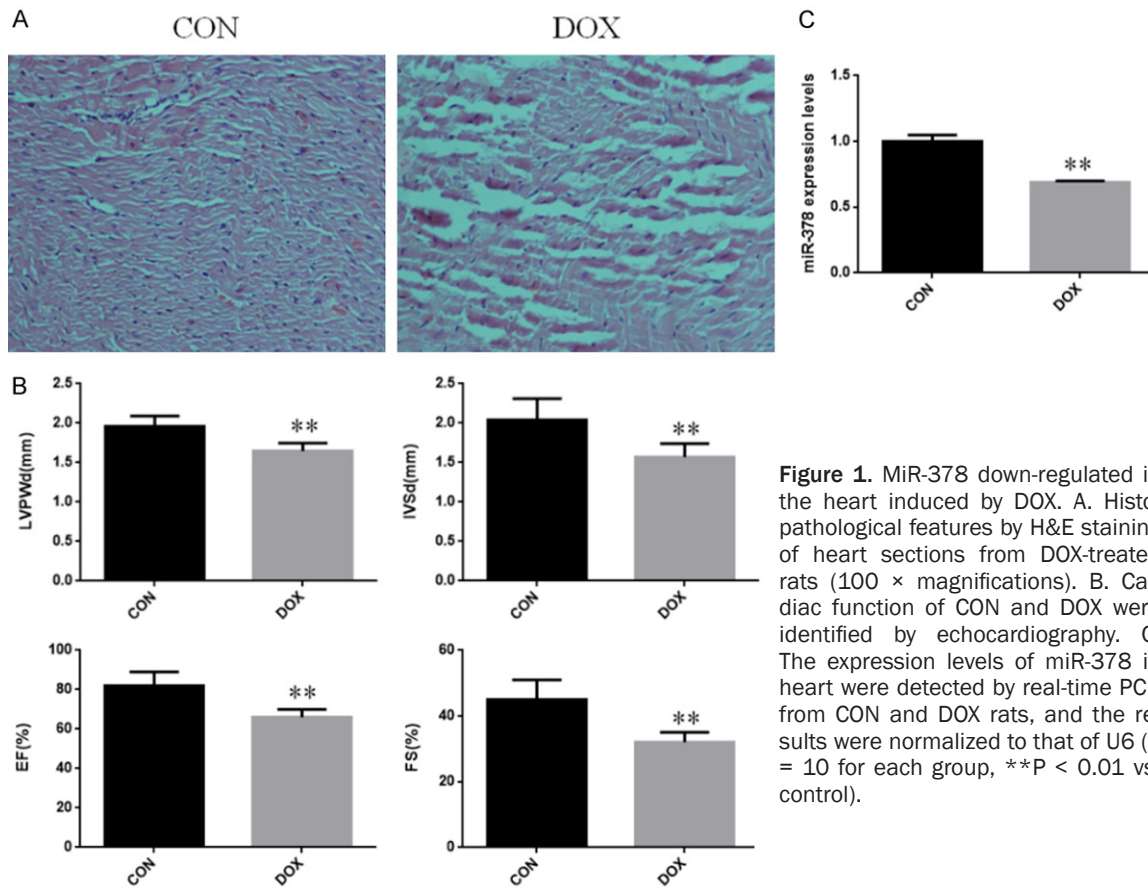
Figure 1. MiR-378 down-regulated in the heart induced by DOX. A. Histopathological features by H&E staining of heart sections from DOX-treated rats (100 × magnifications). B. Cardiac function of CON and DOX were identified by echocardiography. C. The expression levels of miR-378 in heart were detected by real-time PCR from CON and DOX rats, and the results were normalized to that of U6 (n = 10 for each group, **P < 0.01 vs. control).

TUNEL assay

Following the manufacturer's instructions, TUNEL staining was used to detect DNA fragmentation of apoptotic cells. The results were detected by microscopy.

JC-1 fluorescence analysis

Neonatal mice cardiomyocytes were subjected to different treatments. Cardiomyocytes were stained with JC-1 to determine the mitochondrial membrane potential. J-aggregates in the mitochondrial matrix were stained with red fluorescent tags and monomers in the cytoplasm were stained with green fluorescent tags. The JC-1 assay was performed per the manufacturer's instructions. The results were detected by microscopy.

Western blotting

The 10-uL samples of protein were subjected to SDS-PAGE, then transferred to a PVDF membrane, incubated with the primary antibodies (anti-LDHA: 1:500, anti-GRP78: 1:500, anti-bcl-2: 1:1000, anti-Bax: 1:1000, anti-ATF4: 1:1000, anti-PPIA: 1:1000, and anti-CHOP: 1:1000), and then incubated with the secondary antibody (1:5000). ECL substrate luminescence was used to visualize the blot.

Statistical analysis

Experimental results are shown as means ± SEM. SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Comparisons among multiple groups were performed using one-way analysis of variance, followed by Fisher's least significant difference test (for equal variances assumed) or Dunnett's T3 test (for equal variances not assumed) to compare individual group differences. P < 0.05 was considered to indicate a statistically significant difference.

Results

MiR-378 is regulated in cardiotoxicity induced by DOX treatment

To study whether miR-378 was related to the regulation of cardiotoxicity, we first measured the expression of miR-378 in the heart of SD
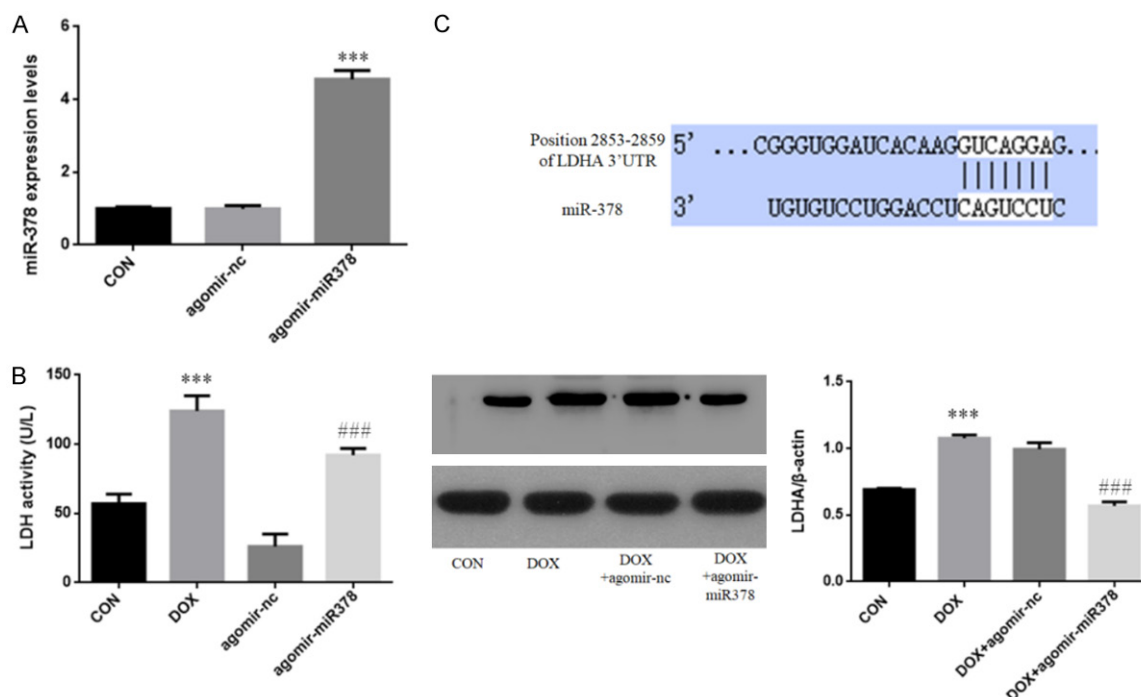
Figure 2. MiR-378 regulated energy metabolism via targeting LDHA expression. A. Overexpression of miR378 in suckling mouse cardiomyocytes (n = 3, ***P < 0.001 vs. control). NC: negative control. B. Overexpression of miR378 attenuated LDH activity in cardiomyocytes (n = 6, ***P < 0.001 vs. control; ###P < 0.001 vs. DOX). C. Expression levels of LDHA in cardiomyocytes of CON and DOX within/out miR-378 by Western blotting (n = 3, ***P < 0.001 vs. control; ###P < 0.001 vs. DOX).

rats treated with DOX. After 10 weeks, the hearts revealed the formation of cytoplasmic vacuole and myofibrillar loss (**Figure 1A**). The left ventricular posterior wall thickness of end diastole (LVPWd), interventricular septal thickness (IVSd), ejection fraction (EF), and % FS were significantly lower in the DOX group than in the control group (**Figure 1B**). The expression levels of miR-378 were repressed following DOX treatment (**Figure 1C**).

MiR-378 inhibited cardiomyocyte damage induced by DOX treatment through energy metabolism

To explore the function of miR-378 *in vitro*, adenoviral miR-378 was used to achieve the overexpression of miR-378 in mouse cardiomyocytes (**Figure 2A**). We found that LDH levels of cardiomyocytes increased after DOX treatment and were relieved by miR-378 (**Figure 2B**). This result indicated that miR-378 affects energy metabolism. Since miRNAs suppress gene expression at the post-transcriptional level by directly binding to the complementary sequences in the 3'UTRs of target genes, we presumed

miR378 had at least one target gene. LDHA was selected as a putative miR-378 target (**Figure 2C**). To identify whether LDHA is a direct target of miR-378, we investigated the effects of miR-378 on the protein expression of LDHA. The results showed the protein levels were correspondingly decreased when miR-378 was overexpressed (**Figure 2C**).

MiR-378 inhibited DOX-induced cardiotoxicity through anti-apoptosis

As shown in **Figure 3**, DOX treatment increased cell apoptosis and cardiomyocyte injury. In contrast, the up-regulation of miR-378 attenuated the increase in DOX-induced cardiomyocyte apoptosis.

MiR-378 regulated cardiomyocyte apoptosis by ER stress

Through target gene screening, PPIA was considered to be the target of miR-378 (**Figure 4A**). Western blotting analysis was carried out to investigate the effects of miR-378* on PPIA protein expression after induction by DOX. The
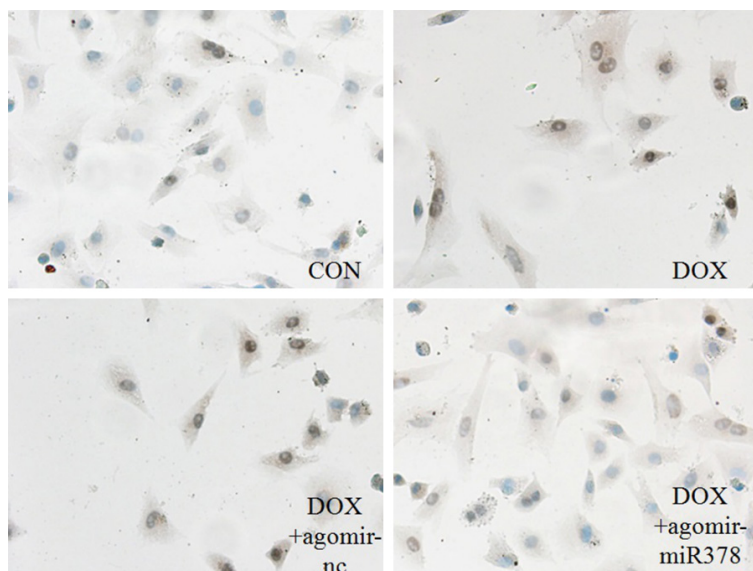
Figure 3. Up-regulation of miR378 attenuated the apoptosis of suckling mouse cardiomyocytes. Apoptosis was examined by TUNEL assay.

results showed that protein levels correspondingly declined when miR-378 was overexpressed after induction by DOX (**Figure 4A**). This indicated that miR-378 regulated PPIA expression in DOX-induced injury of suckling mouse cardiomyocytes. We had previously shown ER stress could induce cardiomyocyte apoptosis [15]. PPIA has also been shown to regulate protein folding and be triggered by the unfolded protein response (UPR) [8]. The expression of ER stress-related genes and mitochondria-related genes were examined by Western blot. As expected, the overexpression of miR-378 significantly inhibited the increase of GRP78, ATF4, and CHOP and the ratio of Bax/Bcl-2 induced by DOX (**Figure 4B-E**).

MiR-378 enhanced cardiomyocyte mitochondrial membrane potential Δψm

JC-1 analysis was used to test cardiomyocytes subject to different treatments. The green-to-red ratio of cardiomyocytes was markedly increased in DOX treatment, indicating the loss of Δψm, while miR-378 decreased the green-to-red ratio (**Figure 5**).
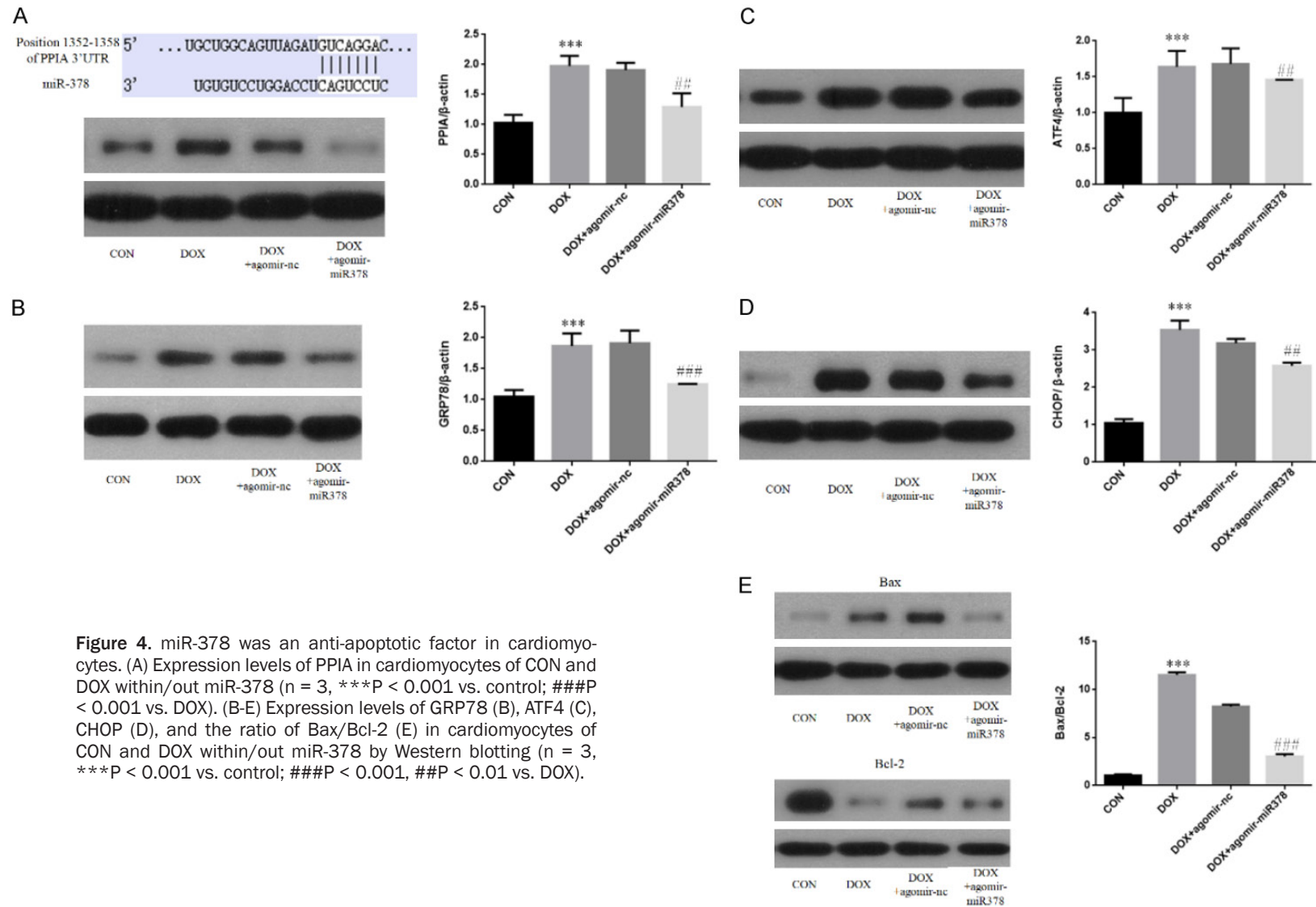
Discussion

This present research demonstrates that cardiomyocytes transfected with miR-378 show a higher viability and a lower apoptosis rate under DOX treatment. Moreover, the energy metabolism was improved. The expression of ER stress and mitochondria-related genes were decreased in injured cardiomyocytes with DOX treatment after miR-378 transfection. Our results will provide new insights into understanding the pathogenesis of DOX-induced cardiotoxicity.

As one of most effective chemotherapeutic drugs, DOX is widely used in the treatment of cancer. However, cardiotoxicity has restricted its clinical application. As the major target in DOX-induced subcellular damage in the heart, mitochondria have been analyzed, and the evidence suggests that its dysfunction is involved with cardiotoxicity [16, 17]. Recent studies about miRNAs have demonstrated that a number of miRNAs are able to affect cardiotoxicity. The overexpression of miR-146a reduced cardiomyocyte injury due to DOX treatment by targeting ErbB4 [18]. MiR-532-3p-mediated suppression of ARC is a substantial causal mechanism of DOX-induced cardiac toxicity [14]. Downregulation of miR-30 in cardiomyocytes triggered by DOX drives alterations in β-adrenergic signaling and enhances apoptosis [19]. In this research, we found expression of miR-378 was regulated in DOX-induced cardiotoxicity *in vivo*.

Preservation of mitochondrial integrity is essential for maintaining energy production and preventing cell death. Mice genetically lacking miR-378 have enhanced mitochondrial fatty acid oxidation [20]. Our results showed that overexpression of miR-378 could decrease LDH levels of cardiomyocytes, which were enhanced with DOX treatment (**Figure 2**). This suggested that miR-378 is related to energy metabolism of mitochondria. Next, we investigated the target genes of miR-378 and identified LDHA as a target. When overexpressed, miR-378 reduced the expression level of LDHA. The results made it apparent that LDHA is the target gene of miR-378. In the glycolytic enzymes, LDHA is unique as it is essential to maintain the rapid regeneration of NAD+ [21].
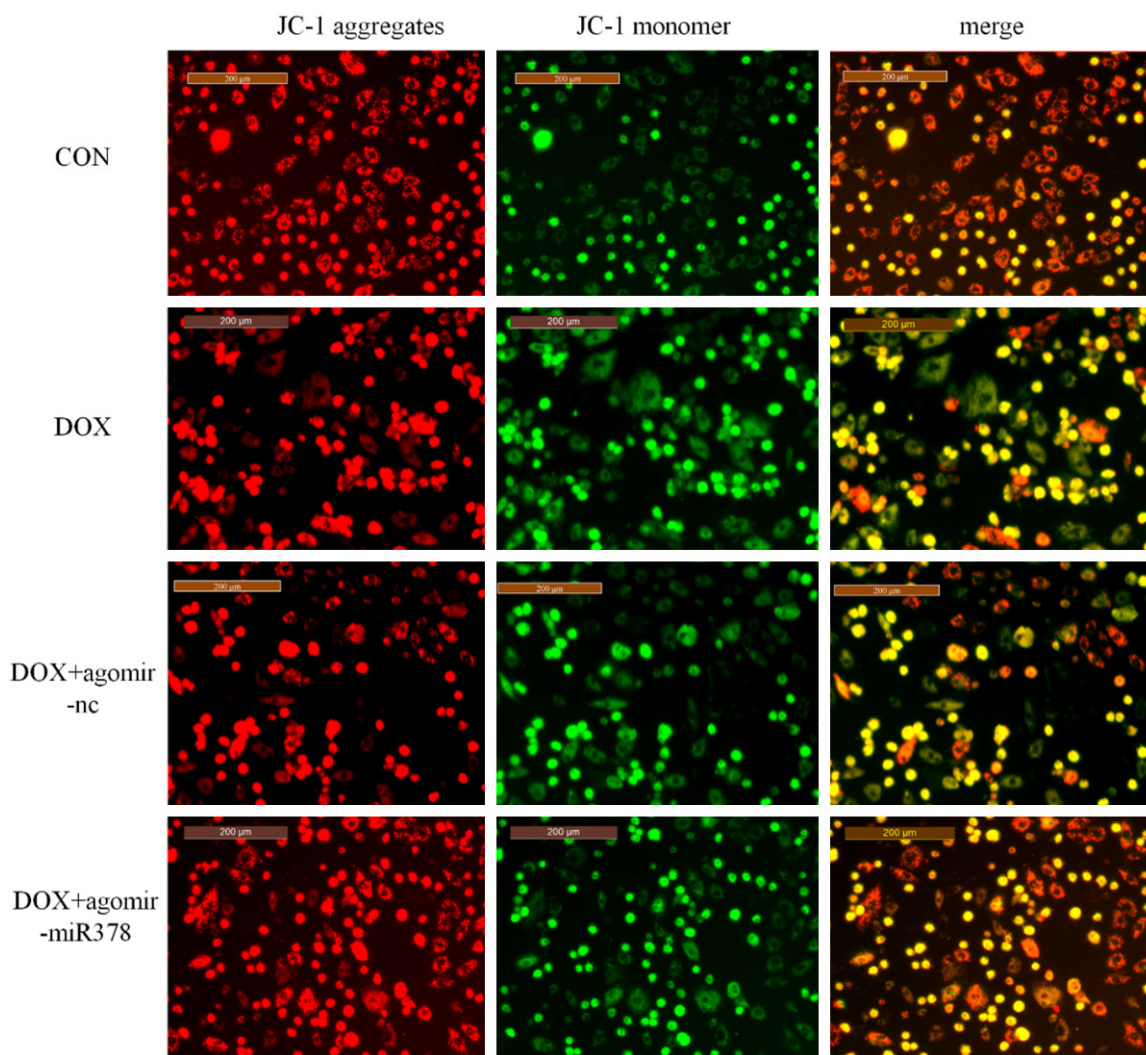
Figure 4. miR-378 was an anti-apoptotic factor in cardiomyocytes. (A) Expression levels of PPIA in cardiomyocytes of CON and DOX within/out miR-378 (n = 3, ***P < 0.001 vs. control; ###P < 0.001 vs. DOX). (B-E) Expression levels of GRP78 (B), ATF4 (C), CHOP (D), and the ratio of Bax/Bcl-2 (E) in cardiomyocytes of CON and DOX within/out miR-378 by Western blotting (n = 3, ***P < 0.001 vs. control; ###P < 0.001, ##P < 0.01 vs. DOX).

Figure 5. miR-378-overexpressing cardiomyocytes inhibited mitochondrial membrane potential Δψm. Mitochondrial membrane potential was examined by JC-1 analysis.

Inhibition of LDHA activity enhanced mitochondrial respiration and decreased mitochondrial membrane potential, leading to apoptosis [22]. LDHA was inhibited by miR-378 in our results. It limits the conversion of pyruvate into lactate, resulting in inhibited oxidative phosphorylation and preventing maximized ATP production [23]. The mitochondrial depolarization is often cited as an early indicator of the onset of apoptosis. We used JC-1 dye to analyze Δψm. In our results, the green-to-red ratio of cardiomyocytes markedly increased in DOX treatment, indicating the loss of Δψm in cardiomyocytes induced by DOX, while miR-378 inhibited this phenomenon.

Some reporters have revealed that miR-378 inhibited apoptosis. The overexpression of miR-378 could promote MSC survival and vascularization under hypoxic-ischemic conditions by inhibiting apoptosis [11]. MiR-378 targeting CLU and down-regulating Bcl-2, pCas-3, pErk1/2, and pAkt has also been reported [10]. In the present research, PPIA was considered to be one of the target genes of miR-378. Mitochondrial injury could induce ROS, ROS-stimulated cyclophilin A (PPIA) secretion, and then inhibition apoptosis [24]. Down-regulation of PPIA could induce UPR, an inductor of ER stress [9]. In the present results, the overexpression of miR-378 inhibited the expression of PPIA. ER stress related proteins were also analyzed. The overexpression of miR378 significantly inhibited the increase of GRP78 and ATF4 induced by DOX. It also decreased the ratio of Bax/Bcl-2, which suggested mitochondrial-induced apop-
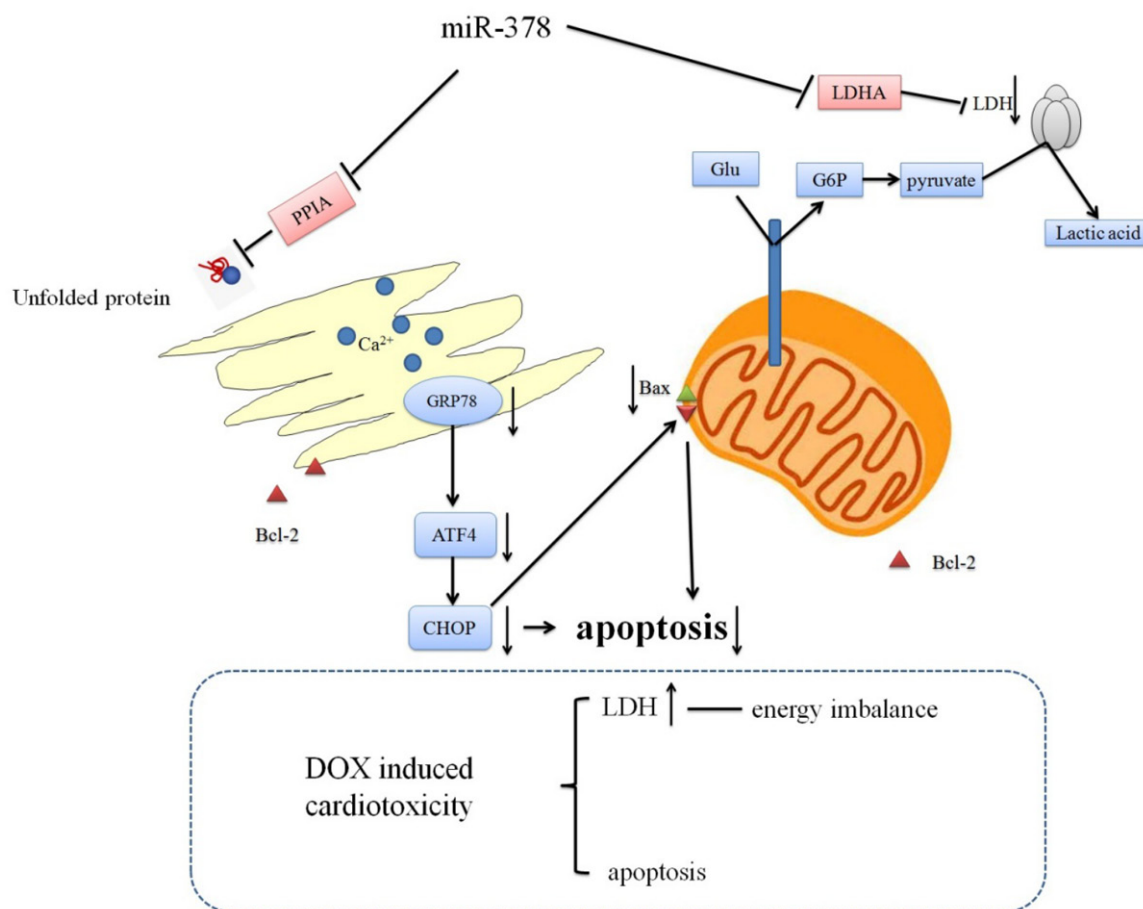
Figure 6. Schematic diagram of miR-378 regulatory effects in cardiomyocytes following DOX exposure.

tosis was inhibited and CHOP downregulated in overexpressed miR-378 conditions to relieve ER stress and mitochondrial-induced apoptosis.

Conclusions

In summary, the present work identified that miR-378 inhibits DOX-induced cardiotoxicity *in vitro*. Furthermore, we revealed that miR-378 targets LDHA to regulate energy metabolism and targets PPIA to regulate apoptosis (**Figure 6**). Our work provides a novel molecular pathway for regulating cardiotoxicity in the heart. It highlights miR-378 as a promising candidate for overcoming the cardiotoxicity of DOX-based chemotherapy.

Acknowledgements

Disclosure of conflict of interest

None.

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