Ultrasound-targeted Microbubble Destruction Promotes Myocardial Angiogenesis and Functional Improvements in Rat Model of Diabetic Cardiomyopathy

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Abstract

Objective: Microvascular insufficiency takes a critical role in the development of diabetic cardiomyopathy (DCM), therapeutic angiogenesis has been mainly used for the treatment of ischemic diseases. The present study sought to investigate the preclinical performance of SonoVue microbubbles(MBs) combined ultrasound(US) treatment on myocardial angiogenesis in a rat model of DCM and investigate the optimal ultrasonic parameters.

Methods and Results: After the DCM model was established, the cardio protective effect of SonoVue MBs using US techniques was examined by histology, morphometry, and echocardiography evaluations. From morphologic observation and echocardiography, the DCM rats had a set of structural abnormalities in the heart compared to the normal rats. The US-MB groups exerted cardio protective effect in DCM rats, improved reparative neovascularization and increased cardiac perfusion, while the 26 cycle group showed significantly therapeutic effects on the cardiac functions in DCM rats.

Conclusion: This strategy using SonoVue MB and US can significantly improve or even reverse cardiac dysfunction and pathological abnormalities, especially using the 26 cycle parameters. Under further study, this combined strategy might provide a novel approach for early intervention of DCM in diabetic patients.

Introduction

Diabetes mellitus (DM) is a common metabolic disorder characterized by high blood glucose owing to a deficiency in insulin secretion, resistance to insulin, or both. DM can cause many acute and chronic complications, leading to the decline of people's quality of life, loss of labor force, and so on. Among the complications, diabetic cardiomyopathy (DCM) is characterized by the morphological, functional, and metabolic changes in the heart, which is a common cardiovascular complication independent of coronary artery disease and hypertension[1]. It has been receiving extensive attention due to the associated morbidity and fatality rates[2, 3]. However, DCM is often neglected in the clinical process because of no special symptoms in the early stage and lack of specialized treatment strategies. Therefore, early detection and timely intervention therapy are of great significance for the prognosis of DM patients.

Microvascular insufficiency takes a critical role in the development of DCM[4]. Accumulating evidence has demonstrated an impaired angiogenic response to chronic ischemia that may lead to decreasing myocardium perfusion, which finally contributes to interstitial fibrosis, tissue hypoxia, and heart failure[5, 6]. Therefore, promoting cardiac angiogenesis and improving microcirculation function has become an important strategy for the treatment of DCM.

Recently, therapeutic angiogenesis has been mainly used for the treatment of ischemic diseases [7, 8]. However, the reported administration of proteins and genes, including an intracoronary, intrapericardial or myocardial administration, are inconvenient and high risk in clinical applications. At the moment, an emerging technique, ultrasound-targeted microbubble destruction (UTMD) has been proposed for a noninvasive and target specific approach in angiogenesis therapy of cardiovascular disease.

Previous studies have shown that combining a non-viral vector with UTMD technique is an effective strategy to deliver basic fibroblast growth factor (bFGF) to the heart, and the resulting growth factor therapy has demonstrated potential to reverse the progress of DCM by restoring the cardiac functions and even the structure of damaged cardiac tissues[9]. However, the lack of an efficient and safe delivery system limits FGF1 application in vivo.

As an ultrasound contrast agent, SonoVue (Bracco International BV, Amsterdam, Netherlands) showed high safety, good contrast-enhanced images and relatively low cost. The purpose of this study was to investigate the effects of SonoVue /UTMD combined treatment on myocardial angiogenesis in a rat model of DCM and inspect the optimal ultrasonic parameters.

Materials And Methods

Microbubble preparation

An ultrasound contrast agent named Sonovue (Bracco Diagnostics, Princeton, NJ, USA) was used as microbubbles (MBs). It was dissolved in 5 ml physiological saline to make a SonoVue solution, which contained 59 mg SF₆ fluoride sulfur gases and 25 mg lyophilized power to form the microbubble suspension. The microbubble (MB) concentration in the solution was measured by cell counting microscopic method. The MB concentration in the solution was about 2×10^8 bubble/mL with an average diameter of 3.4 μ m. The diluted solution of microbubble suspension (1 mL microbubble suspension in 19 mL normal saline, which was diluted by 20 times) was used in therapy. The therapeutic dose was about 1 mL/ piece, and the continuous injection lasted for 20 min. Oscillation shaking of the MBs was performed and shaking was continued in the process of treatment.

DCM Animal Models

The study was approved by the Animal Care and Use Committee of Xinxiang Medical College. All animal-handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Diabetes was induced in male Sprague-Dawley (SD) rats (7-8 weeks, 160-180 g) by intraperitoneal injection of streptozotocin (STZ, Sigma Corporation, USA) once at 65 mg/kg after 12 h of fasting. STZ was prepared as 0.1 mol/L citrate buffer (pH 4.0-4.5). The control rats were injected with the same volume of 0.1 mol/L citrate buffer. After one week, the DM rats were fed with high fat diet (basal diet added with 18% W/W fat oil and 20% W/W glucose) for 8 weeks, while the control group was fed with a basal diet. On the 3rd day, 7th day and 9th week after administration of STZ, the fasting blood glucose was measured from the tail tip. Only the rats with fasting blood glucose levels exceeding 16.7 mmol/L were selected as DM rats using in the following study [10-12].

Groups and Treatments of Animals

Before and 2 weeks after the intervention, all the animals underwent an echocardiographic study (VINNO 70, China). The animals were anesthetized with an intraperitoneal injection of 30 mg/kg sodium pentobarbital. They were placed in the supine position and the thoracic region was shaved. The VINNO 70 color Doppler diagnosis system (VINNO Suzhou, China) combined with VFLASH (a software which can manipulate microbubble cavitation) was utilized to generate the UTMD effect. The linear array transducer (Vinn X4-12 probe, 14 MHz) was placed over the heart of the rat (short axis view; depth 3.0-4.0 cm), and the coupling agent (ultrasound gel) was filled between the probe and the skin. M-mode images were obtained at the papillary muscle level from two-dimensional (2D) short-axis views (Figure 1). The left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVIDd), interventricular septum dimensions (IVS) and left ventricular fraction shortening (LVFS) were measured by conventional M-mode echocardiography in all rats.

After satisfactory images were obtained, a dosage of 1 mL diluted SonoVue solution was administered into the tail vein continuously over 20 minutes. At the same time, the VFLASH mode of the scanner was switched on for UTMD (frequency: 4 MHz, pulse frequency: 20 Hz, exposure time = 1.2 s, US exposure duration per time = 2 s) and the heart was in the region of interest (ROI) during the experiment.

The experimental rats were randomized into three groups: (1) Control group: non-diabetic rats were administered 1 mL normal saline; (2) DCM group: DCM rats were administered 1 mL normal saline. (3) MB+UTMD group: DCM rats were treated with 1 mL SonoVue solution combined with US. The third group was divided into four subsets according to different pulse lengths (PL) (8 cycles; 18 cycle; 26 cycle; 36 cycle). For all animals, the second treatments were processed after one week of first treatments.

Histology and morphometry

Three rats from the control group and DCM group were sacrificed after modeling to observe the pathological changes of the pancreas and myocardium. After all the experiments were completed, the rats were sacrificed. The left ventricular tissues were fixed in 10% paraformaldehyde, embedded in paraffin, and sectioned transversely (5 μ m). Sections of papillary muscle were stained with hematoxylin and eosin (HE) for histopathology.

CD31 immunohistochemistry was used to identify capillaries to measure myocardial capillary density (MCD). MCD was measured by counting the number of brown-stained capillaries under 20 visual high power fields (400 $\times$), and presented as the mean number of blood vessels per high power field (n/hpf). [13]

Statistical Analysis

Data analysis was performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). The normal distribution of the measurement indicators was expressed as the mean $\pm$ SD. The multivariate sample means were compared for variance homogeneity test. The mean comparison between groups was analyzed by one-way ANOVA. The LSD-t test was used to compare the two groups. Dunnett's T3 test was performed on the variance, and the difference was statistically significant at $P < 0.05$.

Results

General Condition of Experimental Rats

After all intervention, 8 rats in the DCM model group, 36 cycle group and 8 cycle group died, 6 rats in the 18 cycle group died, 7 rats in the 26 cycle group died, and 2 rats in the normal control group died. The final number of rats in each group were: 12 in the DCM model group, 12 in the 8 cycle group, 14 in the 18 cycle group, 13 in the 26 cycle group, 12 in the 36 cycle group, and 14 in the normal control group.

For blood glucose analysis, rats fasted overnight and blood samples were collected. Serum was separated and levels of glucose were measured. The blood glucose values after modeling were higher than 16.6 mmol/L, and they were significantly increased compared to the normal control groups ($P < 0.01$, Table 1). The glucose values after modeling were also significantly increased compared to those before modeling ($P < 0.05$) for all groups except the control group.

Histological evaluation

After modeling, the histological changes of the pancreas are shown in Fig.3. The islet cells in the pancreas of the normal rats (Fig.3A) were oval and round, while the islet cells of the DCM rats (Fig.3B) were destroyed and distributed in a loose arrangement with deformed shapes.

After all interventions, the histological changes of the heart are shown in Fig.4. The cardiac myocytes of the normal group (Fig.4A) had a clear cell structure and stained nuclei with an organized arrangement, while the cardiac myocytes of the model group (Fig. 4B) had a hypertrophied and deformed shape and were distributed in an altered and disorganized arrangement with obvious vacuolization, the nuclei were also deeply stained. The DCM rats had a set of structural abnormalities in the heart, and therefore, the US+MBs treatment groups (Fig. 4D, 4E, 4F) showed decreased hypertrophy, fibrosis and cardiomyocyte lesions.

Electron Microscopic Findings

To evaluate the changes of microvascular under different intervention conditions, the ultrastructural changes of the cardiac microvascular were examined under electron microscope. As shown in Figure 5, in the normal group, the surface of cardiac microvascular was smooth, there was no obvious convexity, and the end structure of blood vessels were intact, while the surface of cardiac microvascular in the model control group were more burred and continuity was missing, the endothelial cell membrane was incomplete, and the heterochromatin of the inner nucleus increased and agglomerated, which indicated the pathological state of these microvessels. In the 36 cycle group, myocardial arterioles were not smooth, continuity was missing, endothelial cell membrane was incomplete, nuclear heterochromatin increased and agglomerated, showing a state of injury. In the 8 cycle group, 18 cycle group and 26 cycle group, the surface of cardiac microvascular were smooth and continuous, the endothelial cell membrane was basically intact, and the cytoplasmic mitochondria were angiogenesis, while the myocardial samples in the 26 cycle group showed the relatively well-integrated ultrastructures compared with other US-MBs treatment and DCM group.

Echocardiography Evaluation

After 12 weeks of intervention, two-dimensional parameters, including LVIDd, IVs, LVEF, LVFS and HR were measured by transthoracic echocardiography to monitor the improvement of global myocardial contractile function. As shown in Table 1, the LVEF, LVFS and HR in the DCM model group were significantly lower than those in the normal control group ($P < 0.05$). Compared with the DCM model group, there was a certain extent of improvement in the levels of the LVEF, LVFS and HR of the UTMD intervention group. The LVEF and LVFS in the US+MB subgroup were significantly increased compared with the DCM model group (all $P < 0.05$). Among all the US+MB groups, the 26 cycle group showed the highest LVEF and LVFS after treatment and had no

statistical significance when compared to the normal group ($P < 0.05$). There was no statistical significant difference in HR in the 8 cycle group and DCM group after intervention, but a distinct difference was observed between the DCM group and other US-MB groups in HR with a P value < 0.05 . There was a significant increase in the level of HR in the US-MB group but they still did not reach the levels of the normal control rats. There was no statistical significant difference in IVS and LVIDd in UTMD group and DCM model group with a P value > 0.05 after intervention.

Table 1 Basic Echocardiographic Data of the rats in each group.

Characteristics	US+MB Subgroups					
	Normal	DCM model	8 cycle	18 cycle	26 cycle	36 cycle
LVIDd(mm)	7.42±0.21	7.44±0.41	7.36±0.48	7.23±0.69	7.41±0.78	7.61±0.69
IVS(mm)	1.61±0.16	1.54±0.19	1.51±0.14	1.62±0.17	1.55±0.18	1.59±0.18
LVEF(%)	73.51±4.44#	57.76±6.03*	64.48±5.12*#	65.59±6.27*#	68.97±4.84#	63.49±5.34*#
LVFS(%)	35.06±3.15#	27.70±3.24*	29.94±3.55*#	31.88±4.28*#	34.01±3.97#	30.78±3.87*#
HR	378.30±14.44#	288.78±15.82*	301.67±16.68*	321.21±29.58*#	331.13±20.95*#	307.42±23.81*#

P.S. LVIDd: left ventricular internal dimensions; IVS: interventricular septum dimensions; LVEF: left ventricular ejection fraction; LVFS: percentage of left ventricular fractional shortening; HR: heart rate; *: $P < 0.05$, VS normal group; #: $P < 0.05$, VS model group;

Myocardial Capillary Density

CD31 immunohistochemical staining showed that the vascular endothelial cells were brown. The MCD of myocardial tissue in the DCM model group was significantly decreased than that in the normal control group ($P < 0.05$). The MCD in each US-MBs intervention group was higher than that in the DCM model group, but the increase of MCD in 8 cycle group had no statistical significance when compared to the DM model group ($P > 0.05$), while the other US-MB groups were significantly higher than that in the DCM model group ($P < 0.05$). Among all the US-MB groups, the level of MCD in the 26 cycle group showed no statistical significant difference compared to the normal group ($P > 0.05$).

Discussion

Microcirculatory dysfunction is believed to play an important role in DCM. Accumulating evidence has demonstrated a progressive reduction of the microvasculature and an impaired angiogenic response to chronic ischemia with the development of diabetes[14]. Recently, low-intensity ultrasound in combination with microbubbles has been showed to stimulate endogenous vascular growth factor, inducing angiogenesis, used in ischemic diseases [15-17]. As gas-filled colloidal materials, SonoVue MBs consisted of an inert gas core and a shell composition of phospholipid, polymer with a typical mean diameter of 2.5µm. It is a biocompatible and biodegradable material that has been extensively utilized in clinics with sufficient bio-safety. In this study, we evaluated the in vivo therapeutic effects of SonoVue /UTMD combined treatment in a rat model of DCM and explored optimal parameters of ultrasound devices in different group settings.

In this study, the rat model was established by intraperitoneal injection of STZ. After modeling, both the blood glucose levels(Fig.2) and histological changes of the pancreas and hearts (Fig 3,4) proved that the DCM rats were successfully induced. Then the rats were divided into six groups according to the different interventions. After all treatments, as shown in Table 1, the LVEF, LVFS and HR in the DM model group were significantly lower than those in the normal control group ($P < 0.05$). Consistent with previous report[18, 19], the diabetic rats left untreated for 12 weeks were characterized by a declined systolic myocardial performance and had a set of structural abnormalities in the heart compared to the control group. Therefore, it is very important to prevent the occurrence of DCM by intervening before the obvious pathological changes in myocardium in DCM rats.

All of the echocardiography evaluation criteria (Table 1) showed that there were very significant improvements in the levels of LVEF and LVFS, along with a good change in HR for the US-MBs treatment group as compared to the DM model group, while LVEF, LVFS and HR in the 26 cycle group were significantly higher than those in the other US-MB intervention groups. The results indicated that UTMD combined with Sonovue MBs could significantly restore the cardiac functions in DCM animals, especially when the US pulse length parameter is 26 cycles. The histochemical staining data presented in Fig.4-6 showed signs of moderate structural recovery in the US+MB treatment group compared to the DCM model group, further suggested that the improved functions were the results of structural remodeling of the cardiac tissues and thus may bring longer lasting therapeutic benefit.

US-MBs intervention could increase microvascular density, reverse the ultrastructures of myocardial microvascular injury, improve reparative neovascularization and increase cardiac perfusion. As the diabetes disease progresses, the microangiopathy exerts changes in the morphology and density of microvasculature[20]. As shown in Fig.5, alterations in the surface of cardiac microvascular and destruction in endothelial cell membrane were observed in the myocardial samples by electron microscopy in DCM rats compared with the normal control ones. There were similar alterations in the ultrastructures of the surface of cardiac microvascular, as observed by electron microscope, in the group with UT-MBs treatment compared with the DCM group (Fig.5). In our study, the CD 31 immunohistochemical assay (Fig 6) also confirmed comparable therapeutic effect against the DCM-related microangiopathy. The complex mechanism of angiogenesis using US-MBs treated in DCM model remains to be explored, it may be related to two factors. First of all, cavitation and mechanical effects of blasting could increase oxidative stress and induce local inflammatory factors, and then release vascular growth factor to activate angiogenesis[21]. Secondly, increasing the blood flow using the US-MBs treatment are most likely mediated by cavitation-related increases in shear and activation of endothelial nitric oxide synthase[22]. Huang et al.[23] suggested that the PI3K/ Akt signal pathway might participate in modulating the activity of eNOS. Akt signaling protects against myocyte apoptosis induced by cardiac ischemia-reperfusion injury and DCM[24] [25] . The protective mechanism of US-MBs promoting angiogenesis may be associated with the activation of the PI3K/Akt signaling pathway by the up-regulation of expression of phosphorylated Akt protein to activate eNOS[24, 26].

Among various US-MBs treated groups, we found that different pulse length could achieve different intervention results. There were well-integrated ultrastructures in the myocardial samples in the 26 cycle group compared with other US-MBs treatment and DCM group, while it also demonstrated the highest effectiveness in increasing density of microvasculature. Furthermore, the routine echocardiography (Table 1) showed that the 26 cycle group significantly restored the cardiac functions in DCM rats. Therefore, this method can be used as an effective strategy to prevent the deterioration of cardiac function in DCM rats.

The limitations of this study are as follows: First, the improved functional and reversed pathological changes in the 26 cycle group still did not reach the levels of the control rats, which may related to the short intervention. Therefore, increasing intervention time and further optimize the parameters should be considered in the future studies. Second, because the pathogenesis of DCM is complex and the effect of UT-MBs alone is limited, whether it is necessary to combine other drugs to improve efficacy needs further study.

Conclusion

In conclusion, while using non-viral vectors Sonovue MBs combined with UTMD technique can significantly improve or even reverse cardiac dysfunction and pathological abnormalities, especially using the 26 cycle parameters. Under further study, this combined strategy might be a promising option for early intervention of DCM in diabetic patients.

Declarations

Ethics approval and consent to participate

All study protocol and image procedures have been approved by the Animal Care and Use Committee of Xinxiang Medical College

Consent for publication

Consent for publication obtained by all the authors.

Availability of data and materials

All data and methods are available from the corresponding author by request.

Competing Interests

The authors have declared that no competing interest exists

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Author Contributions

XZ and HZ: manuscript writing; XZ , NZ, HZ and YY: ultrasound treatment and data anlysis; YJ and ZF : collection of the samples, animal model and histology examination; PL, JY and XT: Design of this study, interpretation of data and critical revision of the manuscript.

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Abbreviations

DM:diabetes mellitus; DCM:diabetic cardiomyopathy; UTMD:ultrasound-targeted microbubble destruction ; bFGF: basic fibroblast growth factor; MBs: microbubbles ; MB: microbubble ; STZ :streptozotocin ; LVEF :left ventricular ejection fraction; LVId :left ventricular end-diastolic diameter; IVS: interventricular septum dimensions; LVFS: left ventricular fraction shortening; HE:hematoxylin and eosin; MCD:myocardial capillary density; HR: heart rate

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Figures

Figure 1

Contrast ultrasonography of SonoVue MB in rat heart. (A: 2D echocardiograms B: Ultrasound images before the filling of MB. C: left ventricular was filled with MB)

Figure 2

Blood glucose levels of rats after modeling Comparing with normal group, blood glucose in model group and treatment group increased significantly($P < 0.05$).

Figure 3

The histological changes of the pancreas after modeling. A: the normal group; B: the DCM model group

Figure 4
 The histological changes of the heart after intervention. A: the normal group, B: the DCM model group, C: 8 cycles group, D: 18 cycle group, E: 26 cycle group, F: 36 cycle group

Figure 5
 Representative pictures of electron micrographs (10000×) of cardiac microvascular from the rats of each group. (A) normal control group; (B) DCM model group; (C) 8 cycle group; (D) 18 cycle group; (E) 26 cycle group; F: 36 cycle group

Figure 6

Representatives of CD31 immunohistochemical staining of MCD and the semi-quantitative analysis for all groups(400×). (A) Normal control group; (B) DCM model group; (C) 8 cycle group; (D) 18 cycle group; (E) 28 cycle group; (F) 36 cycle group (G)Statistical histogram. a: $P < 0.05$ vs the normal control group, b: $P < 0.05$ vs the DM model group Abbreviations: DCM, diabetes mellitus; SD, standard deviation; MCD, myocardial capillary density; UTMD, ultrasound-targeted microbubble destruction; n/hpf, number of blood vessels per high-power field.