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RAPID COMMUNICATION

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Protective effect of miR378* on doxorubicin-induced cardiomyocyte injury via calumenin

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Doxorubicin (Dox) is a highly effective antitumor antibiotic, however myocardial toxicity severely limits its use clinically. The pathogenesis of doxorubicin-induced cardiomyopathy is unclear. In Dox cardiomyopathy mice, there is a decline in cardiac function, a change in myocardial pathology and a reduction in miR378* expression. Expression changes in calumenin, an endoplasmic reticulum stress (ERS) chaperone protein and pathway factor, as well as apoptosis, were observed in cardiomyocytes after doxorubicin-induced injury. However, miR378* increased calumenin expression, eased ERS, and reduced cardiomyocyte apoptosis, while, silencing miR378* reduced calumenin expression, aggravated ERS, and increased cardiomyocyte apoptosis. The above results indicate that miR378* alleviates ERS and inhibits the activation of the ERS-mediated apoptosis signaling pathway in cardiomyocytes via regulating calumenin expression, thereby reducing cardiomyocyte apoptosis after doxorubicin-induced injury. Increasing miR378* expression may be a new way to improve cardiac function and quality of life in patients with Dox cardiomyopathy.

KEYWORDS
apoptosis, calumenin, doxorubicin, ERS, miR378*

1 | INTRODUCTION

Doxorubicin (Dox), an anthraquinone antibiotic, is highly effective and widely used. Unfortunately, cumulative, dose-dependent myocardial toxicity, leading to irreversible myocardial damage that can ultimately result in congestive heart failure, severely limits the clinical use of Dox (Lipshultz et al., 2005). The pathogenesis of cell injury induced by Dox is not yet clear, but appears to be closely related to the apoptosis of

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cardiac muscle cells (Senju et al., 2007; Sun et al., 2016). The mechanism of apoptosis is complex, and endoplasmic reticulum stress (ERS)-induced cell apoptosis is a relatively newly discovered signal transduction pathway (Xin, Li, Lu, Niu, & Cai, 2011).

calumenin is a calcium-binding protein that regulates calcium homeostasis in mammalian cardiac muscle cells. Sahoo and Kim (2010) pointed out that calumenin inhibits SERCA2 activity and the calcium release channel, RyR2, to regulate the release, uptake, and storage of calciumin in cardiac muscle cells, maintain calcium circulation homeostasis, and regulate the contraction and relaxation of cardiac muscle cells. In previous studies, it was reported that calumenin alleviates ERS and reduces ERS-mediated apoptosis in cardiac myocytes (Yu et al., 2017).

MicroRNAs (miRNAs) are non-coding single stranded RNA molecules containing 22 nucleotides. MiRNAs promote degradation of target mRNA or inhibits target mRNA expression after transcription via the incomplete complementation in the 3′ non-coding region (Owen et al., 2015). The miRNA precursor has a hairpin structure and the miR378-378* hairpin is highly expressed in cardiac cells. The miR378-378* is produced during the process of maturation when miR378 and miR378* are formed (Lee, Deng, Wang, & Yang, 2007). However, the functions of miR378 and miR378* in the heart have not yet been described. In breast cancer cells, the miR378-378* hairpin is overexpressed and miR-378* is more abundant than miR-378, which promotes glycolytic metabolism instead of mitochondrial oxidative phosphorylation and increases lactate production; a phenomenon known as the Warburg effect (Eichner et al., 2010). MiR378* decreases calumenin expression in H9c2 cardiac muscle cells, but there was no further study (Mallat et al., 2014). The aim of the study is to evaluate whether miR378* regulates ERS and ERS-mediated cardiomyocyte apoptosis via calumenin expression a Dox-injured myocardial cell model.

2 | MATERIALS AND METHODS

2.1 | Animals and drug preparation

Neonatal SD rats (1- to 3-day-old) and SD rats were purchased from the Jilin University Basic Medical School Animal Center (license number: scxk (Ji) 2011–0004); Type II collagenase was purchased from Thermo Fisher Scientific (Waltham, MA), trypsin was purchased from Beyotime (Shanghai, China); and super m-mlv reverse transcriptase was purchased from BioTeke (Beijing, China). The RNA simple total RNA Kit was obtained from Tiangen and the primers were synthesized by SANGON Biological Engineering (Shanghai, China). Lentiviral transfection plasmids were synthesized by the Shenyang WANLEI Life Science Company (Shenyang, China). Dox was obtained from Shenzhen Wandong Pharmaceutical (Shenzhen, China). Animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory animals and all study protocols were approved by the Institutional Animal Care and Use Committee of Inner Mongolia University for the Nationalities (NM-Y-2016-03-13-15-003).

2.2 | Animal model

A total of 20 normal male SD rats, weighing 200 g, were randomly divided into two groups (n = 10) as follows: a control group and a model group. Rats in the model group were given 2 mg/kg Dox via intraperitoneal injection (i.p) once/week, for 4 weeks). Rats in the control group were given the same amount of 0.9% NaCl solution, administered as above.

2.3 | Echocardiography

After 4 weeks of treatment, each rat underwent transthoracic echocardiography examination using GE Vivid E9 color ultrasound with a transthoracic high frequency ultrasound ML6-15 probe (13 MHZ frequency and 3.5 cm image depth). Rats were then anesthetized via i.p. injection of 0.3 ml/100g 2% pentobarbital sodium, guided by two-dimensional ultrasound. M type ultrasound was used to measure interventricular septal thickness at diastole (IVSd), left ventricular internal diameter at en D-systole (LVIDs), and left ventricular diastolic end diameter (LVIDd). Each measured value was calculated using the average values of four consecutive and complete cardiac cycles.

After high frequency echocardiography and hemodynamic examination, the rats were euthanized and the heart was exposed and removed. The upper 1/3 of cardiac tissue was used for HE staining.

2.4 | Isolation and culture of neonatal SD rats cardiomyocytes

The heart was isolated from each neonatal SD rats and washed repeatedly three times with PBS. The heart tissue was then cut into 1–3 mm^3 pieces. A 0.1% mixture of type II collagenase and 0.1% trypsin was then added and the pieces were digested at 37 °C for 25 min. The tissue was then placed in a 15 ml tube, centrifuged at 1,500 r/min for 7 min and the supernatant discarded. The precipitate was suspended in PBS, pipetted repeated, and centrifuged at 1,000 r/min for 5 min. The cells were then resuspended in culture medium and a 200 mesh screen was used to remove chunks of tissue. The cells were washed twice with PBS, centrifuged at 1,000 r/min for 10 min, the supernatant was removed and the precipitate was preserved. The cells were again resuspended in 1 ml culture medium, placed onto a culture plate and counted. Because the adherent speed of fiber cells is faster, a large number of fiber cells can be removed in 2 hr, thus pure myocardial cells remained in the supernatant suspension. Cells were placed on a culture plate for 24 hr. The cells were cultured to a density of about 90% and then washed 2 times with PBS. The cells were then placed in 6-well plates at a density of 5×10^4/ml and the plates were incubated overnight at 37 °C and 5% CO_2. After 24 hr the cells were transfected. Transfecting the target cells with lentivirus. After transfecting for 24 hr, medium containing lentivirus was replaced with complete medium. Then cell were treated with 3 mg/L DOX with 24 hr, collected, and used for subsequent experiments.

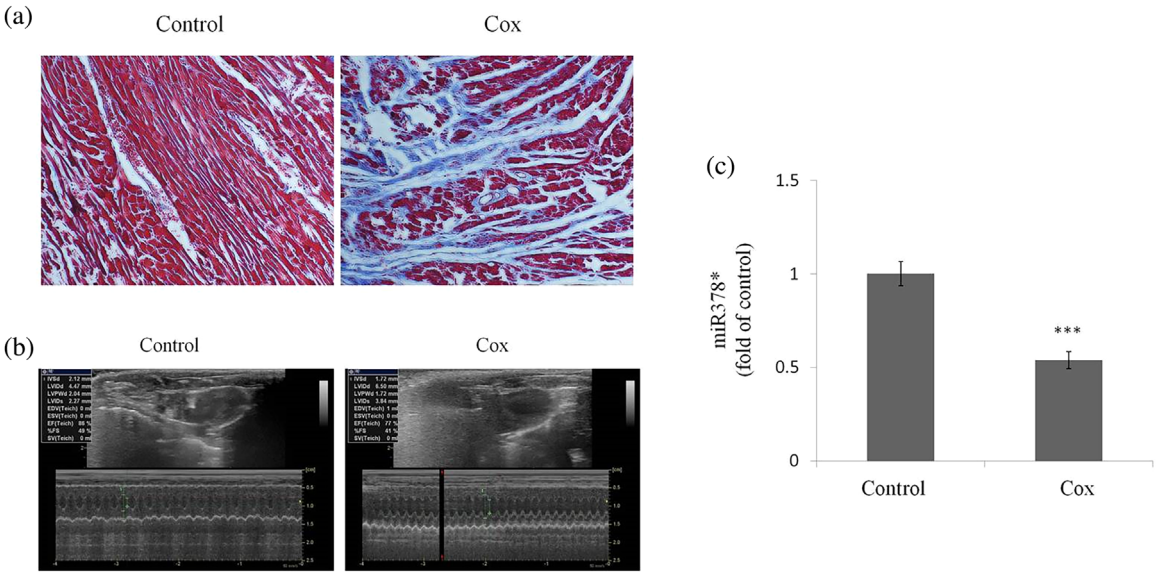

FIGURE 1 miR378* is downregulated in Dox-induced myocardial injury in rats. (a) The histological changes in cardiac tissues (100 × magnification). (b) Changes in high frequency echocardiography in rat cardiac tissue. (c) The expression of miR378* was decreased in the myocardium of rats treated with Dox (n = 10 for each group, ***p < 0.001 vs. control)

2.5 | TUNEL assay

Following the manufacturer's instructions, DNA fragmentation of apoptotic cells was detected by TUNEL staining and the number of apoptotic cells, as defined by chromatin condensation of nuclear fragmentation (apoptotic bodies) was counted.

2.5.1 | Real-time PCR

The RNA of cardiomyocytes was isolated by RNA extraction kit (TIANGEN, Beijing, China), and then the RNA was transcribed. The obtained cDNA was used for real-time PCR.

The primers of calumenin, GRP78, PERK, eIF2a, GAPDH gene fragments were designed following::

calumenin-F: 5′ ACACTTTCTCAATCCCTTACC 3′
calumenin-R: 5′ CTGGGCCTGTGACAACTG 3′
GRP78-F: 5′ GATAATCAGCCCACCGTAA 3′
GRP78-R: 5′ TTGTTTCCTGTCCCTTTGT 3′
PERK-F: 5′ F TCAGCAAGCCAGAGGTGTT
PERK-R: 5′ R GGAAGATTCGAGCAGGGAC
eIF2a −F: 5′ TGGAGCATGTTTGAAATCG
eIF2a −R: 5′ GGTTGGGTTCCAGGTGATA
GAPDH F: 5′ CGGCAAGTTCAACGGCACAG 3′
GAPDH R: 5′ CGCCAGTAGACTCCACGACAT 3′

Amplification was performed in duplicate on FTC-3000 Real-Time PCR system thermocycler by using SYBR Green PCR Master Mix (TIANGEN). The reaction condition was 95 °C for 15 min and following 40 cycles: denaturation (95 °C for 10 s), annealing and elongation (60 °C for 60 s). The relative mRNA expression level of the gene was normalized to the level of GAPDH in the same sample.

2.5.2 | Western blot analysis

Western blot was performed as described previously (Wang et al., 2017). Atibodies for anti-calumenin (1:500) was obtained from Bioss (Beijing, China) and anti-GRP78 (1:400), anti-PERK (1:400), anti-p-PERK (1:400), anti-eIF2a (1:400), anti-p-eIF2a (1:400) were purchased from Boster (Wuhan, China). Protein was extracted and mixed in loading buffer, and then equal amounts were fractionated on gel and transferred onto Hybond-C Extra nitrocellulose membrane using a semidry transfer apparatus. Last the protein was blocked with nonfat dry milk, added antibodies, and detected with supersignal west pico chemiluminescent substrate.

2.6 | Statistical analysis

Experimental results are shown as mean ± SEM. SSPS11.5 statistical analysis software was used for data analysis, paired t test was used to compare different groups. The number of stars (*/#) indicate the p value range: *p-value <0.05, **p-value <0.01, ***p-value <0.001; #p < 0.05, ##p < 0.01, ###p < 0.001.

3 | RESULTS

3.1 | miRNA378* is regulated in Dox induced cardiotoxicity

To determine the cardiotoxicity induced by Dox, changes in cardiac structure and function were examined in SD rats treated with doxorubicin. After 10 weeks of treatment, the hearts from rats in the Dox-treated group revealed the formation of cytoplasmic vacuoles and myofibrillar loss when compared with control group rats

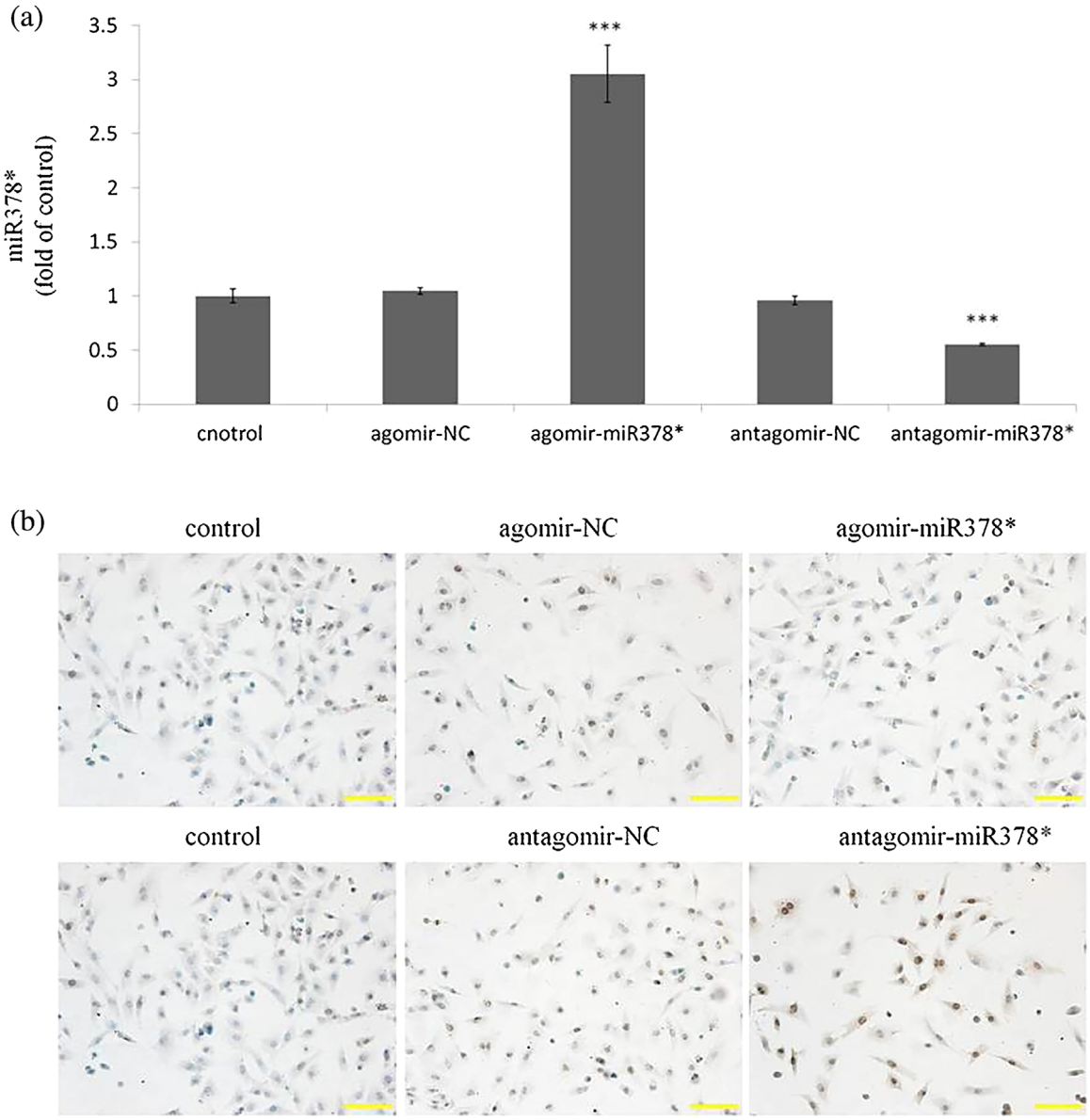

FIGURE 2 miR378* relieved Dox-induced cardiotoxicity by apoptosis. (a) Overexpression or knockdown of miR378* in neonatal SD rats cardiomyocytes ($n = 3$, ***$p < 0.001$ vs. control). NC: negative control. (b) upregulation of miR378* attenuated the apoptosis of neonatal SD rats cardiomyocytes

(Figure 1a). Left ventricular posterior wall thickness of end-diastolic (LVPWd), interventricular septal thickness (IVSd), ejection fraction (EF), and %FS were significantly lower in the DOX group than in the control group (Figure 1b). A high expression of miR378-378* hairpin in cardiac cells has been reported. In this study, miR378* expression was analyzed by real-time PCR. Validation experiments confirmed that miR378* was down-regulated in cardiac injury induced by Dox (Figure 1c).

3.2 | Overexpression of miR378* attenuated Dox-induced myocardial apoptosis

To explore the role of miR378* in myocardial injury, neonatal SD rats cardiomyocytes were transfected with agomir-miR378* and antagomir-miR378* to overexpress and knock down miR378*. The expression of miR378* was analyzed by QPCR (Figure 2a).

As shown in Figure 2, Dox treatment increased cell apoptosis and myocardial injury. In contrast, over-expression of miR378* attenuated the increase in cell apoptosis induced by Dox (Figure 2b). However, down-regulation of miR378* expression did not accelerate the increase in cell apoptosis in other groups compared to that induced by Dox.

3.3 | calumenin may be the target of miR378*

During previous research, it was reported that miR378* decreased the expression of calumenin in H9c2 myocardial cells (Mallat et al., 2014). Real-time PCR and Western blotting analysis were carried out to investigate the effects of miR378* on both calumenin mRNA

and protein expression after injury induced by Dox. The yield showed that the mRNA and protein levels of calumenin were increased when miR378* was over-expressed after treatment with Dox (Figures 3a and 3b), while the protein level decreased when miR378* was down-regulated after treatment with Dox (Figure 3b), which indicated that miR378* regulates the expression of calumenin in neonatal SD rats cardiomyocytes after Dox-treatment.

3.4 | miR378* inhibited Dox-induced ER stress by calumenin

Previous research has demonstrated that calumenin prevents cell apoptosis by inhibiting ER stress (Wang et al., 2017). In the current study the effect of miR378* on ER stress induced by Dox was investigated. As expected, overexpression of miR378* significantly inhibited the increase of GRP78, p-PERK, and p-eIF2a in myocardial

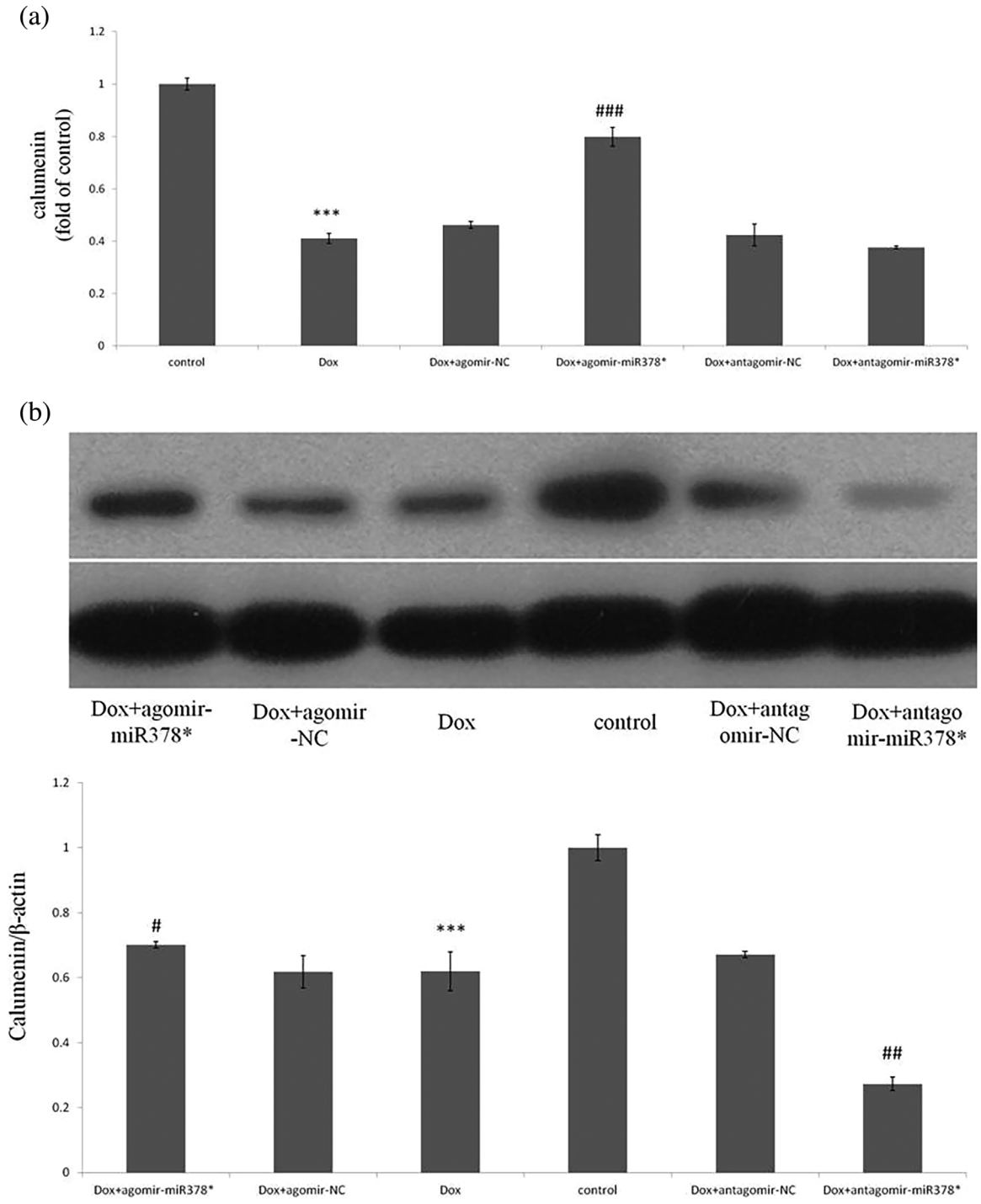

FIGURE 3 Calumenin is the target gene of miR378*. (a/b) Levels of calumenin mRNA and protein were upregulated in Dox-induced cell apoptosis ($n = 3$, ***$p < 0.001$ vs. control; ###$p < 0.001$, ##$p < 0.01$, #$p < 0.05$ vs. Dox). NC, negative control

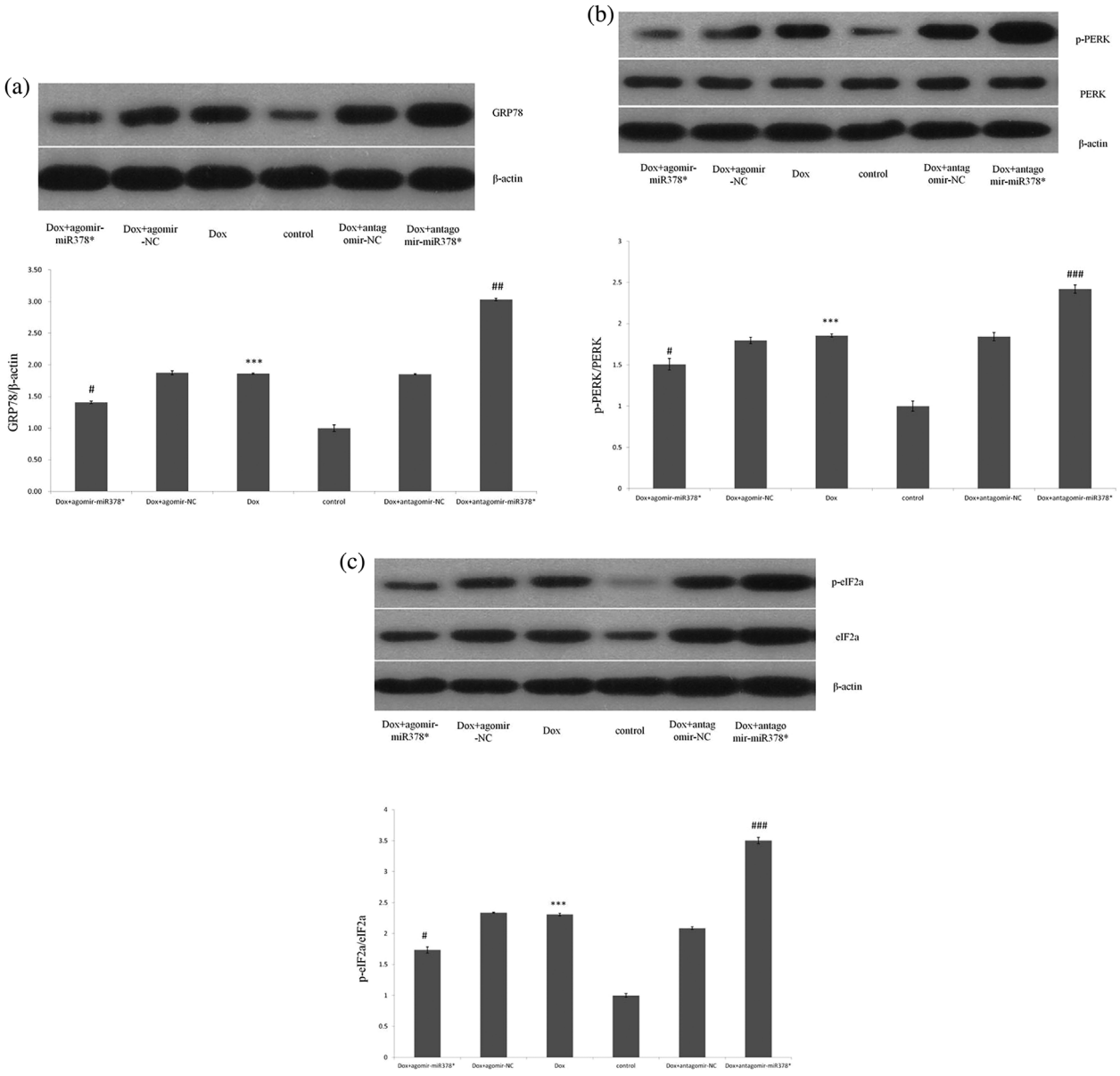

FIGURE 4 miR378* regulates Dox-induced ER stress in neonatal SD rats cardiomyocytes. (a/b/c) The phosphorylation of p-PERK, p-eIF2a and expression of GRP78 were observed using Western blotting ($n = 3$, ***$p < 0.001$ vs. control; ###$p < 0.001$, ##$p < 0.01$, #$p < 0.05$ vs. Dox). NC: negative control

cells injured by Dox (Figure 4). However, down-regulation of miR378* enhanced the expression of GRP78, p-PERK, and p-eIF2a in neonatal SD rats cardiomyocytes injured by Dox.

4 | DISCUSSION

In this study, it was demonstrated that miR378* might affect the ERS and ERS-mediated cell apoptosis through regulating the expression of calumenin. The real-time PCR results were consistent with the results of the Western blot, which adds to the precision of this study. Discussing the pathogenesis of cardiac disease on a genetic level may provide new treatments for Dox-induced myocardial injury.

In this study, the cardiac function declined, pathology of cardiac muscle changed significantly, and expression of miR378* decreased, after Dox treatment, indicating that a change in miR378* expression may be involved in the progression of Dox-induced cardiomyopathy. In order to clarify the effect of miR378* in the progression of Dox-induced cardiomyopathy, myocardial apoptosis was first analyzed. Cell apoptosis is an active programmed cell death mode under the control of the self-gene and is different from the physiological death process of cell necrosis (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). Myocardial cell apoptosis is involved in many cardiovascular diseases (Chen et al., 2014; Fang et al., 2012). In this study, the number of Dox-injured myocardium cells that underwent apoptosis increased significantly and miR378* significantly reduced the apoptosis number of Dox-injured cardiomyocytes.

It is important to explore the molecular biological mechanism of miR378* involved in protecting myocardial cells from Dox injury. calumenin is a calcium ion (Ca^{2+}) binding protein that belongs to many EF-hand structures in the CREC family in mammalian cardiomyocytes (Mazzorana, Hussain, & Sorensen, 2016). Studies have reported that miR378* inhibits calumenin's expression in H9C2 cardiomyocytes (Mallat et al., 2014). The rich expression of calumenin in human cardiomyocytes suggests that calumenin may be closely related to miR378*. In order to explore whether there is a regulatory relationship between the two, the change of calumenin expression by over-expressing and silencing miR 378* was examined. It was found that expression of miR378* promoted calumenin's expression, while silencing miR378* significantly reduced calumenin's expression, suggesting that miR378* may inhibit the apoptosis of myocardial cells by regulating calumenin's expression.

To further explore the protective mechanism of miR378* on Dox-injured myocardial cells, the expression changes of plasma reticulated stress partner protein GRP78 and the expression of its mediated apoptosis signaling pathway factor PERK, eIF2 alpha, were examined. Cardiomyocyte apoptosis induced by ERS is a relatively newly discovered signal transduction pathway (Okada et al., 2004). The expression of GRP78 was increased after calumenin was injured by Dox in the myocardium, the expressions of ATF6 and IRE1 were increased (Zhu et al., 2015), and myocardial apoptosis was increased (Lakshmanan et al., 2013). In this study, it was found that miR378* relieved ERS and inhibited the expression of the apoptosis signaling pathway, thus reducing the apoptosis of Dox-injured myocardial cells.

In conclusion, miR378* may reduce myocardial apoptosis by increasing calumenin expression, relieving ERS, and inhibiting the activation of ERS-apoptosis signaling factor in myocardial cells damaged by Dox. Increasing the expression of miR378* may be a new way to improve cardiac function and quality of life in patients with Dox cardiomyopathy.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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