Up-regulating autophagy by targeting the mTOR-4EBP1 pathway: a possible mechanism for improving cardiac function in mice with experimental dilated cardiomyopathy

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Abstract

Background: Autophagy plays a crucial role in the pathological process of cardiovascular diseases. However, little is known about the pathological mechanism underlying autophagy regulation in dilated cardiomyopathy (DCM).

Methods: We explored whether up-regulating autophagy could improve cardiac function in mice with experimental DCM through the mTOR-4EBP1 pathway. Animal model of DCM was established in BALB/c mice by immunization with porcine cardiac myosin. Both up- or down-regulation of autophagy were studied by administration of rapamycin or 3-MA in parallel. Morphology, Western blotting, and echocardiography were applied to confirm the pathological mechanisms.

Results: Autophagy was activated and autophagosomes were significantly increased in the rapamycin group. The collagen volume fraction (CVF) was decreased in the rapamycin group compared with the DCM group (9.21 ± 0.82 % vs 14.38 ± 1.24 %, P<0.01). The expression of p-mTOR and p-4EBP1 were significantly decreased in rapamycin-induced autophagy activation, while the levels were increased by down-regulating autophagy with 3-MA. In the rapamycin group, the LVEF and FS were significantly increased compared with the DCM group (54.12 ± 6.48 % vs 45.29 ± 6.68 %, P<0.01; 26.89 ± 4.04 % vs 22.17 ± 2.82 %, P<0.05). As the inhibitor of autophagy, 3-MA aggravated the progress of maladaptive cardiac remodeling and declined cardiac function in DCM mice.

Conclusions: The study indicated a possible mechanism for improving cardiac function in mice with experimental DCM by up-regulating autophagy via the mTOR-4EBP1 pathway, which could be a promising therapeutic strategy for DCM.

Background

Dilated cardiomyopathy (DCM) is the most common cardiomyopathy worldwide characterized by ventricular cavity expansion and decline in contraction function, which is associated with congestive heart failure [1–3]. As the highly conserved pathway, autophagy plays a crucial role in the pathological process of cardiovascular diseases. Previous studies indicated that autophagy is activated in maladaptive cardiac remodeling of chronic heart failure [4–6]. However, it is still uncertain whether modulating autophagy can improve the cardiac function by pharmacological interventions. Moreover, the exact mechanisms underlying autophagy regulation are unclear in DCM. There are still many unanswered questions and points of confusion that have yet to be resolved. Therefore, an in-depth investigation into the molecular mechanism is vital to therapeutic interventions in the field [7].

In the current study, we hypothesized that up-regulating autophagy contributed to improve cardiac function in mice with experimental DCM through the mTOR-4EBP1 pathway. Both up- or down-regulation of autophagy were investigated by administration of rapamycin or 3-methyadenine (3-MA) in parallel. To the best of our knowledge, little information is available to identify whether up-regulating autophagy could improve cardiac function in DCM mice. Furthermore, autophagy activation could be a potential

therapeutic target to minimize myocardial injury and optimize the restoration of cardiac function [8, 9]. Therefore, we explored the possible mechanisms underlying autophagy activation in cardio-protection in DCM mice.

Methods

Animal model of DCM and experimental design

All animal experiments were approved by the Animal Care and Utilization Committee of Fudan University (201802021S). We obtained the male BALB/c mice aged 6 weeks from Fudan University Experimental Animal Center. The animal model of DCM was established in BALB/c mice by immunization with porcine cardiac myosin (Sigma). Cardiac myosin was emulsified with an equal volume of complete Freund's adjuvant (Sigma) to the concentration of 2 mg/ml. The solution was subcutaneously injected into the groin of BALB/c mice at days 0 and 7 respectively. The mice in control group were injected with complete Freund's adjuvant alone [10]. The mice were randomly divided into the following four experimental groups as follows: control group, DCM group, rapamycin group, and 3-MA group (n = 8 mice per group). Rapamycin was then administered at a dose of 2 mg/kg/d for 2 weeks. The mice in 3-MA group received 3-MA at a dose of 15 mg/kg/d as previously described [11]. Finally, all mice were sacrificed by cervical dislocation while anesthetized.

Histopathology

After sacrifice, myocardial tissues were fixed in 4% formaldehyde, embedded in paraffin and cut into 5μm thick slices. Specimens were stained with picrosirius red, and microscopic images were evaluated. The collagen volume fraction (CVF) was determined by quantitative morphometry of specimens with IMS Cell Image Analysis System. Images were viewed under confocal scanning microscope (Leica, TCS-SP2, Germany). For quantitation of fibrosis areas, five random fields of view were evaluated for CVF analysis across the left ventricular section.

Transmission electron microscopy

Transmission electron microscopy (TEM) for morphological evaluation was performed at Electron Microscopy Core Laboratory, Shanghai medical college, Fudan University, according to standard operating procedures. As previously reported for morphological TEM [12], cardiac tissues were fixed in 2.5% glutaraldehyde in phosphate buffer overnight at 4°C. After sample preparation, 90-100nm thick sections were mounted onto a 200 mesh copper grid and examined under a Philips CM120 electron microscope. Cardiomyocyte nucleus, mitochondria, myocardial fibers, and autophagosomes were evaluated under TEM respectively.

Western blotting

Proteins were extracted from the myocardial tissues homogenized in RIPA Lysis (Beyotime) and Extraction Buffer with a protease inhibitor cocktail, and proteins were quantified using the bicinchoninic

acid method according to the manufacturer's instructions. Samples of 25μg protein were loaded into 8% SDS-PAGE gels for electrophoresis then transferred to PVDF membranes over night at 30V. Antibodies specific for LC3 II (Cell Signaling), p-mTOR (Cell Signaling), and p-4EBP1 (Cell Signaling) were incubated at 4˚C overnight, and GAPDH (Santa Cruz) was used as a loading control to normalize gel loading and protein expression. HRP-conjugated secondary antibodies plus ECL were incubated at 37˚C for 1 hour for protein visualization. The densitometric values of immunoreactive bands were measured using Image J.

Echocardiography

M-mode transthoracic echocardiography was performed using a 30-MHz imaging transducer to evaluate the cardiac function. The mice were anesthetized with 2% isoflurane and their chests were epilated. M-mode images were obtained at the level of papillary muscles in the long-axis view. The left ventricular ejection fraction (LVEF), fractional shortening (FS), left ventricular end-diastolic dimension (LVEDD), and left ventricular end-diastolic volume (LVEDV) were measured, which were acquired by the technician who was blinded to the present experimental groups.

Statistical analysis

The data are presented as mean ± standard deviation. Values of P less than 0.05 were considered statistically significant. Normal distribution was confirmed in all experimental groups, and differences in means between two groups were analyzed by unpaired Student's t test when the data were normally distributed. Multiple group comparison was performed by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Results

General characteristics

The animal model was successfully established in male BALB/c mice, and twenty-four DCM mice were randomly divided into DCM group, rapamycin group, and 3-MA group equally. Eight mice in the control group were administered with Freund's adjuvant alone. No significant difference was found in the body weight, although a tendency was found that the body weight was slightly decreased in the 3-MA group, it did not reach the statistically significant level.

Modulating autophagy and morphological evaluation

The experimental model of DCM was established in BALB/c mice by immunization with porcine cardiac myosin. Histochemical analysis with picrosirius red staining indicated that there was a significant increase of CVF in the DCM group compared with the control group, revealing myocardial fibrosis in DCM mice. Figure 1 indicated that the CVF was significantly decreased in the rapamycin group than the DCM group (9.21±0.82 % vs 14.38±1.24 %, P<0.01). However, the CVF was increased to 17.68±1.81 % by down-regulating autophagy in the 3-MA group compared with the DCM group (P<0.05).

For morphological TEM, normally arranged myofibrils within the sarcomeres with defined Z-bands were observed in the control group. Autophagy was significantly activated and autophagosomes could be confirmed in mice with experimental DCM, and sarcomeric disarray and myofibrillar lysis could be observed. As shown in Figure 2, double membrane autophagosomes were significantly increased in the rapamycin group compared with the DCM group (P<0.001). We inhibited the autophagy activation by 3-MA and verified that the number of autophagosomes was statistically decreased compared with the DCM group, and the sarcomeric disarray failed to get reversed.

Modulating autophagy and mTOR-4EBP1 pathway

The conversion of LC3 I to LC3 II form is recognized as indicators of autophagy activation. To validate the relationship of autophagy and mTOR-4EBP1 pathway, the p-mTOR and the downstream molecule of p-4EBP1 were measured. Autophagy and mTOR-4EBP1 pathway were regulated in mice with experimental DCM by administration of rapamycin or 3-MA in parallel. Our study indicated that rapamycin-induced inhibition of mTOR-4EBP1 pathway, shown as decreased p-mTOR and p-4EBP1 expression compared with the DCM group. The increased expression of LC3 II confirmed the activation of autophagy in the rapamycin group. With the administration of 3-MA, protein levels of p-mTOR and p-4EBP1 were significantly increased, whereas the expression of LC3 II was decreased in the 3-MA group (Fig. 3).

Modulating autophagy and cardiac function

We next examined whether the cardiac function was improved in mice with experimental DCM by up-regulating autophagy. Therefore, M-mode images were obtained at the level of papillary muscles in the long-axis view (Fig. 4). As summarized in Figure 5, cardiac function differed significantly among the four groups. In the DCM group, the LVEF and FS significantly deteriorated compared with the control group. In the rapamycin group, the parameters were significantly increased compared with the DCM group (54.12 ± 6.48 % vs 45.29 ± 6.68 %, P<0.01; 26.89 ± 4.04 % vs 22.17 ± 2.82 %, P<0.05), although cardiac function was still lower than the control group. Furthermore, the LVEDD and LVEDV significantly decreased following down-regulation of mTOR-4EBP1 pathway to activate autophagy. However, 3-MA-induced inhibition of autophagy provided a negative effect to promote the maladaptive cardiac remodeling, possibly in part, by up-regulation of mTOR-4EBP1 pathway involved in the pathological process of DCM.

Discussion

For the first time we reported the cardio-protection effects of rapamycin-induced autophagy activation, which contributed to improve cardiac function in DCM mice via regulating the mTOR-4EBP1 pathway. The biological effects were tested in experimental DCM mice by administration of rapamycin or 3-MA respectively. We confirmed that autophagy was directly activated by down-regulating the mTOR-4EBP1 pathway, which increased the expression of LC3 II and the formation of autophagosomes. Our study indicated that up-regulating autophagy could be a promising therapeutic strategy to improve cardiac function for the pathological progression of DCM.

Autophagy is a highly conserved cellular recycling process, which not only plays an important role in cellular homeostasis but also participates in physiological processes [13, 14]. Autophagy degrades the recycling material in the cell while the former makes it through the formation of double-membrane vesicles that fuse with the lysosomal [15, 16]. It plays the major role of catabolic mechanism degenerating and recycling long-lived protein and organelles involving in physiological and pathological process. Accordingly, dysfunction of this process contributes to the pathological process of cardiovascular diseases.

The mTOR pathway is a well-known negative regulator of autophagy activity, which has been established to regulate cell growth, proliferation, and metabolism [17–19]. Our previous study confirmed that autophagic activity was up-regulated in a rat model of early-stage dilated cardiomyopathy [20]. As the mTOR inhibitor, rapamycin can dephosphorylate the downstream effectors such as 4EBP1. Furthermore, 4EBP1 is a translation regulator, its dephosphorylation by mTOR inhibitors suppresses overall cellular protein synthesis and induces autophagy [21]. In our present study, the rapamycin-induced autophagy activation successfully reversed myocardial fibrosis and improved cardiac function in DCM mice. In contrast, down-regulating autophagy inhibited the formation of autophagosomes in the 3-MA group, which induced severe myocardial fibrosis and decreased cardiac function.

Echocardiography showed that the LVEF and FS significantly decreased in the DCM group, which is consisted with the pathological development of DCM. With the administration of 3-MA, cardiac function failed to improve compared with the DCM group. In the rapamycin group, cardiac function significantly improved compared with the DCM group, although the LVEF and FS were still lower compared with the control group. Meanwhile, the LVEDD and LVEDV statistically reduced by down-regulation of mTOR-4EBP1 pathway to activate autophagy.

The molecular mechanisms of autophagy regulation remain unclear, an in-depth study of mTOR-4EBP1 pathway might thus contribute to provide an exciting therapeutic strategy for DCM [22–24]. Our data indicated that rapamycin down-regulated the mTOR-4EBP1 signaling pathway in DCM mice. To confirm the effect of autophagy inhibition, 3-MA was employed into the present study, which decreased the formation of autophagosomes and activated the pathway as indicated by increase of p-mTOR and p-4EBP1 expression. Data in all demonstrated that directly targeting on the mTOR-4EBP1 pathway was a possible mechanism in the regulation of autophagy in DCM.

Our study was carefully designed and conducted in animal experimental center of Fudan University. Both up- or down-regulation of autophagy were studied by administration of rapamycin or 3-MA in parallel. Some limitations of this study should be acknowledged. We preliminary explored the mechanisms for improving cardiac function induced partially by regulating the mTOR-4EBP1 pathway, so other aspects of critical molecular mechanisms should be focused in the future research and is needed to develop better pharmacological interventions. Through further research, a more complete picture of the molecular mechanism and regulation of autophagy will strengthen our understanding of the pathological process.

Conclusions

The present study indicated that autophagy activation was involved in the pathological progress of experimental DCM. As a possible mechanism, up-regulating autophagy contributed to improve cardiac function in part through mTOR-4EBP1 pathway, which could be a promising therapeutic strategy for DCM.

Declarations

Acknowledgements

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Authors' contributions

BJ and BWW provided the hypothesis, and handled funding and

supervision. HMS, JZ and GSQZ collected and analyzed the data. BWW drafted and revised the manuscript. All authors approved the final version to be published.

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Availability of data and materials

All relevant data is presented in the manuscript and supporting material.

Ethics approval and consent to participate

All animal studies were approved by the Animal Care and Utilization Committee of Fudan University (201802021S).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

CVF
collagen volume fraction; DCM:dilated cardiomyopathy; FS:fractional shortening; LVEDD:left ventricular end-diastolic dimension; LVEDV:left ventricular end-diastolic volume; LVEF:left ventricular ejection fraction; 3-MA:3-methyadenine; SD:standard deviation; TEM:transmission electron microscopy

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Figures

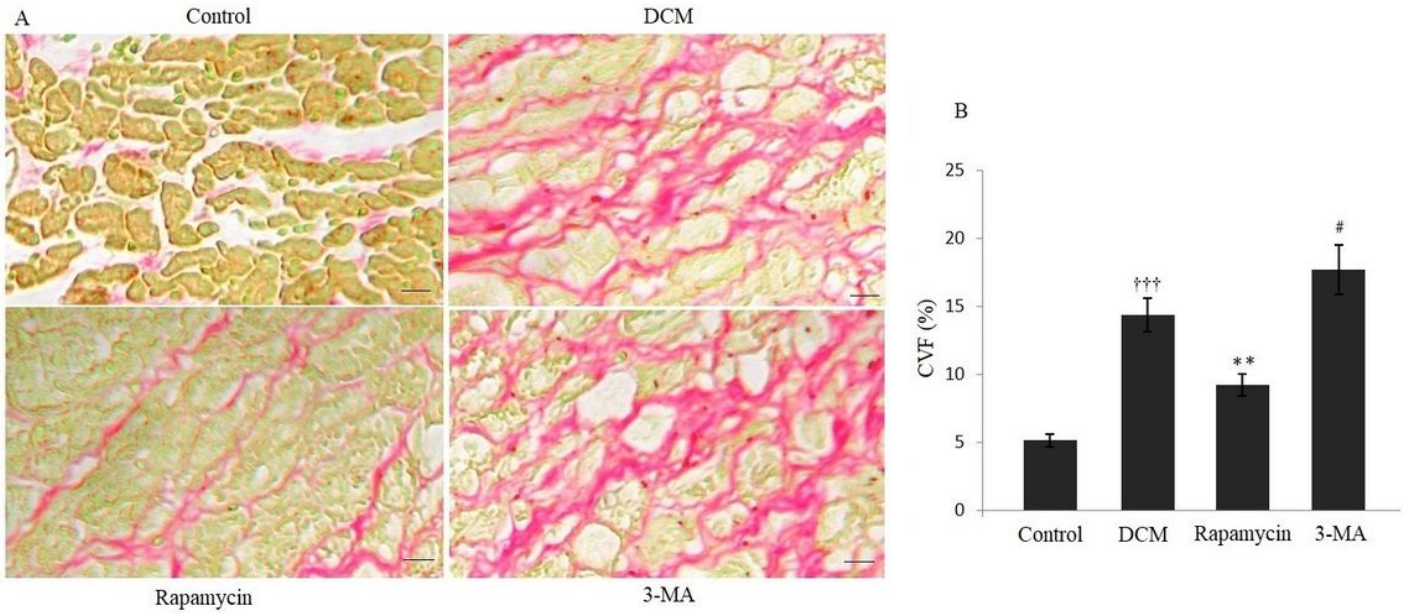

Figure 1

Modulating autophagy and cardiac matrix remodeling of DCM. (A) Picrosirius red staining indicated significantly changes of collagen distribution in the four different groups. (B) Histochemical analysis showed that there was a significant increase of collagen distribution in the DCM group compared with the control group. Quantitative assessment demonstrated that the CVF was significantly decreased in the rapamycin group, and it was increased in the 3-MA group compared with the DCM group. †††P < 0.001 vs Control, **P < 0.01 and #P < 0.05 vs DCM. Scale bar = 100μm.

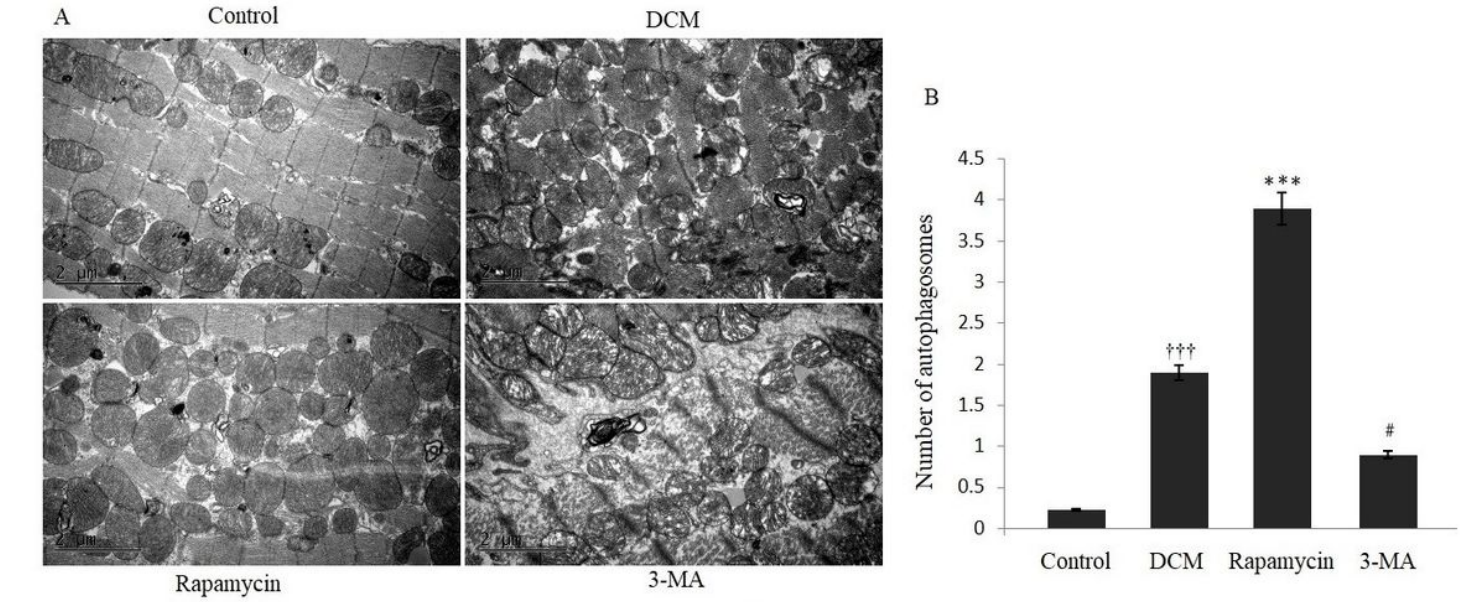

Figure 2

Transmission electron microscopy assessment for modulating autophagy. (A) Transmission electron microscopy indicated significant changes of autophagosomes in the four different groups. (B) Transmission electron microscopy showed that there was a significant increase of autophagosomes in the DCM group compared with the control group. Quantitative assessment demonstrated that autophagosomes were significantly increased in the rapamycin group, and they were decreased in the 3-MA group compared with the DCM group. †††P < 0.001 vs Control, ***P < 0.01 and #P < 0.05 vs DCM.

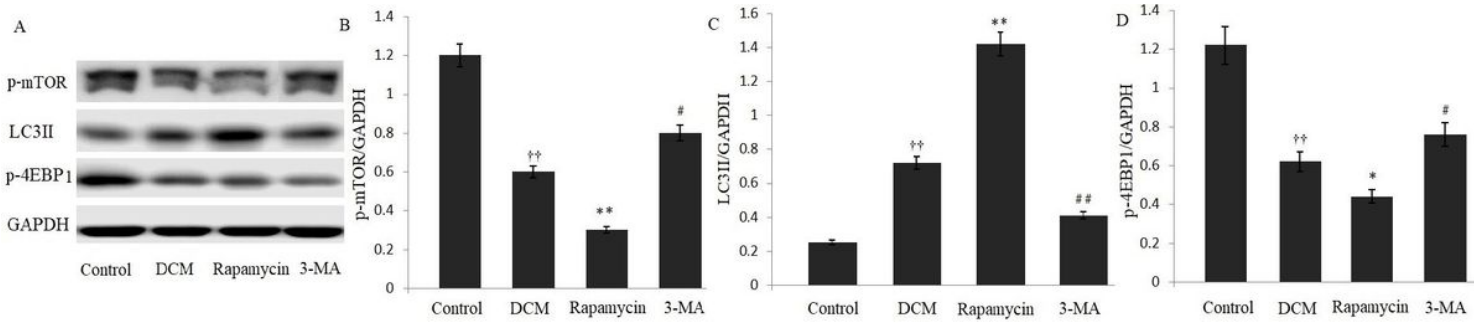

Figure 3

Modulating autophagy and the mTOR-4EBP1 pathway. (A-D) The expression levels of p-mTOR and p-4EBP1 were significantly decreased in rapamycin-induced autophagy activation, and the effects were significantly increased by down-regulating autophagy with 3-MA. The increased expression of LC3 II indicated the activation of autophagy in the rapamycin group, whereas the expression of LC3 II was decreased in the 3-MA group. ††P < 0.01 vs Control, **P < 0.01, *P < 0.05, ##P < 0.01, and #P < 0.05 vs DCM. Each experiment was conducted 3 times in triplicate.

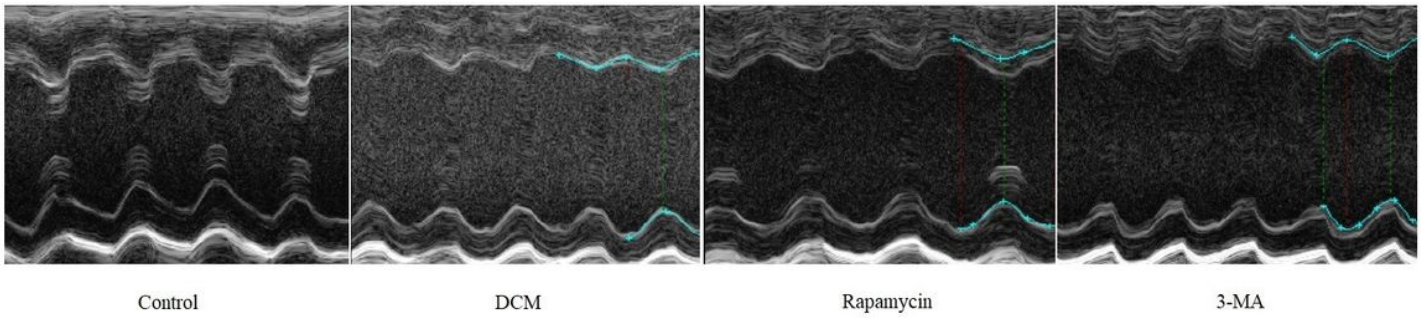

Figure 4

M-mode ultrasound images at the level of papillary muscles in the long-axis view. The M-mode echocardiography evaluation included the following four experimental groups as follows: control group, DCM group, rapamycin group, and 3-MA group. Each group, n = 8.

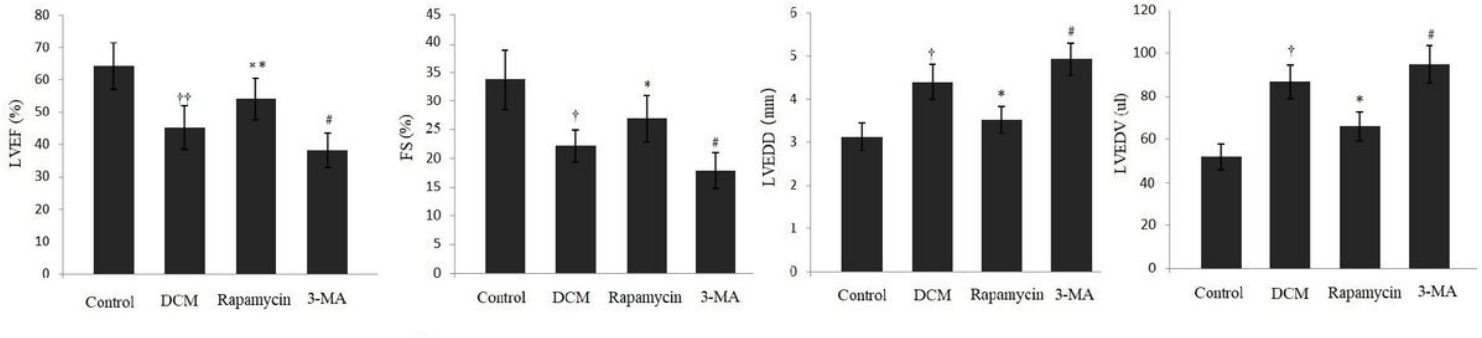

Figure 5

Modulating autophagy and cardiac function. (A) and (B) Cardiac function differed in the four groups, and the LVEF and FS significantly improved in the rapamycin group compared with the DCM group. (C) and (D) The LVEDD and LVEDV were significantly decreased in the 3-MA group compared with the DCM group. ††P < 0.01 and †P < 0.05 vs Control, **P < 0.01, *P < 0.05, and #P < 0.05 vs DCM. Each group, n = 8.