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Influences of ALDH2 on Cardiomyocyte Apoptosis in Heart Failure Rats Through Regulating PINK1-Parkin Signaling Pathway-Mediated Mitophagy

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ABSTRACT

The study aimed to investigate the influences of aldehyde dehydrogenase 2 (ALDH2) on cardiomyocyte apoptosis in heart failure (HF) rats through regulating the PTEN induced putative kinase 1 (PINK1)-Parkin signaling pathway-mediated mitophagy. The rat model of HF was established, and the rats were randomly divided into model group (HF model, n=20) and ALDH2 group (intervention with ALDH2, n=20), with a normal group (n=20) set. After successful modeling, MRI and ECG were applied to detect the cardiac function indexes of the rats. The myocardial function index creatine kinase (CK) was measured, the status of myocardial tissue injury was determined using hematoxylin and eosin staining, and the apoptosis was observed via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The activity of ALDH2 was detected, and the expression levels of genes and proteins were measured through quantitative polymerase chain reaction (qPCR) and Western blotting assay. The model group had notably decreased fractional shortening (FS) and ejection fraction (EF) and remarkably increased left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) compared with the normal group (p<0.05). The activity of ALDH2 declined obviously in the model group. The myocardial tissue injury was severer in the model group, and the number of apoptotic cells in myocardial tissues was greater in the model group than that in other groups (p<0.05). The model group manifested higher expression levels of Caspase-3 and light chain 3 (LC3) than the ALDH2 group (p<0.05) but significantly lower expression levels of PINK1, Parkin and B-cell lymphoma-2 (Bcl-2) (p<0.05). In comparison with those in the model group, the protein expression levels of PINK1, Parkin and Bcl-2 in myocardial tissues were prominently higher in the ALDH2 group (p<0.05). ALDH2 can inhibit cardiomyocyte apoptosis in HF rats by activating the PINK1-Parkin signaling pathway-mediated mitophagy, which is conducive to the recovery of HF.

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Introduction

Heart failure (HF), caused by cardiac abnormalities, can result in decreased cardiac output and/or increased ventricular pressure, whose etiologies can be divided into three categories, namely diseased myocardium, abnormal load and arrhythmia. With the high prevalence rate and long survival time of HF patients, this chronic disease is becoming relatively common among adults, with a high death rate. The prevalence rate of HF among adults is about 1-2% in developed countries (1, 2). There is evidence that a majority of patients with cardiovascular diseases, especially those with HF, are the aging population, and the prevalence rate of HF among people aged >75 years old is 8.4% (3). Apparent cardiomyocyte apoptosis and cell loss induced are observed in the process of senescence, which causes a persistent decline in ventricular function, finally leading to fatal dilated cardiomyopathy. Therefore, anti-apoptosis may be a potential and beneficial prevention and treatment strategy for heart diseases in elderly people (4). Increased oxidative damage and accumulated mitochondrial dysfunction have been observed in many heart diseases. Mitochondria participate in maintaining cellular homeostasis ranging from energy production to regulation of reactive oxygen species (ROS) signal and intra-cellular death pathway (5). Mitochondrial dysfunction is widely associated with age-related heart diseases, and mitochondrial injury, as an early event of HF, has become a feature of cardiac aging (6). It has been proven that cardiomyocyte apoptosis occurs in multiple

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cardiovascular diseases. An experimental model of HF showed that increased cardiomyocyte apoptosis and cardiac hypertrophy are detected in patients with advanced HF (7). Mitochondrial fission mediates the cardiomyocyte apoptosis in hypertensive cardiomyopathy (8). However, the specific mechanism remains elusive. Currently, mitochondrial morphology is regarded as an important determinant of the energy status in mitochondria. The mitochondria are subjected to fusion and fission constantly, which is crucial to the fidelity of organelles (9). The cardiomyocyte apoptosis is a common pathological manifestation of such heart diseases as ischemic heart disease, cardiomyopathy and HF. It can induce declined pump function, serious HF and even death in some cases (10). Cardiomyocyte apoptosis has diversified functions and complex activation mechanisms, and it is a result of various programmed pathways that ultimately lead to cardiomyocyte death (11).

As a mode of programmed cell death, autophagy is a process of self-degradation regulated by cellular contents and a vital pathway for maintaining cellular homeostasis (12). It refers to a metabolic pathway in which the excess impaired or misfolded proteins and organelles in cells are finally delivered to the lysosomes for degradation by means of autophagic degradation. Harmful substances in cells can be cleared by autophagy which reacts relevantly to the invasion of cell bodies, and autophagic response is initiated rapidly in case of lethal threats. As a defender of the body, autophagy can maintain cell stability (13). No conceptual summary of autophagy was proposed at first, but the phenomenon has become one of the hotspots of studies in the biological field following apoptosis under the current international trend. Autophagy can regulate the metabolic process of nerve cells under stress, not only protecting the cell survival but also serving as a death mode. However, the mechanism of autophagy in physiological metabolism in organisms has not been completely defined at present, but the elaborated mechanisms of action and routes can serve as important guidelines for related clinical diseases such as tumor and infectious neuropathy (14, 15). There is a close relationship between autophagy and apoptosis, and different associations to be explored are produced due to changes in stimulating factors and variations in test environments. The apoptosis is triggered on the premise of autophagy which plays an assistant role in cell apoptosis. In addition, autophagy can delay the occurrence of apoptosis and inhibit cell apoptosis as an antagonist, thus protecting cell survival and increasing the survival rate. Autophagy and apoptosis can also work as alliances to induce cell death in a coordinated way. Both cell autophagy and apoptosis maintain the dynamic balance in the body and manifest very complex relations (16, 17). Moreover, they can contain each other, that is, the inhibition on autophagy can promote apoptosis, while the activation of autophagy can repress apoptosis (18). There is an intricate association between the mechanisms of autophagy and apoptosis, but the mutual regulatory mechanism has not been completely clarified yet, which still needs to be explored by in-depth trials.

The PTEN-induced putative kinase 1 (PINK1)/Parkin is a signaling pathway that mediates mitophagy (19). The mitochondria are the most abundant in the heart, whose dynamic balance is destroyed during a cardiomyopathy attack. Nevertheless, the exact functions of the PINK1/Parkin signaling pathway and mitophagy regulated in cardiomyocyte injury have not been elaborated so far. This research aims to investigate the role of aldehyde dehydrogenase 2 (ALDH2) in cardiomyocyte apoptosis and its impacts on the PINK1-Parkin signaling pathway in HF rats, but there are few studies on whether the PINK1-Parkin signaling pathway is involved in the pathogenesis of HF in rats and regulates cardiomyocyte apoptosis. Therefore, in order to investigate the potential role of the PINK1/Parkin in cardiomyocyte apoptosis in HF rats, in-vivo experiments and multiple molecular biological techniques were adopted in this research to elaborate its impacts on cardiomyocyte apoptosis in HF rats. The levels of cardiac function indexes and expressions of pathway-related proteins in the rat model of HF were detected after intervention with ALDH2, so as to provide important experimental support for the treatment of HF with ALDH2, thus offering theoretical and experimental references for the treatment of cardiomyocyte apoptosis in HF rats with similar drugs in subsequent studies.

Materials and methods

Establishment of animal model

After adaptive feeding, a total of 40 Sprague-Dawley rats were used to establish the HF model via intraperitoneal injection of adriamycin (3 mg/kg), while those in the normal group were intraperitoneally injected with an equal volume of normal saline. Then 20 rats were selected from the model group and injected with ALDH2 for treatment. The clinical manifestations of all the rats were observed regularly every day. The changes were timely recorded in detail, and at 48 h after the last medication, the blood and tissue samples were collected and preserved for subsequent experiments. A portion of cardiac tissue was used for hematoxylin and eosin (HE) staining, and the other portion was stored at -80°C for the measurement of the expression levels of genes and proteins. All the animals in this research were fed under standard conditions and provided with water and food at any time. All the animal experiments were conducted according to the clauses of the Animal Protection Law and approved by the Laboratory Animal Committee. The study was approved by the ethics committee of Zhongshan Hospital affiliated to Fudan University.

Detection of myocardial function

Abnormalities of the myocardial function will occur in the case of HF, so the detection of creatine kinase (CK), a myocardial function index, can provide a critical reference for the early diagnosis and prediction of HF. The venous blood samples were drawn routinely, centrifuged at 4°C for 10 min and separated to collect the serum, followed by an examination of indexes using a biochemical analyzer.

Measurement of physiological function indexes of rat heart

The left ventricular function of all the rats was measured through a Philips 7500 ultrasonic machine (Philips Healthcare, Amsterdam, Nederland), MRI and ECG systems. Each rat to be checked was fixed in the supine position, and the electrocardiogram examination (probe frequency: 10 MHz) was performed, including left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), ejection fraction (EF) and fractional shortening (FS), in accordance with the specific instructions of the instruments.

Detection of ALDH2 activity

The ALDH2 activity in tissues can be determined by the OD value at the wavelength of 340 nm. The myocardial tissues of the rats were taken under sterile conditions and broken using a homogenizer containing prepared tissue lysis buffer after being flushed clean. Subsequently, the tissues were centrifuged and separated to collect the supernatant, and the absorbance in each group was measured using a 200 μL reaction system and a microplate reader. Finally, the curves were plotted, and the ALDH2 activity was calculated according to the formula.

HE staining

The cardiac tissues were fixed in 10% neutral buffered formalin for 7 d, washed with running water for 24 h and dehydrated with graded alcohol. Later, the tissues were sliced into 5 μm-thick conventional sections using a microtome. After deparaffinization, the sections were hydrated in ethanol with gradually decreased concentrations, followed by clearing, dipping and embedding in paraffin. Next, the paraffin-embedded blocks were used to prepare pathological sections. Finally, the thin sections were baked dry for HE staining, followed by mounting and histological observation under a light microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay

The paraffin-embedded sections prepared were utilized to determine the cardiomyocyte apoptosis by virtue of the TUNEL apoptosis assay kit (Roche). After fixation, rinsing, permeabilization with 0.1% Triton X-100 and preparation of paraffin-embedded sections, the mounted section samples were subjected to labeling reactions using a fluorescent chromogenic reagent. After that, the FITC-labeled TUNEL-positive cells at the wavelength of 530 nm were challenged with 488 nm fluorescence under a fluorescence microscope, which was counted in 10 fields of vision later.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen) was applied to extract the total ribonucleic acid (RNA) in the myocardial tissues of rats in each group. After meeting the purity and concentration, the total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) strands, with attention to the use of isopropyl alcohol. Later, the primer amplification was performed using a 20 μL system (2 μL of cDNA, 10 μL of Mix, 2 μL of primer and 6 μL of ddH_2O) for 40 cycles, and then PCR amplification was conducted. The primer sequences of target genes and internal reference β-actin were designed according to those on GenBank (Table 1). The expression levels of target genes were detected via qRT-PCR, and the mRNA expression levels in the myocardial tissues of rats in each group were calculated using the $2^{-\triangle\triangle Ct}$ method.

Western blotting assay

The cardiac tissues of the rats were cut into pieces, weighed and added with RIPA lysis buffer at a ratio of 100 mg: 1 mL for tissue homogenization. Then the total proteins in the myocardial tissues of rats in each group were extracted using strong lysate, and the concentration was measured via a BCA protein assay kit (Pierce). After that, samples and gel were prepared, the loading buffer was added for electrophoresis, and the proteins were transferred onto a membrane and sealed, followed by incubation with primary antibody overnight and secondary antibody. The freshly prepared ECL mixture was added for image development in a dark room, and then the bands were processed with software. The protein bands were scanned and quantified using an Odyssey scanner, and the level of proteins to be detected was corrected via GAPDH. Image Lab software was employed to quantify the bands of Western blotting. The expression levels of corresponding proteins in each group were calculated.

Statistical analysis

All the raw data recorded during experiments were processed using SPSS 20.0 software and were subjected to multiple comparisons. The experimental results obtained were presented as mean ± standard deviation ($\bar{\chi}$±SD), and $p<0.05$ suggested significant differences. The histograms were plotted by means of GraphPad Prism 8.0.

Results and discussion

Detection of myocardial function

The test result of the myocardial function index CK (Figure 1) showed that the CK content was increased markedly in the model group compared with that in the normal group ($p<0.05$), while it was decreased notably in the ALDH2 group ($p<0.05$), implying that the myocardial function index is increased significantly during the occurrence and development of cardiomyocyte apoptosis in HF, providing an important reference for early diagnosis.

Detection of rat's cardiac function indexes

According to Table 2, the model group had lower FS and EF but remarkably larger LVEDD and LVESD compared with the normal group ($p<0.05$), indicating that the rat model of HF was successfully selected.

HE staining for myocardium in each group

The results of HE staining manifested that the cardiomyocytes were arranged irregularly, and the myocardial fibers were thickened in the model group (Figure 2A). The HF-induced myocardial injury was alleviated after the treatment with ALDH2 (Figure 3B).

Apoptosis level of cardiomyocytes in rats detected via TUNEL staining

According to the results of TUNEL staining (Figure 3), there was almost no cardiomyocyte apoptosis in the normal group (Figure 3A) but massive apoptosis in model group (Figure 3B). After the treatment with ALDH2, cardiomyocyte apoptosis was reduced prominently in the ALDH2 group (Figure 3C).

Detection results of ALDH2 activity

The results of ALDH2 activity detected in each group are shown in Figure 4. The activity of ALDH2 declined obviously in the model group ($p<0.05$), but it was increased significantly in the ALDH2 group ($p<0.05$), basically close to that in the normal group.

Expressions of apoptosis- and pathway-related genes detected via RT-PCR

As shown in Figure 5, in the ALDH2 group, the expression levels of Caspase-3 and LC3 were lower (p<0.05), and those of Bcl-2, PINK1 and Parkin were prominently higher (p<0.05) than those in the model group, illustrating that the cardiomyocyte proliferation is promoted, while the apoptosis is repressed after the treatment with ALDH2.

Expressions of apoptosis- and pathway-related proteins detected via Western blotting

The expression levels of Bcl-2, PINK1 and Parkin proteins were elevated markedly in the ALDH2 group (p<0.05), while the opposite results of those expression levels were detected in the model group, suggesting that the cardiomyocyte apoptosis is repressed after the treatment with ALDH2 (Figure 6).

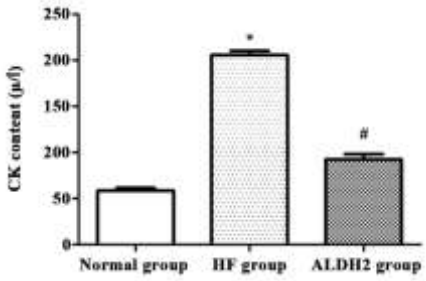

Figure 1. Results of biochemical examination. The CK content is decreased notably in the ALDH2 group, implying an abnormal myocardial function index. *p<0.05 vs. normal group, #p<0.05 vs. model group.

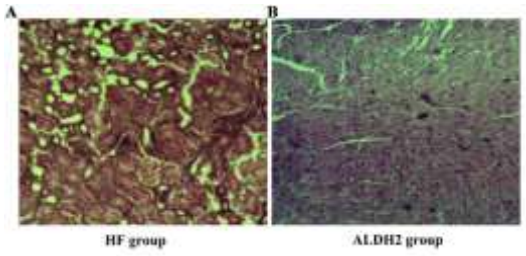

Figure 2. HE staining for rat heart. A: Model group (*10), B: ALDH2 group (*10). The cardiomyocytes are arranged irregularly, and the myocardial fibers are thickened in the model group, while the myocardial injury is hardly observed in the ALDH2 group.

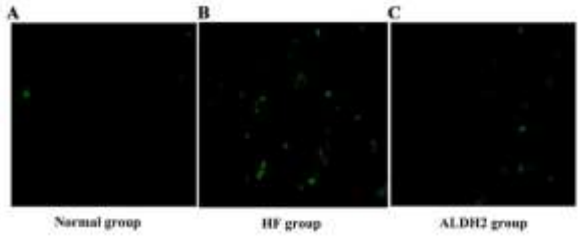

Figure 3. Apoptosis level detected via TUNEL staining. A: Normal group, B: HF group. C: ALDH2 group

There is massive cardiomyocyte apoptosis in the model group, which is reduced prominently in the ALDH2 group.

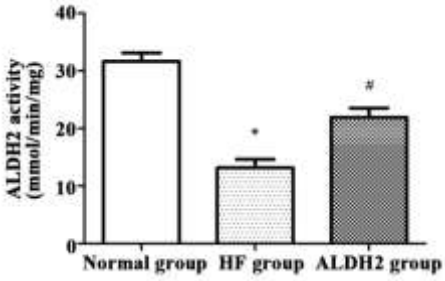

Figure 4. ALDH2 activity. The activity of ALDH2 declines obviously in the model group (p<0.05), but it is increased significantly in the ALDH2 group (p<0.05), basically close to that in the normal group. *p<0.05 vs. normal group, #p<0.05 vs. model group.

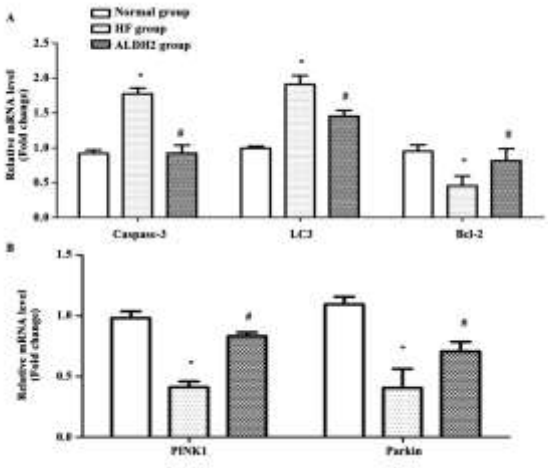

Figure 5. Expressions of apoptosis- and pathway-related genes. A: In the ALDH2 group, the expression levels of Caspase-3 and LC3 are lower (p<0.05), B: those of Bcl-2, PINK1 and Parkin are prominently higher (p<0.05) than those in the model group. *p<0.05 vs. normal group, #p<0.05 vs. model group.

Table 1. PCR primers

Target gene	Primer sequence
β-actin	F: 5'-CAGTGCCAGCCTCGTCTCAT-3' R: 5'-AGGGCCATCCACAGTCTTC-3'
Caspase-3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
B-cell lymphoma-2 (Bcl-2)	F: 5'-GGTGCTCTTGAGATCTCTGG-3' R: 5'-CCATCGATCTTCAGAAGTCTC-3'
light chain 3 (LC3)	F: 5'-ACATGAGCGAGTTGGTCAAG-3' R: 5'-GTTCATAGATGTCAGCGATG-3'
PINK1	F: 5'-CTTGGCATCCGCACTCTG-3' R: 5'-GTGAAGCCTGGCAACCTG-3'
Parkin	F: 5'-ACAAGCTTTTAAAGAGTTTCT-3' R: 5'-AGGCAATGTGTTAGTACACA-3'

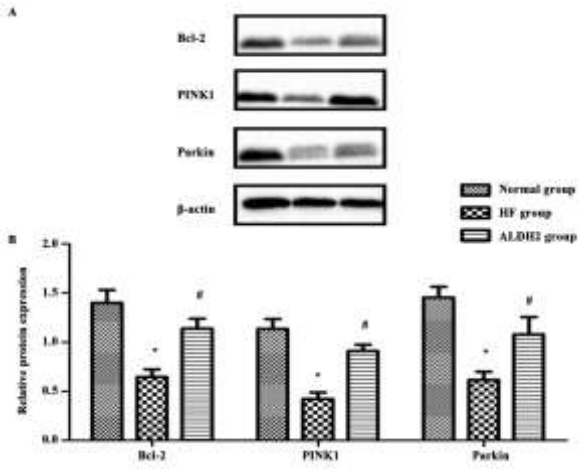

Figure 6. Expression levels of apoptosis- and pathway-related proteins. A: Western blot of protein expression. B: Quantification analysis of protein expression. The expression levels of Bcl-2, PINK1 and Parkin proteins are elevated markedly in ALDH2 group ($p<0.05$). *$p<0.05$ vs. normal group, #$p<0.05$ vs. model group.

Table 2. Detection of cardiac function indexes

Group	LVEDD (mm)	LVESD (mm)	EF (%)	FS (%)
Normal group	4.98±0.86	4.68±0.28	62.2±3.9	55.7±3.1
Modelgroup	9.45±0.15[a]	7.51±0.26[a]	47.6±3.6[a]	39.8±2.8[a]
ALDH2 group	5.64±0.23[b]	4.99±0.69[b]	59.4±2.9[b]	51.6±1.9[b]

Note: Model group has lower FS and EF but remarkably larger LVEDD and LVESD compared with the normal group. [a]$p<0.05$ vs. normal group, [b]$p<0.05$ vs. model group.

The deterioration of HF is a complicated process involving the adrenergic nervous system and neurohormone as well as a variety of biochemicals and signaling pathways, thus resulting in oxidative stress, abnormal mitochondrial function, cell apoptosis, autophagy, hypertrophy, fibrosis and inflammation (20). Different individuals will manifest varying susceptibility, prognosis and treatment response to HF even though the major clues to the etiology of HF are the same, which is probably associated with genetic variation (21). Cardiomyocyte apoptosis is a common pathological manifestation of heart diseases such as ischemic heart disease, cardiomyopathy and HF. It can lead to decreased cardiac function and even death sometimes. Besides, cardiomyocyte apoptosis has various functions and complex activation mechanisms, and it is a result of various programmed pathways that ultimately cause cardiomyocyte death. It has been confirmed that ALDH2 is closely correlated with the incidence rates of coronary heart disease, hypertension and diabetes in the Asian population, and there is growing evidence that it has potential effects on HF (22, 23). In this research, the rat model of HF was established to investigate the influences of ALDH2 on the cardiomyocyte apoptosis in HF rats through regulating the PINK1-Parkin signaling pathway-mediated mitophagy. The detection of the myocardial function index CK displayed that its content was increased markedly in model group, while it was decreased notably in the ALDH2 group, suggesting that the myocardial function index will be raised significantly during the occurrence and development of cardiomyocyte apoptosis in HF, thereby providing an important reference for early diagnosis. Additionally, the rheological indexes of the heart were observed, and it was manifested that the FS and EF were lower, but the LVEDD and LVESD were remarkably larger in the model group compared with those in normal group ($p<0.05$), elaborating that the rat model of HF meeting the requirements for laboratory animals in this experiment was successfully selected, which could be used for subsequent studies. The results of HE staining manifested that the model group had irregularly arranged cardiomyocytes and thickened myocardial fibers, and the HF-induced myocardial injury was alleviated after the treatment with ALDH2. The results of ALDH2 activity detected in each group showed that the activity was attenuated obviously in the model group ($p<0.05$), but it was enhanced significantly in the ALDH2 group ($p<0.05$), basically close to that in the normal group. All the above results are similar to those in previous studies (24, 25), revealing that ALDH2 is capable of prominently ameliorating cardiac function and pathological changes in HF.

As a mode of programmed cell death, autophagy can react correspondingly when the cell body is

invaded, and remove the harmful substances in cells. Currently, apoptosis and autophagy have become the hotspots of studies in the biological field (13). Autophagy controls the metabolic process of nerve cells under stress, which can not only protect the survival of nerve cells but also act as a mode of cell death. However, the mechanism of autophagy in physiological metabolism in organisms has not been completely defined so far, but the elaborated mechanisms of action and routes can serve as important guidelines for relevant clinical diseases such as tumor and infectious neuropathy. Autophagy has a close correlation with apoptosis, that is, apoptosis is triggered on the premise of autophagy, and autophagy plays an assistant role in cell apoptosis. In addition, autophagy can postpone the occurrence of apoptosis and inhibit cell apoptosis as an antagonist. Hence, the relationship between autophagy and apoptosis is very complex (26, 27). There is an intricate association between the mechanisms of autophagy and apoptosis, but the mutual regulatory mechanism has not been completely clarified yet, which still needs to be explored by in-depth trials. The results of the TUNEL assay indicated that the apoptosis level in the model group was remarkably higher than that in the other two groups, and apoptosis is regulated by apoptosis-related genes and proteins, including Bcl-2 and Caspase-3. In this research, the results of apoptosis-related genes and Western blotting assay also indicated that the expression level of apoptosis-related protein Caspase-3 was raised markedly, while that of Bcl-2 was lowered notably in the model group, suggesting apparent cardiomyocyte apoptosis in HF. Similar results in consistence with those in this research are also obtained in previous studies (26, 27).

PINK1 and Parkin are key factors regulating cell autophagy and mitochondrial biogenesis (28). Under physiological conditions, PINK1 is introduced into the mitochondria and degraded via proteolysis. Moreover, it can recruit Parkin into the mitochondria to trigger the process of mitophagy (29). PINK1/Parkin can interact with mitochondria, modulate the occurrence of mitophagy and maintain homeostasis (30). The pathological cardiac hypertrophy and ventricular dysfunction occur in PINK-/- mice aged 2 months old, accompanied by oxidative stress and mitochondrial dysfunction. The transgenic mice with overexpressed Parkin have stronger resistance to age-related changes in mitochondria due to the increased generation of ROS. Research has discovered that mice with Parkinson's disease are more sensitive to dose-induced cardiomyopathy and myocardial infarction (31). An in-vivo study demonstrated that DOX up-regulates p53, decreases the translocation from Parkin to mitochondria and reduces autophagy (32, 33). The results of RT-PCR in this research showed that the expression level of LC3 declined, while those of PINK1 and Parkin rose notably in the ALDH2 group, but the model group exhibited the opposite results, illustrating that the cell autophagy is promoted, and cardiomyocyte apoptosis is inhibited after the treatment with ALDH2. According to the results of the protein assay, the expression levels of PINK1 and Parkin were elevated remarkably in the ALDH2 group, suggesting that cardiomyocyte apoptosis is repressed after the treatment with ALDH2, which is consistent with the aforementioned findings. In this research, through establishing the rat model of HF, it was testified that ALDH2 has beneficial effects on the cardiomyocyte apoptosis in HF rats by regulating the PINK1-Parkin signaling pathway-mediated mitophagy. However, there were still deficiencies. For instance, no in-vitro experiments were conducted to further verify such effects (34).

In conclusion, ALDH2 probably has protective effects on HF rats and affects cardiomyocyte apoptosis, and such effects are exerted mainly through the PINK1/Parkin-mediated mitophagy. This research provided theoretical bases for the prevention and treatment of cardiomyocyte apoptosis in HF.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MA wrote the manuscript. MA and HZ were responsible for the construction of the animal model. WY and YZ performed PCR and Western blot. JZ detected myocardial function. AS and JG contributed to the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Shanghai Xuhui Central Hospital affiliated to Fudan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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