SUPPLEMENTARY MATERIAL

2. MATERIAL AND METHODS

2.1 Fabrication of phospholipid-based microbubbles

Hydrogenated phosphatidylcholine (HPC, HPC>99%, Doosan Corporation Biotech BU, Kyonggi Do, Korea), polyethylene glycol 1500 (Qingming Chemical Plant, Zhejiang Province, China), and Poloxamer 188 (Shenyang Chemical Plant, Liaoning Province, China) were dissolved in analytical-grade butanol (Beijing Chemical Plant, Beijing, China) and sonicated at 30°C using a JY 92-II ultrasonic processor (KunShan US Instrument Inc., KunShan, China) at a frequency of 40 kHz and power of 160 W for 3 minutes. The solution was stored at 0°C for 30 minutes and at –20°C for 1 hour. Then, the coagulated solution was lyophilized at 5×10^{-4} Pa for a total of 20 hours with primary drying at -48°C for 15 hours and then gradual temperature increase to 10 °C in 5 hours. PMB lyophilized powder was put in 10 mL vials (200 mg/vial) and saturated with electronic grade perfluoropropane (C_3F_8)(Institute of Special Gas, Tianjing, China). PMB solution was obtained by adding 2 mL 0.9% w/v NaCl solution in lyophilized PMB. The PMB concentration was 2×10^9 bubble/mL, with an average diameter of 3.4 μm as measured by a Coulter counter (Coulter Corporation, Hialeah, FL).

2.2 Preparation and characterization of bFGF-lip

bFGF-lip preparation First, bFGF was dissolved in 1 ml 20% w/v poloxamer 188–grafted heparin copolymer solution. Then, the solution was added into 2 mL of 2.0% w/v gelatin solution to produce a homogeneous mixture. The mixture solution was then sonicated (110 w, 15 °C, 15 s) using a probe sonicator to disperse bFGF. The emulsion was lyophilized to obtain the bFGF mixture powder. Then, lyophilized bFGF powder was dispersed in tert-butyl alcohol solution containing hydrogenated soybean phospholipids (HSPC) and cholesterol. By sonication (90 w, 20 s) at 25℃, the mixture suspension was then lyophilized to gain lyophilized powder containing bFGF-lip, which was

reconstituted in double-distilled water to form the bFGF liposome suspension for administration.

Blank liposomes, i.e., liposomes using gelatin solution instead of bFGF gelatin solution, and bFGF solution, i.e., bFGF dissolved in 0.9% NaCl solution, were also prepared for the experiments. The final bFGF concentration in bFGF-containing solutions was 2 mg/mL.

Characteristics of bFGF-lip The blank and bFGF-lip morphologies were observed by transmission electron microscope (TEM) (1230, Jeol Jem Company, Tokyo, Japan) using negative staining with 1% phosphotungstic acid. Each sample was diluted with 5% trehalose solution. A drop of the diluted sample was placed on the surface of a copper grid. The diluted sample was stained with 1% phosphotungstic acid and dried by air. The blank and bFGF-lip were examined by TEM. Zeta potential of the blank and bFGF-lip was determined by dynamic light scattering using a Zeta Potential/Particle Sizer Nicomp™ 380 ZLS (PSS. Nicomp, Santa Barbara, CA, USA).

ELISA assay was used to determine the encapsulating efficiency of bFGF-lip. First, 1.5 mL of the bFGF-lip dispersion was centrifuged at 10,000 g for 40 minutes. The supernatant was then collected and diluted for ELISA assay. The analyses were performed in triplicate, and the encapsulation efficiency was calculated as:

Encapsulation efficiency (%) = (total amount of drug − amount of drug in supernatant) / total amount of drug × 100%

NIH-3T3 cells were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) in a 96-well plate (7,000 cells per well) to assess bioactivity. The cells were incubated for 24 hours and placed in 0.1% FBS RPMI 1640 medium for 24 hours. Then, bFGF-lip suspension was added, and the cells were incubated for 72 hours. The number of viable cells was determined by adding 20 ml of methylthiazoletetrazolium (MTT; 5 mg/ml) to each well. The culture was incubated for 5 hours. After removal of the medium, 100 ml dimethyl sulphoxide (DMSO) was added to each well. After being maintained at room temperature for 30 minutes, the absorbance of the solution in the plate was measured at 490/690 nm.

2.4 Experimental design

The experimental design and grouping are summarized in Table S1. After DM was induced, the study animals were randomly divided into seven groups. All treatments were administered through tail vein twice a week for 12 weeks with or without ultrasound treatments. 1) DM (no treatment) group: the animals were administered STZ to create DM but treated with normal saline. (2) Control group: the animals were not administered STZ and were treated with normal saline. (3) bFGF group: bFGF (3 μg/kg) in 1 ml normal saline was administered without PMB and ultrasound treatment. (4) bFGF-lip group: bFGF-lip (3 μg/kg) in 1 ml normal saline was administered without PMB and ultrasound treatment. (5) UTMD group: only 1 ml PMB solution was administered and combined with ultrasound treatment. (6) bFGF + UTMD group: bFGF (3 μg/kg) and PMB mixture dissolved in 1 ml normal saline were administered and combined with ultrasound treatment. (7) bFGF-lip + UTMD group (study group): bFGF-lip (3 μg/kg) and PMB mixture dissolved in 1 ml normal saline and combined with ultrasound treatment.

2.7 Histological and molecular analyses

TUNEL staining An in situ detection kit from Roche Biochemicals was used according to the manufacturer's instructions. Briefly, the sections of myocardial samples were treated with H_2O_2 and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 hour at 37 °C. Labelled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody using 3,3-diaminobenzidine (DAB) as the chromogen. In the control group, TdT was omitted from the reaction mixture. Three ventricular sections (from the apex to the base) of each heart were analysed. Cardiomyocyte nuclei were quantified by randomly counting 10 fields per section. The apoptotic index (percentage of apoptotic nuclei) was calculated as apoptotic nuclei/total nuclei counted ×100%.

Immunohistochemistry staining Four micron thick paraffin sections of the LV were stained with polyclonal rabbit anti-rat CD31, Caspase-3 and pAKT antibodies (1:100)(Santa Cruz

Biotechnology, USA). The staining was visualized by reaction with 3,3-diaminobenzidine (1:20)(DAB; Sigma Chemical Co, USA). The sections were counterstained with Mayer's haematoxylin, dehydrated, and xylene-based mounted under glass coverslips. Brown coloured sites were quantified at a magnification of 200× and 400× with a microscope connected to a video camera.

The intensity of positive Caspase-3 and pAKT immunohistochemistry in tissue sections was analysed by integrated optical density (IOD) using the Image-Pro Plus 6.0 software (NIH). Briefly, four 40 × TIFF-format images from eight individual rats in each group were analysed in a blinded manner. All of the images were taken using the same microscope and camera sets. Image-pro Plus software was used to calculate the average IOD per stained area (μm^2) (IOD/area) for positive staining.

CD31 immunohistochemical staining was used to identify capillaries to measure MCD. Myocardial capillary density (MCD) was measured by counting the number of brown-stained capillaries in 20 visual fields using high power (400×) and presented as the number of blood vessels per high power field (n/hpf).

Western blot assay Proteins were isolated from homogenized tissues with TRIzol reagent (Invitrogen, Carlsbad, CA) using standard Invitrogen protocols. The protein concentration was measured by Braford protein assay. Equal amounts of protein (20 μg) were electrophoresed through a 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore Company, USA). The membrane was blocked with 5% skim milk powder in PBS before overnight incubation with primary antibodies against the following proteins: bFGF, AKT, phosphorylated AKT (pAKT), BCL-2, Bax and β-actin (Santa Cruz Biotechnology, USA). Proteins were then visualized using the secondary antibody goat anti-rabbit IgG-horseradish peroxidase (1:5,000) (Santa Cruz Biotechnology, USA) and visualized with a chemiluminescence system.

3. RESULTS

3.1 Characterization of bFGF-lip

Fig. S1 showed the representative TEM micrographs of the blank liposome and bFGF-lip. Both bFGF-lip and blank liposome showed good elliptical morphology. Characteristics of the blank and bFGF-lip are summarized in Table S2. Dynamic light scattering results demonstrated that the average particle sizes of blank and bFGF liposomes were 105±1.32 nm and 124±1.84 nm, respectively. The polydispersibility index (PI) (<0.3) was observed in both blank and bFGF-lip, indicating that blank and bFGF liposomes approached a monodispersed stable system.

The zeta potential showed both blank and bFGF liposomes possessed a strong negative charge on the surface with the zeta potential value below −15 mV, indicating the physical stability of the novel liposome.

The encapsulation efficiency of bFGF-lip reached 87.6±2.8%. The bioactivity of bFGF-lip was 7.4×10^5 IU/ml, suggesting that the bFGF-lip preparation did not alter the bioactivity of bFGF. There was no significant difference comparing the bioactivity between the bFGF-lip and free bFGF at the same quantity (approximately 7.7×10^5 IU/ml) ($P > 0.05$). These results indicated that the bFGF-lip preparation did not diminish the original bioactivity of bFGF.

SUPPLEMENTAL FIGURES AND TABLES

Fig. S1 Transmission electron microscope (TEM) of (A) blank liposome and (B) bFGF-lip. Liposomes generally show uniformity with good elliptical morphology.

Fig. S2. Inhibition of metabolism abnormalities under bFGF-lip treatment. A: Quantitative analysis of the body weight of rats. B: Quantitative analysis of the heart-to-body ratio (HW/BW) of rats. C: Quantitative analysis of the blood glucose of rats. N=8 per group; *P<0.05 *vs.* normal control group; #P<0.05 *vs.* DM group.

Table S1 Group design of experimental animals (n=8)

Table S2 Characterization of liposome and bFGF- liposome (n=5).

Table S3 Results of LVIDd, LVPW, and LVFS in control and study groups (n=8)

Table S4 Results of peak velocity, strain, and strain rate in control and study groups (n=8)

Table S5 The hemodynamic data in experiment in vivo (mean± SD, n=8)

Fig. S1

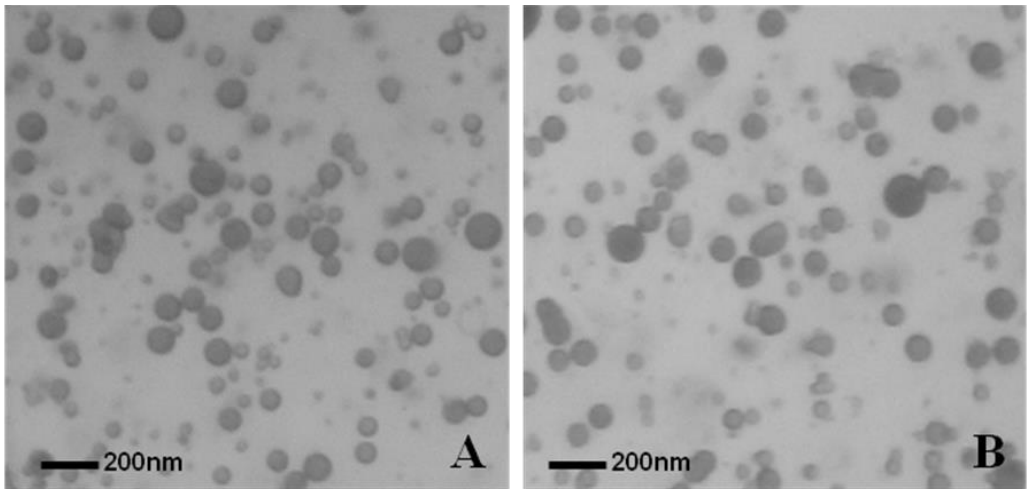

Fig. S1 Transmission electron microscope (TEM) of blank liposome (A) and bFGF-liposomes (B). Liposomes generally show uniformity with good elliptical morphology.

Fig. S2

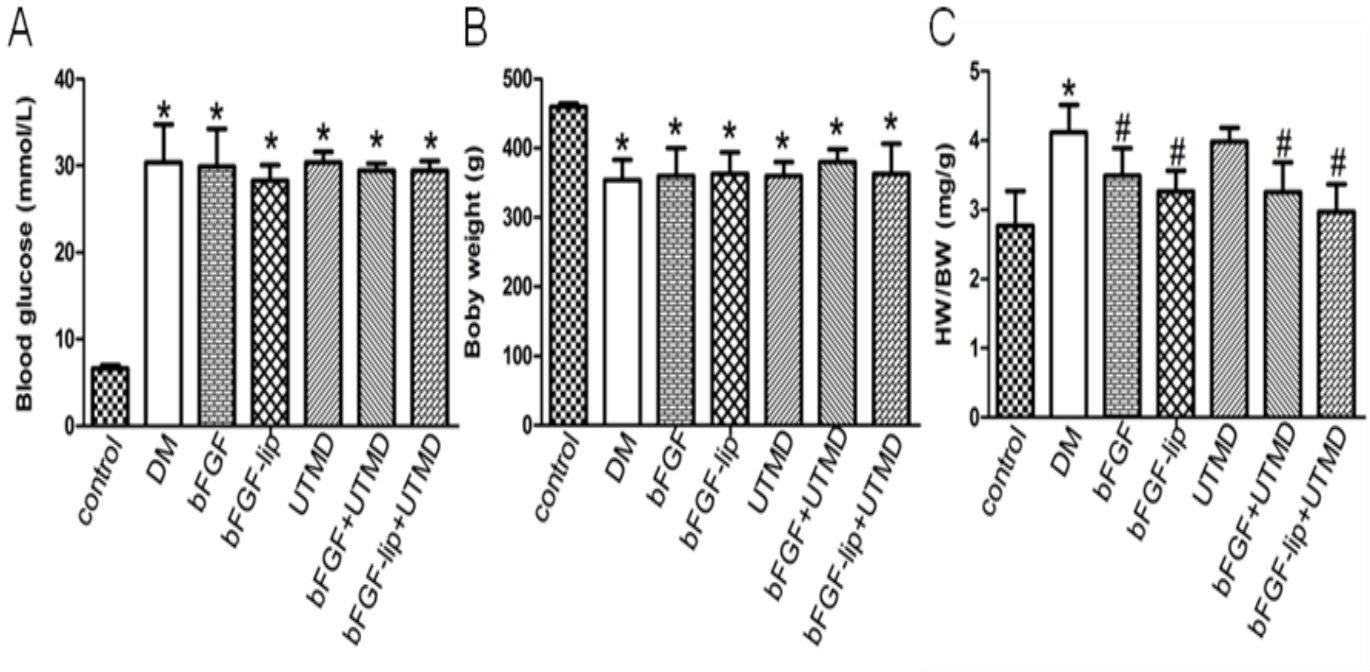

Fig. S2. Inhibition on metabolism abnormalities under bFGF-lip treatment. A: quantitative analysis of the body weight of rats; B: quantitative analysis of the heart-to-body ratio (HW/BW) of rats; C: quantitative analysis of the blood glucose of rats. n=8 per group; *P<0.05 *vs* normal control group; #P<0.05 *vs* DM group.

Table S1 Group design of experimental animals (n=8)

Group				Treatment			
No	Abbreviation	DM rat	Normal rat	Saline	bFGF	bFGF-lip	UTMD
1	Control		√	√			
2	DM	√		√			
3	bFGF	√			√		
4	bFGF-lip	√				√	
5	UTMD	√					√
6	bFGF+UTMD	√			√		√
7	bFGF-lip+UTMD	√				√	√

Notes: the groups administrated microbubble solution were simultaneously received ultrasound exposure; each group has the treatment two times a week for twelve weeks. n=8 per group.

Table S2 Characterization of liposome and bFGF- liposome (n=5).

Group	Particle Size (nm)	PI	Zeta Potential (mV)	Encapsulating efficiency (%)	Bioactivity ($\times 10^5$ IU/ml)
Blank lip	105 ±1.32	0.112	−17.2 ±1.5	/	/
bFGF-lip	124 ±1.84	0.083	−16.1 ±1.6	87.6 ±2.8	7.3 ±0.675

Table S3 Results of LVIDd, LVPW, and LVFS in control and study groups (n=8)

Group	LVIDd		LVPW		LVFS	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
1. Control	**5.22±0.43**	**7.43±0.32**	**1.43±0.15**	**1.59±0.12**	**49.5±2.6**	**51.2±3.5**
2. DM	**5.19±0.36**	**5.92±0.24***	**1.42±0.13**	**1.87±0.09***	**49.5±3.1**	**37.6±4.3***
3. bFGF	**5.35±0.40**	**6.68±0.28$^{\#+}$**	**1.47±0.09**	**1.71±0.11$^{\#+}$**	**50.1±2.5**	**43.5±4.3$^{\#+}$**
4. bFGF-lip	**5.23±0.26**	**6.75±0.25$^{\#+}$**	**1.38±0.08**	**1.72±0.08$^{\#+}$**	**49.3±3.4**	**43.4±3.7$^{\#+}$**
5. UTMD	**5.31±0.37**	**5.88±0.23^{+}**	**1.40±0.11**	**1.86±0.13^{+}**	**48.0±2.5**	**37.5±3.4^{+}**
6. bFGF+UTMD	**5.29±0.36**	**6.73±0.44$^{\#+}$**	**1.39±0.08**	**1.69±0.10$^{\#+}$**	**48.6±2.7**	**42.9±4.2$^{\#+}$**
7.bFGF-lip+UTMD	**5.32±0.39**	**7.38±0.43$^{\#}$**	**1.45±0.10**	**1.59±0.08$^{\#}$**	**48.1±3.4**	**49.7±3.7$^{\#}$**

*Note: LVIDd= left ventricular end-diastolic diameter, LVPW= left ventricular posterior wall, LVFS= left ventricular fraction shortening. Data are Mean ± SD. *P<0.05 vs. control; #P<0.05 vs. DM group; +P<0.05 vs. bFGF- lip +UTMD.*

Table S4 Results of peak velocity, strain, and strain rate in control and study groups (n=8)

Group	*Vs(cm/s)*		*Sc(%)*		*SRc(1/s)*	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
1. Control	0.860±0.060	0.87±0.06	-14.6±1.8	-14.8±1.7	-3.76±0.44	-3.82±0.27
2. DM	0.840±0.050	0.53±0.08*	-14.4±2.0	-10.3±1.5*	-3.69±0.46	-2.46±0.41*
3. bFGF	0.850±0.070	0.67±0.06$^{\#+}$	-14.3±2.2	-12.0±1.6$^{\#+}$	-3.66±0.46	-2.89±0.45$^{\#+}$
4. bFGF-lip	0.830±0.070	0.67±0.06$^{\#+}$	-14.5±1.9	-12.5±1.6$^{\#+}$	-3.69±0.20	-3.01±0.37$^{\#+}$
5. UTMD	0.850±0.080	0.52±0.08*	-14.3±1.8	-10.3±1.3*	-3.72±0.36	-2.49±0.39$^{\#+}$
6. bFGF+UTMD	0.840±0.080	0.68±0.10$^{\#+}$	-14.4±1.7	-12.5±1.1$^{\#+}$	-3.48±0.49	-2.99±0.35$^{\#+}$
7.bFGF-lip+UTMD	0.840±0.110	0.83±0.08$^{\#}$	-14.6±1.6	-14.4±1.5$^{\#}$	-3.73±0.32	-3.71±0.44$^{\#}$

Note: Vs= peak systolic velocity; Sc= peak circumferential strain; SRc= peak circumferential strain rate.

*Data are Mean ± SD. *P<0.05 vs. control group; #P<0.05 vs. DM group; +p<0.05 vs. bFGF-lip+UTMD.*

Table S5 The hemodynamic data in experiment in vivo (mean± SD, n=8)

Group	LVSP(mmHg)	LVEDP(mmHg)	LV +dp/dtmax(mmHg)	LV -dp/dtmax(mmHg)
1. Control	95.3±7.5	2.83±0.43	4.79×10^3±198	4.02×10^3±289
2. DM	72.3±7.1*	3.92±0.45*	3.11×10^3±212*	2.80×10^3±188*
3. bFGF	83.5±7.7#+	3.44±0.31 #+	4.04×10^3±297#+	3.49×10^3±206#+
4. bFGF-lip	83.9±7.1 #+	3.42±0.29 #+	4.11×10^3±340 #+	3.57×10^3±314 #+
5. UTMD	73.7±6.9+	3.87±0.38 +	3.17×10^3±307+	2.84×10^3±248+
6. bFGF + UTMD	83.5±6.1#+	3.45±0.31 #+	3.96×10^3±335 #+	3.58×10^3±235 #+
7. bFGF- lip + UTMD	93.0±7.3#	2.91±0.33#	4.66×10^3±248 #	3.99×10^3±211#

*Notes: LVSP=left ventricular systolic pressure; LVEDP=left ventricular end diastolic pressure; ±dp/dtmax - maximum rate of the rise and fall of left ventricular pressure. Data are Mean ± SD. *P<0.05 vs. control; #P<0.05 vs DM group; +P<0.05 vs bFGF- lips +UTMD.*