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Targeted delivery of isoliquiritigenin by ultrasonic microbubbles attenuate myocardial injury via suppressing inflammation and oxidative stress and activating AMPK/SIRT1/eNOS signaling pathway in rats

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Abstract

To investigate the protective efficacy of ultrasound targeted microbubble destruction (UTMD) combined with Isoliquiritigenin on myocardial injury in rats. The GK rat model of cardiomyopathy was successfully established by the induction of adriamycin. Then these rats with cardiomyopathy were randomly assigned into the model group, isoliquiritigenin microbubbles and ultrasound alone or combination group, using healthy ones as normal control. After 8-week consecutive treatment, the relevance indexes of diabetes, echocardiography as well as the hyperlipidemia, oxidative stress of model animals were examined. In addition, the fibrosis, morphological changes and inflammation response of myocardial tissues were also assessed. After further 4-week intervention, the blood biochemical indexes and the cardiac functions of model rats received the combined treatment were improved (all $P < 0.05$) compare to those received either monotherapy or saline. After chronic treatment, the heart/body weight ratio and serum cardiac index levels in model rats received combined treatment were significantly changed (all $P < 0.05$) compared with others. Furthermore, combination therapy could ameliorate excessive oxidation stress and inflammation response as well as up-regulate the expression levels of AMPK/SIRT1/eNOS signaling pathway. Targeted delivery of isoliquiritigenin by ultrasonic microbubbles can ameliorate the myocardial injury via activating AMPK/SIRT1/eNOS signaling pathways.

Keywords Isoliquiritigenin, Ultrasound targeted microbubble destruction, Cardiomyopathy, Inflammation, Oxidative stress, AMPK/SIRT1/eNOS signaling pathway

Introduction

So far, the number of diabetic patients has jumped to third place in world and has become an important disease endangering human health [1]. Chronic diabetes may induce a variety of symptoms, of which the diabetic cardiovascular diseases are becoming the leading cause of death, mainly including atherosclerosis, coronary heart disease, heart failure and diabetic cardiomyopathy (DCM) [2, 3]. Persistent insulin resistance and chronic hyperglycemia may cause compensatory and decompensated responses in diabetic patients [3–5]. DCM usually

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is independent of heart failure syndrome occurring in other cardiovascular diseases [6]. However, the pathogenesis of DCM is still not clear, of which the metabolic disorders, oxidative stress, mitochondrial dysfunctions, calcium homeostasis disorders and abnormal glycosylation are currently known [7]. At present, how to ameliorate the diabetic myocardial injuries is an urgent medical need to be met.

It has been reported that isoliquiritigenin (ISO) is a flavonoid extracted and isolated from Glycyrrhiza uralensis, and holds many pharmacological activities, including anti-hyperglycemia, lowering blood pressure, and antivirus [8]. Therefore, ISO has gradually become a research hotspot [9]. Huang et al. demonstrated that the ISO could achieve therapeutic activities on the liver injuries in mice by scavenging free radicals and anti-lipid peroxidation in liver tissues [10]. It has also been reported that ISO inhibits ROS generation in epithelial cells and prevents accumulation of VCAM-1 and e-selectin, thereby achieving a reduction in excessive inflammatory responses [11]. Considering the pathogenesis and inflammatory response of DCM, there is a correlation between excessive oxidative response, suggesting that ISO holds potential to ameliorate the myocardial injuries. A variety of drug delivery systems have now been used for the treatment of diseases, such as liposomes, micelles, nanoparticles, etc., but these carriers do not have the targeting of organ tissues and have low delivery efficiency [12]. Ultrasound-mediated microbubble cavitation is a specific, minimally invasive drug delivery system with great promise [13]. Ultrasound microbubble drug carriers can selectively target and release the drug at the site of action under the guidance of ultrasound [13]. In addition, they can also promote the absorption and uptake of the drug by cells, so as to greatly reduce the dose of the drug and reduce side effects without reducing the efficacy [14].

Given the potential benefit of ISO applied to myocardial injury, we wondered whether rupture of ISO-containing microbubbles under the ultrasound irradiation can up-regulate the concentration of drugs around the myocardial tissues and protect myocardial tissue more effective. Current study aims to explore the protective effects of targeted delivery of ISO by ultrasonic microbubbles on myocardial injury in rats with cardiomyopathy, and to explore its mechanism of improving the cardiac functions and injuries, which further provides a new direction for the clinical treatment of DCM.

Materials and methods

Materials

Isoliquiritigenin with the purity over 99% were purchased from Chengdu Herbpurify Co.ltd. (Chengdu, China). The ELISA kits for detecting the cardiac troponin I (cTnI), creatine kinase (CK), lactate dehydrogenase (LDH), endothelin (ET), and calcitonin gene-related peptide (CGRP), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nuclear factor-κB (NF-κB), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were brought from Invitrogen Biotechnology Co., LTD (USA)). All western blot related reagents were purchased from Sigma-aldrich (USA). SonoVue injection kit was purchased from BRACCO INTERNATIONAL BV (Netherlands).

The GK rats (6–8 weeks and 175 ± 10 g) were brought from Shanghai Slack Laboratory Animal Company (Shanghai, China). Rat was housed in SPF cage of three at 25 ± 2 ℃ and 65 to 75% relative humidity. All studies were approved by Ethics Committee of The First People's Hospital of Yuhang District, Hangzhou, and executed according to the standard operating procedures.

ISO microbubbles preparation

According to the kit instructions, 5 mL of 0.9% NaCl (injection grade) containing 0.5 mg ISO (final concentration 0.1 mg/mL) was added to the vial provided in the SonoVue kit, followed by vigorous shaking for 1 min until the lyophilized powder was completely dispersed. Subsequently, the suspension was incubated in a shaking incubator at 25 ℃ for 2 h, and ultrasonic microbubbles co-loaded with ISO (SonaVue-ISO) were obtained by shaking several times.

Animal experiments

GK rats received the intraperitoneal administration of adriamycin at the dose of 2.5 mg/kg once weekly for 6-week, whereas normal Wistar rats received saline once a week for 6 weeks. After the model was successfully established, these rats were randomly assigned into the model control group, UTMD group, ISO group and UTMD + SonaVue-ISO group, and healthy Wistar rats were used as normal control group. Of these, 0.5 mg/kg ISO was administered via tail vein injection to rats in the ISO and UTMD + SonaVue-ISO groups, while rats in the model control group and UTMD only group received the saline at same volume. Subsequently, the rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital 1 mL/kg and the precordial area of the rats was shaved. The precordial region of the rat was irradiated by an ultrasonic therapeutic apparatus (frequency of 1 MHz and intensity of 1 W/cm^2) for 10 min. Body weight, fasting blood glucose level (BGL), oral glucose tolerance, and lipid parameters including triglyceride (TG), total cholesterol (TC), and free fatty acid (FFA) were measured and recorded every 2-week. The procedures of oral glucose tolerance test (OGTT) were performed as previously described [15]. Lipid parameters were measured by an

automatic blood chemistry analyzer (ABX pentraXL80). Left ventricular ejection fraction (LVEF), left ventricular end diastolic diameter (LVEDd), shortening fraction (FS), ratio of peak E to peak A of mitral inflow velocity variables (E/A), and early diastolic E/A ratio (E′/A′) were analyzed and measured by echocardiography. All measurements were performed by the same observer.

After 8-week of intervention, all rats were anesthetized and sacrificed, and then the heart was then removed and the weight of the collected heart was recorded. Blood samples were allowed to stand at room temperature for 30 min, and then centrifuged at 3000 rpm for 10 min to collect serum, which was aliquoted and stored at − 80 ℃ for the detection of serological markers. The TNF-α, IL-1β, NF-κB, MDA, SOD, and GSH-Px levels in myocardial tissues and serum levels of cTnI, ET, CGRP, CK, and LDH were determined by above-mentioned commercial ELISA kits according to the attached instructions.

Histopathologic analysis

Myocardial tissues were washed with normal saline, then half of the heart tissues were stored in liquid nitrogen for further use, and the other half of the tissues were fixed in 4% paraformaldehyde. The fixed heart tissues were routinely dehydrated, waxed, embedded, and sectioned. H&E staining was performed as follows: After routine baking and deparaffinization, the sections were stained with hematoxylin, then rinsed with clean water, stained with eosin, rinsed with clean water, and then dehydrated, cleared, and mounted. Masson staining was performed as follows: sections were deparaffinized to water and stained according to Masson's instructions. The changes of myocardial morphology and structure and collagen fibers were observed under light microscope.

Western blot analysis

Myocardial tissues were placed on ice, lysed in lysis buffer containing PMSF for 30 min, and the supernatant was centrifuged and collected. The protein concentration of supernatant was detected via the BCA measurements, then the supernatant containing 30 μg of protein was added to SDS-PAGE gel electrophoresis. At the end of electrophoresis, PVDF membrane was wet transferred and blocked for 2 h. The antibodies targeting the AMPK, p-AMPK, SIRT1, eNOS and β-actin were added to incubate overnight at 4 ℃. PVDF membranes were incubated with secondary antibodies at 25 ℃ for 1 h. After washing, they were developed in an ECL luminescent automatic exposure machine (Tanon-5200Multi). Quantitative analysis of protein gray values was performed using Image J software.

Statistical methods

SPSS 20.0 and GraphPad Prism 6.0 software was applied for statistical analysis. All quantitative data were showed as Mean ± SD, and t-test was applied for the comparison between two groups. $P < 0.05$ was considered statistically significant.

Results

Long-term effects of UTMD-mediated ISO microbubble on diabetic parameters in DCM rats

Firstly, we evaluated the effect of ISO microbubbles combined with UTMD on the diabetic parameters in GK rats. As shown in Fig. 1, fasting BGL in the model control group and the UTMD group showed a similar trend, namely a continuous increase. Fasting BGL were statistically lower in ISO group and combination group compared with other groups ($P < 0.05$). Moreover, the combination of UTMD and ISO microbubbles resulted in significantly improved glycemic control than the ISO alone group. Moreover, the rats in the control and UTMD groups showed a linear decrease in body weight, while that of the ones received ISO or combination therapy remained basically unchanged or showed a slight upward trend, respectively. In addition, UTMD combined with ISO microvesicles could also significantly ameliorate glucose tolerance and lipid metabolism in DCM rats, and was better than that in ISO monotherapy group, but there was no statistical difference.

Chronic effects of UTMD-mediated ISO microbubbles on cardiac function in DCM rats

In order to explore the effects of ultrasound combined with ISO microbubbles on the diastolic functions of GK rats, LVEF, LVEDd, shortening fraction, E/A and E′/A′ were further evaluated. As shown in Fig. 2, LVEF and shortening fraction were impaired in DCM rats, and E/A and E′/A′ showed a significant decreasing trend. In general, diastolic dysfunction precedes systolic dysfunction in rat hearts. DCM rats in control group had the highest LVEDd, indicating impaired diastolic function. The results of conventional ultrasound showed that the decreasing trends of LVEF, shortening fraction, E/A and E′/A′ were significantly reversed in the ISO treatment group and the combined treatment group after 8 weeks of intervention. Interestingly, the combination therapy group showed a significantly better improvement in cardiac index than the ISO treatment group alone. Moreover, after ultrasound combined with ISO microbubble treatment, LVEDd was significantly shortened in DCM rats, indicating a significant improvement in diastolic function.

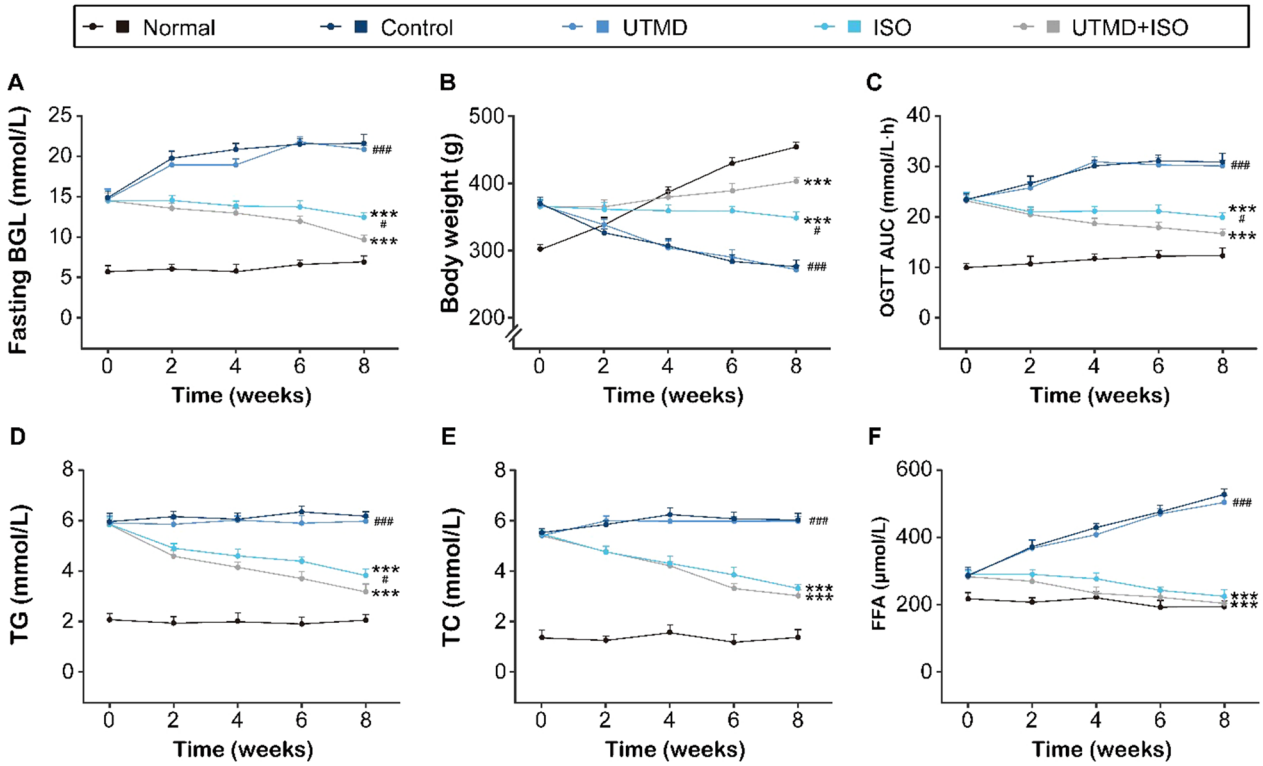

Fig. 1 Long-term effects of combined UTMD with ISO microbubble on diabetic indexes of DCM rats. The **A** fasting BGLs, **B** change of the body weight, **C** oral glucose tolerance, **D** TG, **E** TC and **F** FFA levels of DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

Chronic effects of UTMD-mediated ISO microbubbles on cardiac indicators in DCM rats

To further confirm the improvement on cardiac injury in DCM rats via the treatment of ultrasound in combination with ISO microbubbles, serum myocardial injury-associated proteins were detected. As the results showed in Fig. 3, HW/BW was significantly up-regulated in the DCM rats compare with the healthy ones. In addition, treatment of ultrasound combined with ISO microbubble significantly reduced HW/BW in DCM rats, and better than ISO treatment group alone. Compared with normal rats, cTnI and ET levels were significantly increased and CGRP levels were significantly decreased in the DCM group, while the upregulation or downregulation of these parameters were reversed in the combined treatment group, and the improvement was more significant compared with the two mono-therapy (both $P < 0.05$). In addition, ultrasound combined with ISO microbubble treatment also significantly decreased the serum CK and LDH activities in DCM rats. Above results collectively suggested that combined treatment can ameliorate myocardial injury in DCM rats by reversing biomarkers of myocardial injury.

Chronic effects of UTMD-mediated ISO microbubbles on the cardiomymorphology in DCM rats

We further evaluated the improvement of myocardial tissue in DCM rats by histopathological methods. As showed in Fig. 4A, H&E staining analysis exhibited that the myocardial tissue of DCM rats was fibrolytic, the boundary was blurred, the myocardial cells were irregularly arranged, and obvious lesions and inflammatory cell infiltration occurred. After 8 weeks of intervention, myocardial tissue was significantly improved in DCM rats received the combined treatment. Histopathologic scoring results were showed in Fig. 4B, which demonstrated that both ISO alone and combined treatment can significantly reduce myocardial tissue injury scores, but the combined treatment group showed better improvement. These results collectively indicated that combined therapy can significantly ameliorate myocardial histopathological injury in diabetic rats.

Further analysis of Masson staining demonstrated that the myocardial cells of DCM rats in the control group were disorganized, the myocardial cells were necrotic, and the collagen network structure in the interstitium was destroyed and disorganized, indicating the occurrence of myocardial fibrosis. Long-term intervention

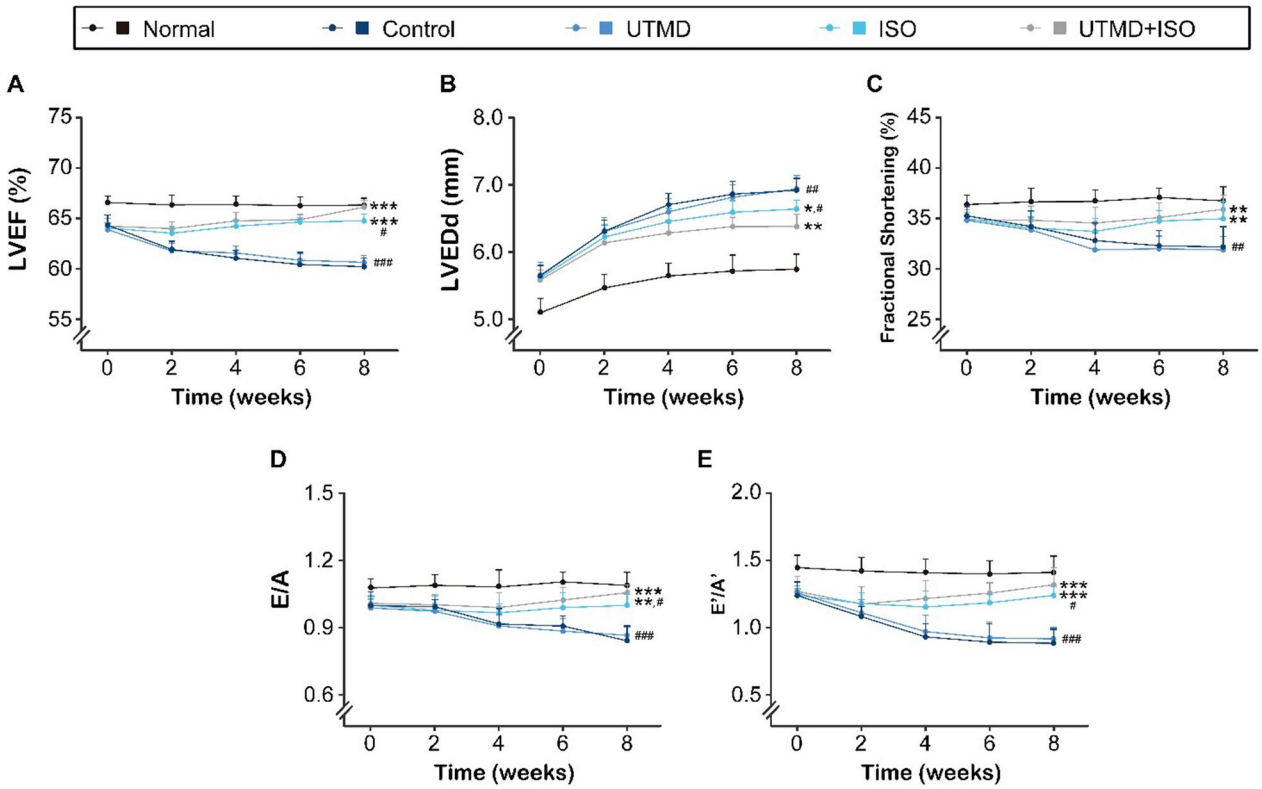

Fig. 2 Long-term effects of UTMD in combination with ISO microbubbles on ultrasound cardiac functions of DCM rats. The **A** LVEF, **B** LVEDd, **C** Fractional shortening, **D** E/A, and **E** E′/A′ levels of DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

with ultrasound combined with ISO microbubbles significantly reversed fibrosis in myocardial tissues of DCM rats, as shown by neatly arranged cardiomyocytes, and only a small amount of bright green collagen fibers was distributed in the myocardial interstitium and around blood vessels (Fig. 4C). Quantification of fibrotic areas showed that myocardial fibrotic areas were significantly lower in the combined treatment group than in model control group and group received either monotherapy. In general, ultrasound in combination with ISO microbubbles could effectively improve the myocardial injuries in DCM rats.

Chronic treatment of UTMD-mediated ISO microbubbles on inflammation and oxidative stress in DCM rats

Inflammation and oxidative stress are markers of myocardial damage in diabetes. To assess the inflammation in myocardial tissues, the levels of related pro-inflammatory factors, including TNF-α, IL-1β and NF-κB, were determined by ELISA. As shown in Fig. 5A–C, oxidative stress-related factors were also detected, including SOD, MDA and GSH-PX. Treatment of ISO alone significantly decreased the levels of the above inflammatory parameters in myocardial tissues compared to DCM rats in the model control group. In contrast, combination therapy exhibited a greater reduction in inflammatory parameters. On the other hand, we further evaluated oxidative stress-related parameters in myocardial tissue of DCM rats. As the results showed in the Fig. 5D–F, the MDA levels were significantly increased, GSH-PX levels were significantly decreased, and SOD activity was significantly decreased in the myocardial tissues of DCM rats in the control group compared with normal ones, suggesting the occurrence of oxidative stress. Long-term treatment with ultrasound combined with ISO microbubbles significantly reversed the changes in SOD, and MDA, GSH-PX levels, which were close to those in healthy rats. Above results collectively revealed that ultrasound combined with ISO microbubble treatment can effectively ameliorate the inflammation and oxidative stress in DCM rats and thus protect damaged heart tissues.

Effects of chronic treatment of UTMD-mediated ISO microbubbles on the activation of AMPK/SIRT1/eNOS signaling pathway in DCM rats

To investigate whether the change of AMPK/SIRT1 signaling pathway related markers are involved in myocardial injury amelioration in DCM rats, western blotting

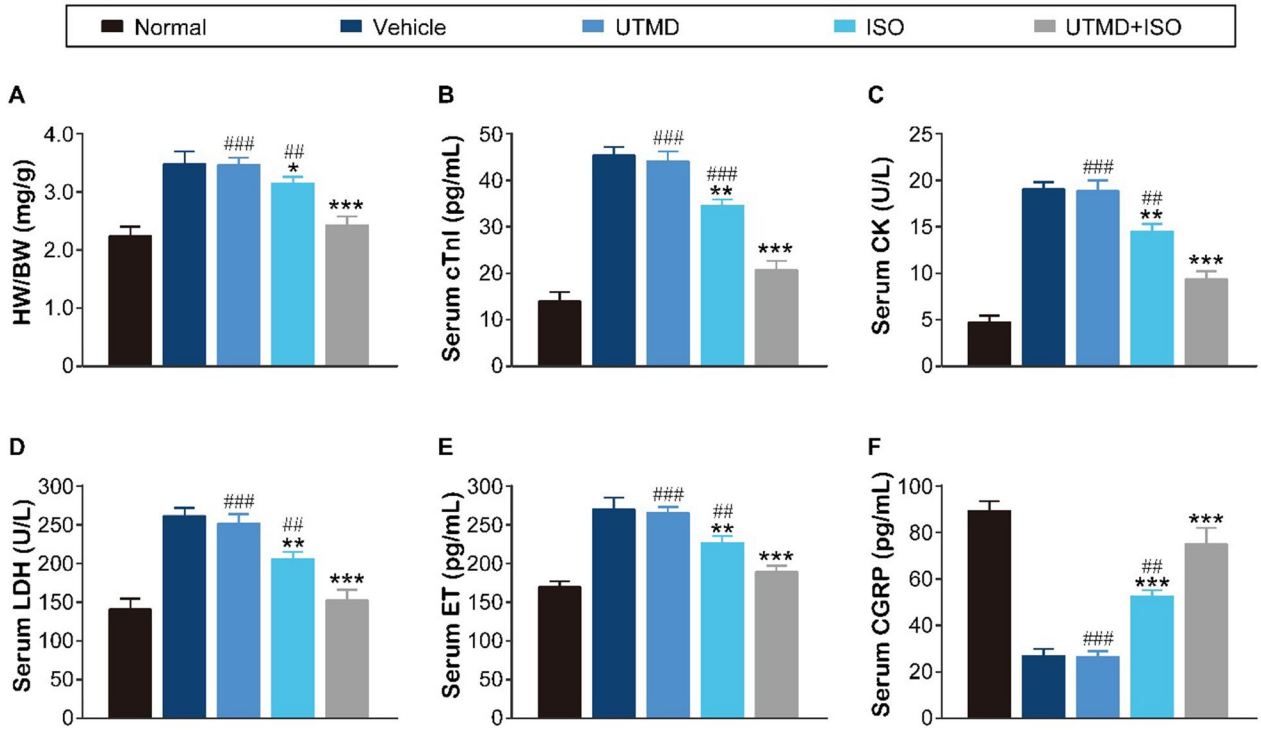

Fig. 3 Chronic effects of combined UTMD with ISO microbubbles on the cardiac indexes of diabetic rats with DCM. The **A** HW/BW and the serum levels of **B** cTnI, **C** CK, **D** LDH, **E** ET, and **F** CGRP of DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

analysis was performed. As shown in Fig. 6, p-AMPK/AMPK levels remained low in the myocardial tissues of DCM rats in model control group. After 8 weeks of ISO alone treatment, the p-AMPK/AMPK signaling pathway was significantly up-regulated ($P < 0.01$). In addition, the ultrasound combined with ISO microbubble treatment group showed a greater degree of p-AMPK/AMPK up-regulation. Previous studies have demonstrated that activation of eNOS can exert beneficial effect on myocardial injuries. In addition, eNOS expression is regulated by SIRT levels [16, 17]. In the present study, the SIRT and eNOS levels were both significantly down-regulated in control DCM rats compared to normal ones. In contrast, SIRT and eNOS expression levels were significantly up-regulated in the myocardial tissue of DCM rats receiving combined treatment anxd were almost comparable to those of normal rats. In addition, significant up-regulation of SIRT and eNOS expression levels was also observed in the ISO monotherapy group ($P < 0.05$).

Discussion

DCM is a serious irreversible chronic cardiovascular complication that occurs in diabetic patients and is characterized by the cardiac hypertrophy, systolic dysfunctions, myocardial fibrosis and may eventually progress to heart failure [2, 6]. The pathogenesis of DCM is complex, and current studies have shown that it is associated with factors such as myocardial tissue fibrosis, oxidative stress and inflammation [6]. DCM occurs mainly due to increased free radical production in a hyperglycemic environment, resulting in mitochondrial damage and increased apoptosis and cardiomyocyte fibrosis [18]. Impaired cardiac function is one of the end-stage events in DCM and severely impacts the prognosis of diabetic patients [18].

ISO has been shown to have antioxidant, anti-inflammatory, anti-diabetic, and cardioprotective functions [19]. According previously published data, ISO has protective effects on diabetic cardiomyopathy models in vitro and in vivo [11]. In diabetic rats, LVEF and LVFS levels decreased, apoptosis levels increased, myocardial hypertrophy was observed by H&E staining, and Masson staining exhibited myocardial fibrosis, while the above findings were reversed after the treatment of ISO [19]. According to previous study, ISO holds potential to be applied for treating DCM [20]. Furthermore, as a bio-macromolecular protein drug, ISO has poor in vivo stability and is easily degraded, and its clinical application is greatly limited. Moreover, it remains unclear whether the protective effect of ISO could be enhanced by other

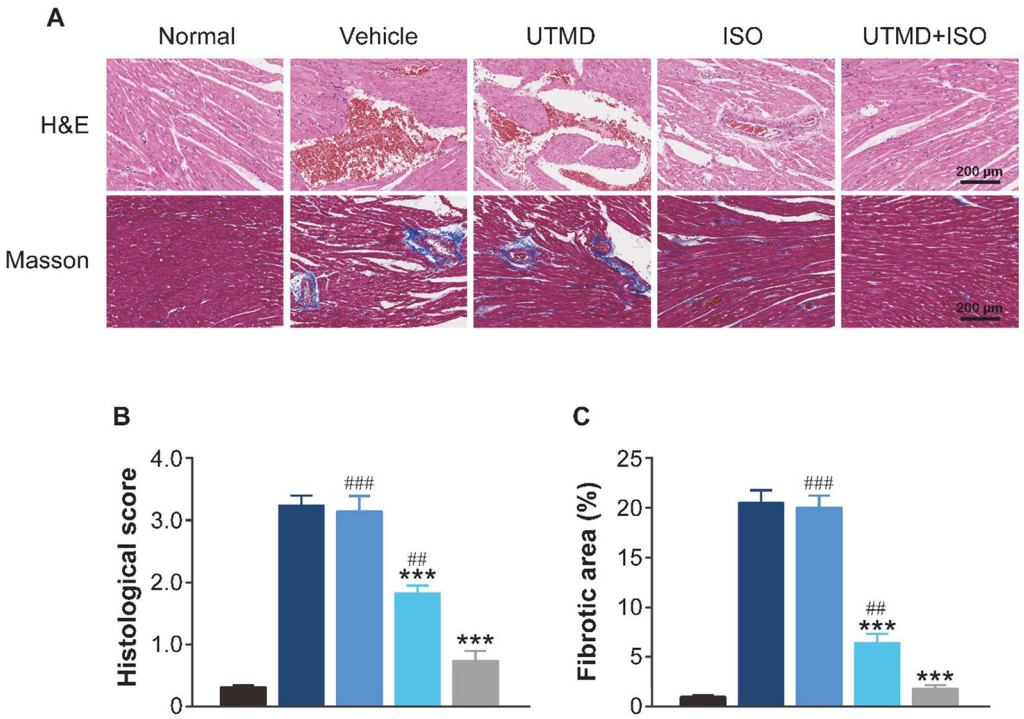

Fig. 4 Long-term effects of combined UTMD with ISO microbubbles on the cardiomymorphology of DCM rats. **A** The representative images of H&E and Masson staining analysis, **B** H&E histological score, and **C** fibrotic area of myocardial tissue from the DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

delivery modalities to suppress diabetic myocardial injury. Therefore, current study aims to investigate the ameliorative effects of ISO on diabetic myocardial injury by ultrasound-mediated microbubble delivery in vivo.

In this study, ultrasound microbubbles were used as carriers of ISO by non-covalent physical combination, and ultrasound energy was targeted to burst microbubbles carrying ISO in myocardial tissue to release and exert its efficacy in myocardial tissue. Firstly, we examined diabetes-related parameters, including the fasting BGL, glucose tolerance, body weight, and lipid metabolism in DCM rats. The fasting BGLs as well as glucose tolerance of DCM rats were significantly higher than those of normal rats, but the above changes were significantly reversed after 8-week treatment with ultrasound in combination with ISO microbubbles. In addition, the occurrence of diabetes causes the decreased insulin secretion and insulin resistance, both of which can lead to poor lipid metabolism [21]. Cardiomyocytes are dependent on increased lipid oxidative energy supply, especially TG and FFA. Increased oxidation of FFA produces accumulation of metabolites that can activate the apoptosis program and promote cardiomyocyte apoptosis [22]. Moreover, the increased metabolism of FFA aggravates myocardial oxygen consumption, resulting in the increased cardiac burden [23]. When FFA continue to increase and cannot be fully utilized, lipid droplet particles accumulate in the myocardium, causing inhibition of enzyme activity in myocardial cells and increased low-density lipoprotein oxidation, which leads to severe stasis of oxidatively modified low-density lipoprotein in the body and damages myocardial cells, resulting in impaired cardiac functions [23, 24]. Chronic treatment with UTMD combined with ISO microbubbles significantly decreased the serum levels of TG, TC and FFA in the DCM rats, suggesting that combined treatment could significantly improve lipid metabolism and thereby reduce myocardial injury in DCM rats.

After 6-week intervention, LVEF, shortening fraction and E/A ratio in combined treatment group were higher than those in the control DCM rats, while LVEDd was significantly smaller, suggesting a significant improvement in the diastolic functions. The cardiac function-associated protein levels were further examined. Serological markers, including the cTnI, LDH, and CK, were considered as markers of myocardial injury [25]. After adriamycin intervention, the levels of the above serological markers all significantly increased, suggesting severe myocardial damage. After 8 weeks treatment of ultrasound combined with ISO microbubbles, the serum

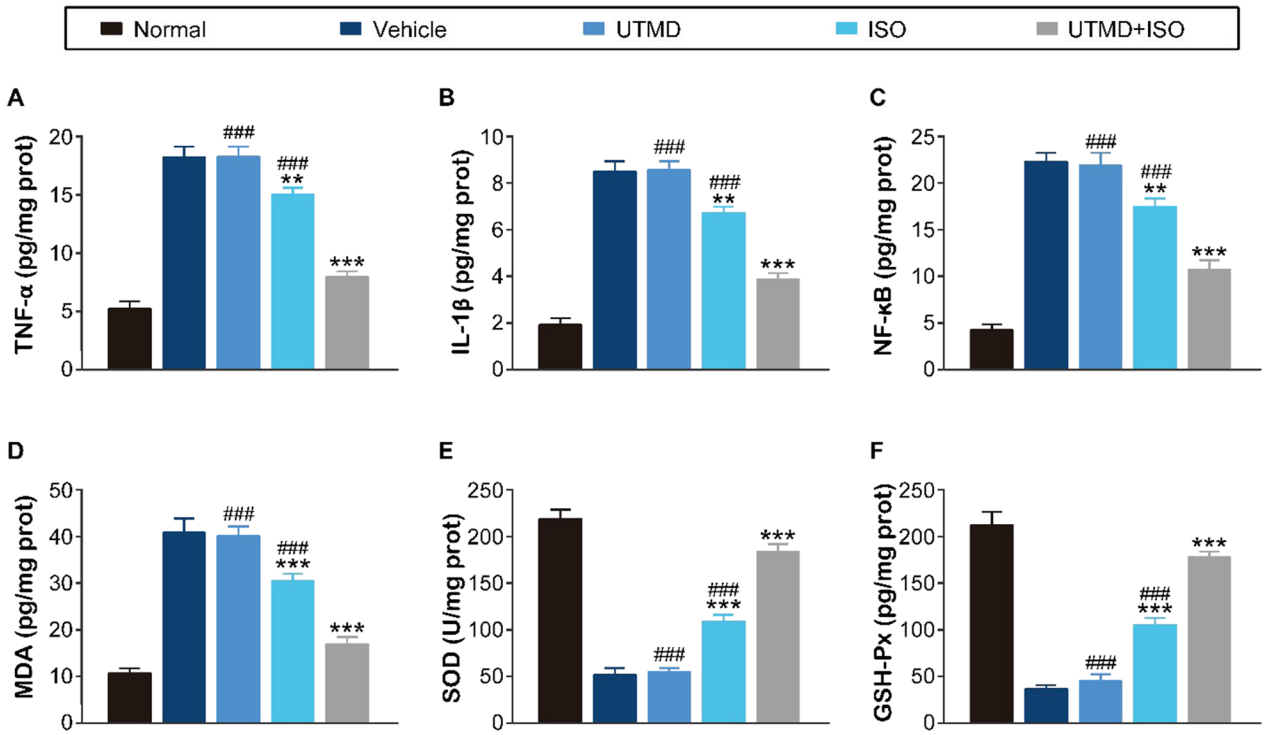

Fig. 5 Chronic treatment of UTMD combined with ISO microbubbles ameliorates inflammation and oxidative stress in DCM rats. The levels **A** TNF-α, **B** IL-1β, **C** NF-κB, **D** MDA, **E** SOD and **F** GSH-Px of myocardial tissue from the DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

cTnI levels and LDH and CK activities in DCM rats were all significantly down-regulated, suggesting a significant improvement of myocardial injury. ET is the most potent and long-lasting vasoconstrictor polypeptide known to date [26]. When cardiac function is impaired, ET secretion doubles and participates in and promotes the development of heart failure [26]. The increase of ET is associated with a decrease in stroke volume, an increase in systemic and pulmonary resistance, and cardiac remodeling, hypertrophy, and fibrosis [27]. CGRP is widely distributed in the cardiovascular system and is involved in the regulation of many cardiovascular functions in the body, which is of great significance for the occurrence, development, prevention and treatment of heart failure [26]. CGRP is currently the strongest known vasodilator polypeptide in vivo [28]. Previous study have shown that CGRP has the effect of enhancing myocardial contractility [28]. Myocardial injury occurred with increased ET levels and decreased CGRP, and there was a significant negative correlation between ET and CGRP levels [29]. Consistent with previous findings, ET levels were significantly increased and CGRP levels were significantly decreased in myocardial tissue of DCM rats, indicating the occurrence of myocardial injury. Interestingly, the combined treatment of UTMD and ISO microbubbles significantly reversed the changes in ET and CGRP, suggesting that combined treatment significantly ameliorated myocardial injury in DCM rats.

Subsequently, we analyzed the improvement of myocardial tissue in DCM rats by histopathology. H&E and Masson staining analysis exhibited that the myocardial cells of DCM rats in the control group were disorganized, the cell staining was uneven, and the collagen fibers in the myocardial tissues were up-regulated. However, UTMD combined with ISO significantly decreased the increase of collagen fibers in myocardial tissue of DCM rats, suggesting that combined treatment prevented myocardial tissue fibrosis and improved myocardial injury.

ISO has been shown to have anti-inflammatory and antioxidant biological functions, whereas diabetic myocardial injury is highly associated with the development of oxidative stress and inflammation [19]. Therefore, the inflammatory and oxidative stress-related proteins levels in myocardial tissues were further detected by ELISA. It has been shown that macrophage infiltration in the adipose tissue of diabetic patients with lipid metabolism disorders were observed, as well as the excessive feedback of fat storage signals activates the chronic inflammatory response [30]. The results of current study showed that the levels of pro-inflammatory factors, including the

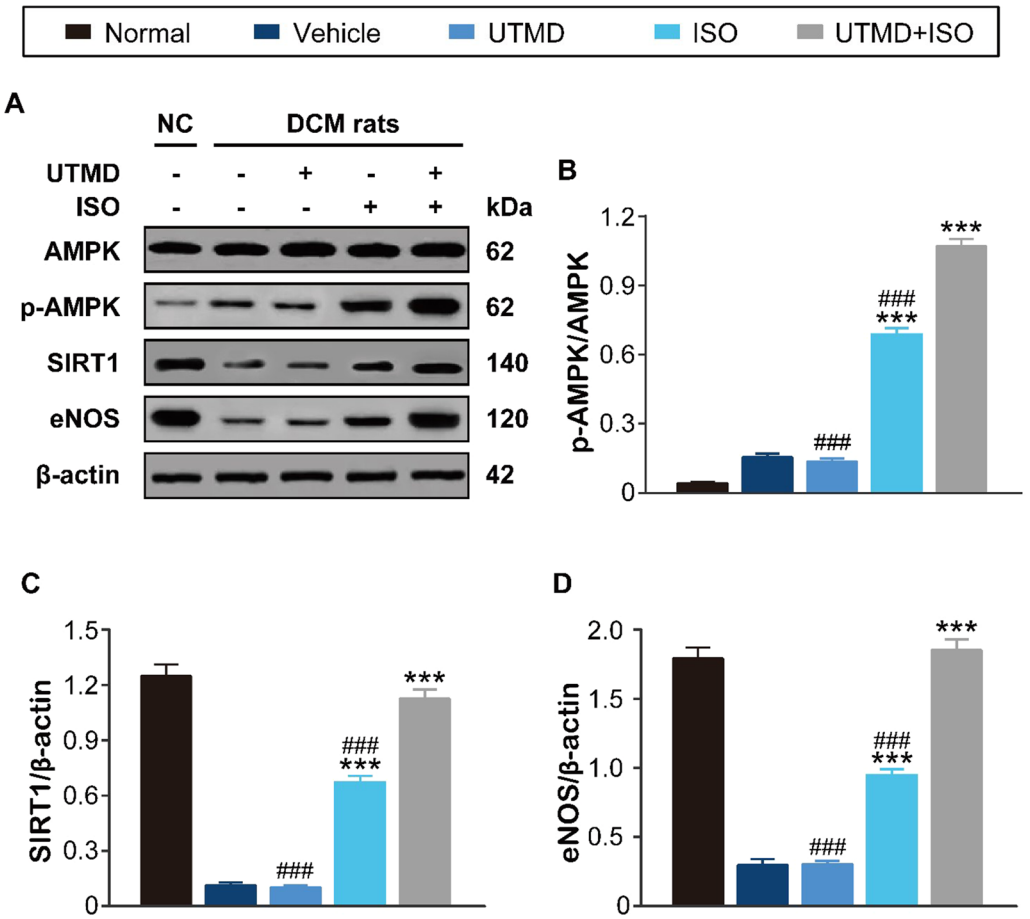

Fig. 6 Chronic treatment of combined UTMD with ISO microbubbles on the protein expression of AMPK/SIRT1/eNOS signaling pathway. **A** The representative images of western blot gel and the expression of **B** p-AMPK/AMPK, **C** SIRT1 and **D** eNOS of myocardial tissue from the DCM rats after chronic treatment for 8 weeks. All results showed as Mean ± SD (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ vs. UTMD + ISO group

TNF-α, IL-1β, and NF-κB, in the myocardial tissue of DCM rats were significantly increased, while the combined treatment of ultrasound and ISO microbubbles up-regulated the levels of these proinflammatory factors. Under normal physiological conditions, the generation of oxygen free radicals is usually under strict regulation [31]. Aerobic organisms have a complete set of perfect free radical scavenging systems, so that the generation and scavenging of free radicals in a dynamic balance [31]. When the dynamic balance of free radical generation and scavenging is disrupted, that is, free radical generation increases, and/or free radical scavenging decreases. Moreover, if there is a large amount of accumulation of free radicals in the body, it will inevitably cause damage to the tissue structure [32, 33]. MDA is an important product of membrane lipid peroxidation, and is also a marker of oxidative stress [34]. SOD catalyzes the disproportionation reaction of superoxide anion and protects cells from toxic oxygen radicals, which is the first line of defense against reactive oxygen species [35]. The main function of GSH-Px is to remove peroxides from various biomacromolecules [36]. While the SOD activity and GSH-PX level were both significantly down-regulated and decreased, respectively, while the MDA level was significantly increased in the myocardial tissues of DCM rats, indicating the occurrence of oxidative stress. Eight weeks of combined treatment significantly reversed these changes in DCM rats, illustrating that ultrasound-mediated ISO microbubbles protect antioxidant metabolism in DCM rats.

Silencing regulatory protein 1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent histone deacetylase [37]. Recent studies have shown that SIRT1 expression is down-regulated, adenylate-activated protein kinase (AMPK) phosphorylation levels are decreased, and endothelial nitric oxide synthase (eNOS) activation is reduced when diabetic myocardial injury occurs, which leads to oxidative stress responses

[38, 39]. In addition, it has been shown that phosphorylation of AMPK can further activate downstream signaling pathways, thereby reducing inflammatory responses and oxidative stress [40]. The expression of SIRT1/AMPK signaling pathway-related proteins in myocardial tissues of DCM rats were analyzed by western blotting method. The results showed that the expression levels of SIRT1 and eNOS were significantly down-regulated in the myocardial tissues of DCM rats in the control group (both $P<0.05$). Combined treatment of UTMD with ISO microbubbles significantly up-regulated the expression levels of SIRT1 and eNOS. Moreover, the combined treatment of UTMD with ISO microbubbles also significantly upregulated the phosphorylation level of AMPK. Current studies collectively indicated that the combined treatment of UTMD and ISO microbubbles can ameliorate myocardial injury in DCM rats by activating SIRT1/AMPK/eNOS signaling pathway and inhibiting inflammatory and oxidative stress responses.

The current results are the first to show that ultrasound-mediated ISO microbubbles protect against diabetic myocardial injury by activating the AMPK/SIRT1/eNOS signaling pathway. Activation of AMPK is a well-known mechanism by which further ameliorates the development of myocardial tissue inflammation and oxidative stress by increasing SIRT1 availability and activating eNOS. Importantly, our results provide compelling evidence that ultrasound-mediated ISO microbubbles can inhibit myocardial injury in doxorubicin-induced diabetic rats. The current study provides new insights into ultrasound-mediated ISO microbubbles as potential medication for the treatment of diabetic myocardial injury.

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Author contributions
SL: conceptualization, supervision, Writing—review & editing; SL: methodology, data acquisition, data analysis, writing—original draft; LZ: data acquisition, data analysis, validation, writing—review & editing.

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Availability of data and materials
The datasets used and/or analyzed during the current study are included in the manuscript.

Declarations

Competing interests
The authors declare no conflict of interest.

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