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Prevent diabetic cardiomyopathy in diabetic rats by combined therapy of aFGF-loaded nanoparticles and ultrasound-targeted microbubble destruction technique

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ABSTRACT

Acidic fibroblast growth factor (aFGF) has shown the great potential to prevent the structural and functional injuries caused by diabetic cardiomyopathy (DCM). The present study sought to investigate the preclinical performance and mechanism of the combination therapy of aFGF-nanoparticles (aFGF-NP) and ultrasound-targeted microbubble destruction (UTMD) technique for DCM prevention. From Mason staining and TUNEL staining, aFGF-NP + UTMD group showed significant differences from the diabetes group and other groups treated with aFGF or aFGF-NP. The cardiac collagen volume fraction (CVF) and cardiac myocyte apoptosis index in aFGF-NP + UTMD group reduced to 4.15% and 2.31% respectively, compared with those in the diabetes group (20.5% and 11.3% respectively). Myocardial microvascular density (MCD) in aFGF-NP + UTMD group was up to 35 n/hpf, much higher than that in the diabetes group (14 n/hpf). The diabetes group showed similar results (MCD, CVF and cardiac myocyte apoptosis index) to other aFGF treatment groups (free aFGF ± UTMD or aFGF-NP). Indexes from transthoracic echocardiography and hemodynamic evaluation also proved the same conclusion. These results confirmed that the abnormalities including diastolic dysfunctions, myocardial fibrosis and metabolic could be suppressed by the different extents of twice weekly aFGF treatments for 12 consecutive weeks (free aFGF or aFGF-NP ± UTMD), with the strongest improvements observed in the aFGF-NP + UTMD group. Western blot and immunohistochemical analyses of heart tissue samples further revealed the high efficiency of heart-targeted delivery and effective cardioprotection with this combination approach. Overall, this study has generated supportive data that are critical for the translation of a promising DCM prevention strategy.

1. Introduction

Diabetic cardiomyopathy (DCM) is described as the structural and functional changes in the myocardium that are associated with diabetes (DM) in the absence of ischemic heart diseases, hypertension, or other cardiac pathologies [1,2]. The structural changes include fibrosis, apoptosis, angiopathy of myocytes and the functional changes include endothelium-myocytes uncoupling, impairment for contractility of cardiomyocytes, decrease in survival and differentiation of cardiac stem cells as well as diastolic and systolic dysfunction [3,4]. DCM has been identified as the leading cause of morbidity and mortality in DM patients. However, up to date there is no effective treatment for this common yet lethal pathological condition.

Acidic fibroblast growth factor (aFGF, also known as FGF-1) is a 15.8 kDa peptide and is also referred to as heparin-binding growth factor 1 because of its affinity for heparin. aFGF induces endothelial and smooth muscle cell proliferation and angiogenesis *in vivo* [5]. In addition, aFGF has shown to be an important pro-survival anti-apoptotic factor in a variety of cell types [6]. Zhang et al. showed that the non-mitogenic aFGF has the therapeutic effects on DCM by the suppression of oxidative stress and damage in diabetes rats [7]. aFGF is thus a potentially valuable therapeutic agent for DCM treatment. However, there is a strong need to optimize the mode of aFGF delivery aiming at minimizing the impact on systemic tissues (*e.g.* liver, spleen,

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lung and kidney), while retaining the aFGF bioactivity on the myocardial tissues. The current strategies for delivery of exogenous aFGF or aFGF gene to the damaged myocardial tissue include direct cardiac injection of bolus dose and delivery by drug carrier. The use of carriers such as nanoparticles (NP) is less risky, and may also improve the stability of aFGF both during storage and in blood circulation. But without additional strategy to increase the selectivity for cardiac tissue, aFGF encapsulated nanoparticles still may not be able to improve the aFGF delivery to the heart without causing unnecessary impact on the other body tissues.

Recently, low-intensity ultrasound (US) in combination with microbubbles has been shown to improve the efficiency and tissue/organ specificity of *in vivo* uptake for nanoparticles [8]. When exposed to the low intensity ultrasound, microbubbles would lead to a stable cavitation (the oscillations of microbubbles) [9]. Such stable oscillations created a liquid flow around the microbubbles, the so-called microstreaming [10]. When these oscillating microbubbles were in close vicinity of cells, these cells would experience shear stress. Consequently, these US induced elevated shear stress levels can enhance cellular uptake of macromolecular drugs [11–13]. Therefore, this ultrasound-targeted microbubble destruction (UTMD) technique, which has been conventionally used as a clinical diagnosis, holds considerable promise as an effective strategy to achieve targeted delivery of aFGF from nanoparticle formulation to the heart.

The present study aimed at determining whether the combination therapy of UTMD technique with novel aFGF-loaded nanoparticles (aFGF-NP) is effective to prevent DCM in a diabetes animal model. In a previous study, we developed Poloxamer 188-grafted heparin copolymer which demonstrated high affinity for aFGF as a result of interaction with its heparin content [14]. This copolymer was therefore chosen for preparation of aFGF-NP in this study. To achieve an in-depth understanding of the therapeutic impact of the aFGF-NP/UTMD technique, a broad range of commonly used pathophysiological indicators of the heart conditions were measured in a DCM rat model induced by streptozotocin (STZ). These measurements allowed thorough preclinical evaluation of the *in vivo* effects of 12 weeks aFGF-NP + UTMD treatment on the cardiac functions and related structural damages. Overall, this study has generated comprehensive data that are critical for the translation of this promising combination therapy of DCM, a frequently occurred and deadly disease.

2. Methods

2.1. Preparation and characterization of aFGF-NP

2.1.1. Preparation of phospholipid-based aFGF-NP

aFGF (20 mg/ml) (Sigma-Aldrich, USA) was dissolved in 1 ml of 20% *w/v* Poloxamer 188-grafted heparin copolymer solution. The resulting solution was added into 2 mL of 2.0% *w/v* gelatin solution to produce a homogeneous mixture. Under sonication (110 w, 15 °C) using a probe sonicator, D, L-glyceraldehyde was injected into the mixture until its final concentration reached 0.1% *w/v*. The mixture solution was kept at 5 °C and aFGF-NP was formed by the cross-linking reaction under magnetic stirring at 2500 rpm for 5 h. Empty nanoparticles (blank nanoparticles, using Poloxamer 188-grafted heparin solution instead of aFGF Poloxamer 188-grafted heparin solution in preparation) and free aFGF solution (aFGF dissolved in 0.9% NaCl solution) were also prepared for comparison. Final aFGF concentration in the aFGF-containing solutions (aFGF-NP or aFGF solution) was 2 mg/mL.

2.1.2. Preparation phospholipid-based microbubbles

Lyophilized phospholipid-based microbubbles (PMB) were prepared by sonication-lyophilization method which was reported in our previous study [15]. The PMB concentration in the solution formed was about 2×10^9 bubble/mL with an average diameter of 3.4 μm as measured by coulter counter (Coulter Corporation, Hialeah, FL).

2.1.3. Characterization of aFGF-NP

The morphologies of aFGF-NP and blank NP were observed by scanning electron microscopy (SEM). Size and zeta potential values of aFGF-NP and blank NP were measured by dynamic light scattering using a Zeta Potential/Particle Sizer Nicomp™ 380 ZLS (PSS. Nicomp, Santa Barbara, CA, USA).

To determine the aFGF encapsulation efficiency of aFGF-NP, aFGF-NP dispersion was centrifuged at 10,000 *g* for 40 min. The supernatant was then collected and diluted for aFGF determination using an ELISA kit [16]. The drug encapsulation efficiency was calculated as indicated below. The analyses were performed in triplicate.

Encapsulation efficiency (%) = (total amount of drugs − amount of drugs in supernatant)/total amount of drugs added initially × 100%.

To evaluate the aFGF bioactivity after encapsulation in aFGF-NP, NIH-3T3 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a 96-well plate (7000 cells per well). The cells were incubated in 0.1% FBS 1640 medium for 24 h and treated with aFGF-NP suspension for 72 h. The number of viable cells was determined by MTT cell proliferation kit in accordance with the manufacture protocol.

To evaluate the stability of aFGF-NP in sonoportation, aFGF-NP encapsulation values before and after sonoporation were compared. 100 μL PMB and 100 μL aFGF-NP were mixed for 5 min in a sealed container and then added into 12-well plates. 200 μL 10% FBS was added into the mixture which was rotated at approximately 30 rpm for 60 s before ultrasound exposure. Sonoportation experiments were performed in a device described in a previous study [17]. A linear array transducer (15LSw.S probe, 12 ~ 14 MHz, Acuson Sequoia 512C system, Siemens) was used to generate the sonoportation. The ultrasound transducer was inserted in a 37 °C water tank and directly faced the bottom of the cell plate. A spongy rubber ultrasound shield was used to focus ultrasound on experimented cells. Each sample received designed ultrasound exposure in the water bath. The 12-well plate was held 4 cm from the submersed transducer (US exposure duration per time: 5 s, 10 s and 15 s respectively; repeat three times with off intervals of 1 s). After ultrasound exposure, the aFGF encapsulation efficiency of aFGF-NP was determined by the ELISA kit mentioned above.

2.2. Animal studies

2.2.1. Type 1 DM animal model

DM was induced in male Sprague Dawley (SD) rats (42 ~ 49 days, 180 ~ 220 g) by intraperitoneal single injection of streptozotocin (STZ, Sigma Corporation, USA) at 70 mg/kg after 12 h of fasting. On the 3rd day, 7th day, and 2nd week after STZ administration, the fasting blood glucose was measured from the tail tip using an autoanalyzer (Surestep, Roche, Germany). Only the rats with fasting blood glucose levels exceeding 16.7 mM and stabilized in the next two weeks were selected as diabetic rats. The normal control rats were injected with STZ-free citrate buffer instead. All animal experiments were performed under the approval and guidance of the Institutional Animal Care and Use Committee of Wenzhou Medical University.

2.2.2. Groups and Treatments of Animals

After the model of diabetic rats was induced, the experimental rats were randomized into seven groups (n = 8 per group): (1) DM group: DM rats were administered 1 ml normal saline. (2) Control: non-diabetic rats were administered 1 ml normal saline; (3) aFGF group: DM rats were treated with free aFGF (15 μg/kg) in 1 ml normal saline without PMB and US; (4) aFGF-NP group: DM rats were treated with aFGF-NP (15 μg/kg) in 1 ml normal saline without PMB and US; (5) UTMD group: DM rats were treated with 1 ml PMB solution only combined with US; (6) aFGF + UTMD group: DM rats were treated with a mixture of free aFGF (15 μg/kg) and PMB in 1 ml normal saline combined with US; (7) aFGF-NP + UTMD group: DM rats were treated