Supplementary for Review file

Telmisartan Ameliorates Cardiac Fibrosis and Diastolic Function in Cardiorenal Heart Failure with Preserved Ejection Fraction

Supplemental Methods

Assessment of Chronic Kidney Disease

Blood was acquired from the canthus vein and 24 h urine samples were collected from all rats in metabolic cages at Baseline before the initial surgery and every 4 weeks after the whole surgery. 24 h urine volume was recorded, and 24 h urine protein excretion was detected through urine samples by a Protein Urine Determination Kit (Beijing Wantai, China). Serum creatinine, blood urea nitrogen and urinary creatinine were measured by the Department of Clinical Laboratory, Zhongda Hospital employing a Synchron autoanalyser (Beckman Coulter, USA). Creatinine clearance (Ccr) was calculated and normalized for body weight using the formula: CCr (mL/min/kg) = (urinary creatinine × 24 h urinary volume)/(serum creatinine × 1,440 × body weight). [1]

Echocardiography

Trans-thoracic echocardiography was performed with a Vevo 2100 ultrasound system (Visual Sonics, Toronto, Ontario) with the animals anesthetized by 2% isoflurane. Both M-mode and two-dimensional short- and long-axis images of the left ventricle were obtained at week 0 before the surgery as a baseline value and at week 16 after the SNX surgery. Parameters including interventricular septal thickness at end-diastole (IVSd), interventricular septal

thickness at end-systole (IVSs), left ventricular (LV) posterior wall thickness at end-diastole (LVPWd), LV posterior wall thickness at end-systole (LVPWs), LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), EF (EF = (LVEDV - LVESV) / LVEDV × 100%), and early to late diastolic transmitral flow velocity (E/A) ratio were measured and calculated according to standard formulas to evaluated LV hypertrophy, systolic function and diastolic function. All image measurements were performed by 2 independent readers who were blinded to the group information.

Cardiac Magnetic Resonance Imaging

In vivo cardiac magnetic resonance imaging (MRI) was performed in a 7.0 Tesla MR Scanner (Bruker PharmaScan, Ettlingen, Germany) with respiratory and electrocardiograph double-gated. The animals were initially anesthetized with 1% isoflurane delivered through a nose cone, and the heart rates were adjusted to approximately 300 bpm with a 2-3% (v/v) isoflurane/air gas mixture. Cine imaging was obtained with a black-blood T1W-Cine FLASH sequence in the axial direction spanning the entire heart. The imaging parameters were as follows: field of view = 5.5 × 5.5 cm, matrix size = 256 × 256, slice thickness = 1.5 mm, number of slices = 1, repetition time = 8 ms, echo time = 2.8 ms, flip angle = 30°, number of averages = 3, and total scan time = 3 min 16 s. 5 to 8 slices were collected from each rat to assess their cardiac function, and Cine FLASH images were acquired in the three groups (Supplementary Movies I-III). For cardiac function analysis, Cine images were transferred to a self-developed workstation based on clinical cardiac Cine imaging analysis techniques. A software analysis package (Matlab Analysis, version 6) was used to depict the endocardial and

epicardial contours around each slice to produce the myocardial volume-time curve of a cardiac cycle (Supplementary Figure I). The IVSd, IVSs, LVEDV, LVESV and EF were derived from the curve. All image measurements were performed by 2 independent readers who were blinded to the group information.

Histology and Immunohistochemistry

At the end of the experiment, all rats were euthanized by an overdose of chloral hydrate (intraperitoneal, i.p.), followed by an intravenous (i.v.) perfusion of 300-400 ml PBS that contained 20 U/ml heparin to clear the blood from the organs. After euthanasia, the organs of kidney, heart, liver, lung and spleen were collected, and half of the tissues were fixed in 4% (w/v) paraformaldehyde. The tissue sections of kidney were stained with Masson trichrome (Masson) for fibrosis and CD68 for macrophages. The tissue sections of heart were stained with hematoxylin & eosin (H&E) and Masson, followed by immunohistochemistry staining with antibodies against α-smooth muscle actin (α-SMA), transforming growth factor (TGF)-β1, connective tissue growth factor (CTGF) and CD68 (Abcam, Cambridge, UK).

Masson trichrome staining: Fixed tissues were embedded in paraffin and cut into 4 μm slides for Masson trichrome staining. After dewaxing and rehydration, the tissue sections were stained with Masson composite staining solution (5 min; Fuzhou Maxim Biotech, Fuzhou, China), 1% (v/v) phosphomolybdic acid (5 min), and 1% (w/v) aniline blue (5-10 min) and treated with 95% (v/v) alcohol (10-15 min) for differentiation. The sections were then washed in acidified water, dehydrated and cemented using neutral gum to reveal fibrosis.

Immunohistochemical staining: The fixed tissues were embedded in paraffin and cut into 4

μm slides for immunohistochemistry staining. Briefly, the paraffin-embedded slides were dewaxed, rehydrated, and treated with 3% (v/v) H_2O_2 for 10 min. The slides were then blocked with 5% (v/v) goat serum for 30 min and incubated with primary α-SMA, TGF-β1, CTGF or CD68 antibodies (Abcam, Cambridge, UK) overnight at 4°C. Biotinylated goat anti-rabbit and goat anti-mouse polyclonal IgG (Sigma-Aldrich, St. Louis, MO, USA) were used as the secondary antibodies followed by horseradish peroxidase (HRP)-streptavidin and visualized with diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA). Secondary antibody only (without primary antibody) was used in each staining batch as a control for the specificity of the primary antibody. For quantification, 10-20 randomly selected microscopic fields were imaged for each section, and the quantification work was conducted using Image Pro-Plus 6.0 (Media Cybernetics., Washington, USA) by 2 blinded readers.

Real-time PCR

The heart tissue samples obtained from the left ventricle were shredded. Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the guidelines of the manufacturer and quantitative estimation of collagen I, collagen III, α-SMA, TGF-β1 and CTGF gene expression was performed with a real-time PCR system (Life Technologies, USA). The expression level of each gene was normalized with GAPDH as an endogenous control and the fold increases in the levels of gene expression were calculated using software provided by the manufacturer.

Western Blot

The other half of the tissues were frozen with liquid nitrogen and stored at -80°C for western blotting analysis. Snap-frozen cardiac tissues were smashed on dry ice and homogenized in lysis buffer that contained 50 mmol/L Tris (pH 7.4), 150 mM NaCl, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mmol/L EDTA, 1% (v/v) Triton X-100, 2 mmol/L sodium pyrophosphate, 25 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 0.5 μg/mL leupeptin, and 10 μL phenylmethylsulfonyl fluoride. The protein concentration was determined with a BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein (30 μg) were electrophoresed through a 10% SDS-PAGE gel and then transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked with 5% (w/v) skim milk (BD Bioscience, USA) in PBS prior to overnight incubation with rabbit anti-rat collagen I, collagen III, α-SMA, TGF-β1 and CTGF antibodies (Abcam, Cambridge, UK) at 4°C. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody (Sigma, NJ, USA). The signal intensity (the protein levels) was quantified with Image J 1.5 software (NIH, Bethesda, MD, USA), and the results were normalized to β-actin as an endogenous control.

Results

Mortality Rates

The overall mortality rate was 10%, and the deaths occurred within 2 weeks after the surgery or near the end of the experiment, possibly due to surgical injury and anesthesia. No infection or severe bleeding occurred, and no dialysis was performed. The survival rates for the Sham, SNX+Veh and SNX+Tel groups were 100%, 75% and 95%, respectively (Supplementary Table I).

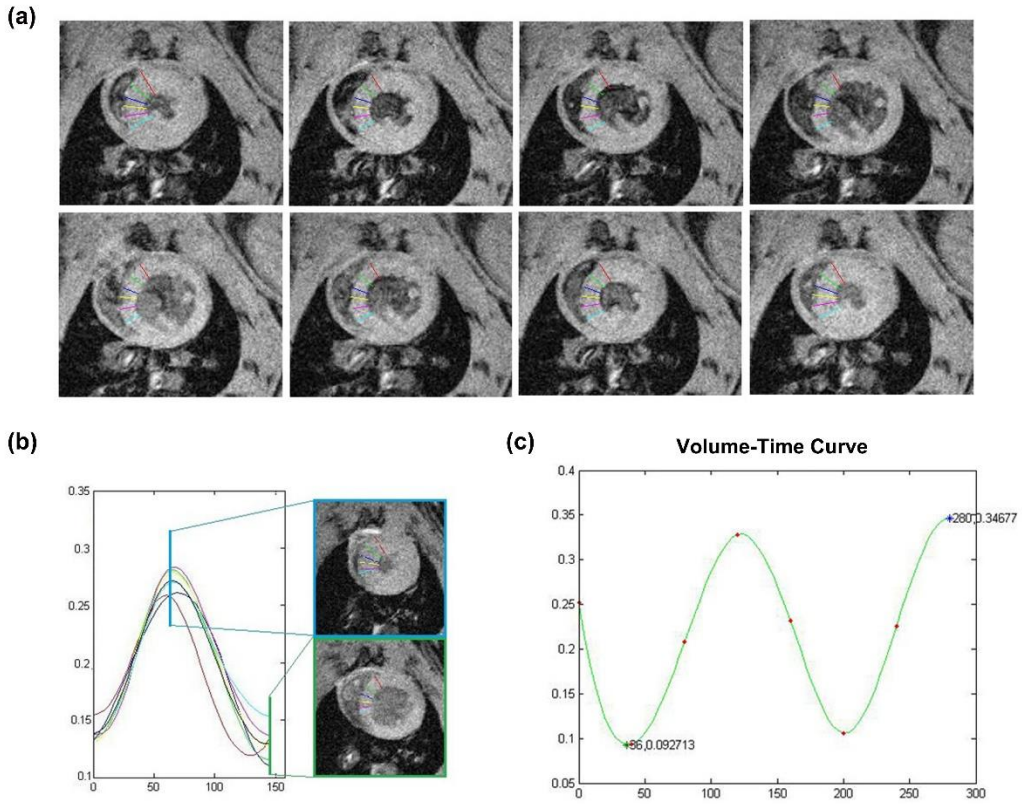
***Supplementary Table I*. Mortality Rates, Animal Characteristics, Echocardiography and Cardiac MRI values at Week 16 After the Surgery.**

	Sham	SNX+Veh	SNX+Tel
Animal number (start-end)	20-20	20-15	20-19
Survival rate (%)	100	75	95
Heart rate (beats/min)	473 ± 96	486 ± 162	469 ± 123
LV mass (mg)	898.2 ± 93.1	1030.0 ± 98.8*	941.5 ± 74.8†
BW (g)	510.9 ± 40.9	398.8 ± 35.5*	452.3 ± 18.9†
HW/BW ratio (mg/mm)	2.54 ± 0.29	3.37 ± 0.41*	2.74 ± 0.33†
Urine volume (mL/24 h)	15.8 ± 4.2	24.6 ± 7.2*	17.8 ± 3.5†
Ccr (mL/min/kg)	2.55 ± 0.4	1.37 ± 0.3*	1.79 ± 0.2†
Echocardiography			
IVSd (mm)	2.01 ± 0.16	2.84 ± 0.35*	2.37 ± 0.21†
IVSs (mm)	3.25 ± 0.37	4.33 ± 0.27*	3.65 ± 0.38†
LVPWd (mm)	2.19 ± 0.11	2.71 ± 0.29*	2.35 ± 0.26†
LVPWs (mm)	3.54 ± 0.28	4.31 ± 0.31*	3.88 ± 0.21†
LVEDV (μl)	319.9 ± 34.9	201.7 ± 42.0*	279.8 ± 36.8†
LVESV (μl)	101.9 ± 35.8	44.2 ± 23.4*	85.9 ± 13.9†
EF	75.8 ± 8.4	80.5 ± 10.3	73.9 ± 10.0
E/A ratio	1.47 ± 0.23	0.81 ± 0.20*	1.31 ± 0.27†
Cardiac MRI			

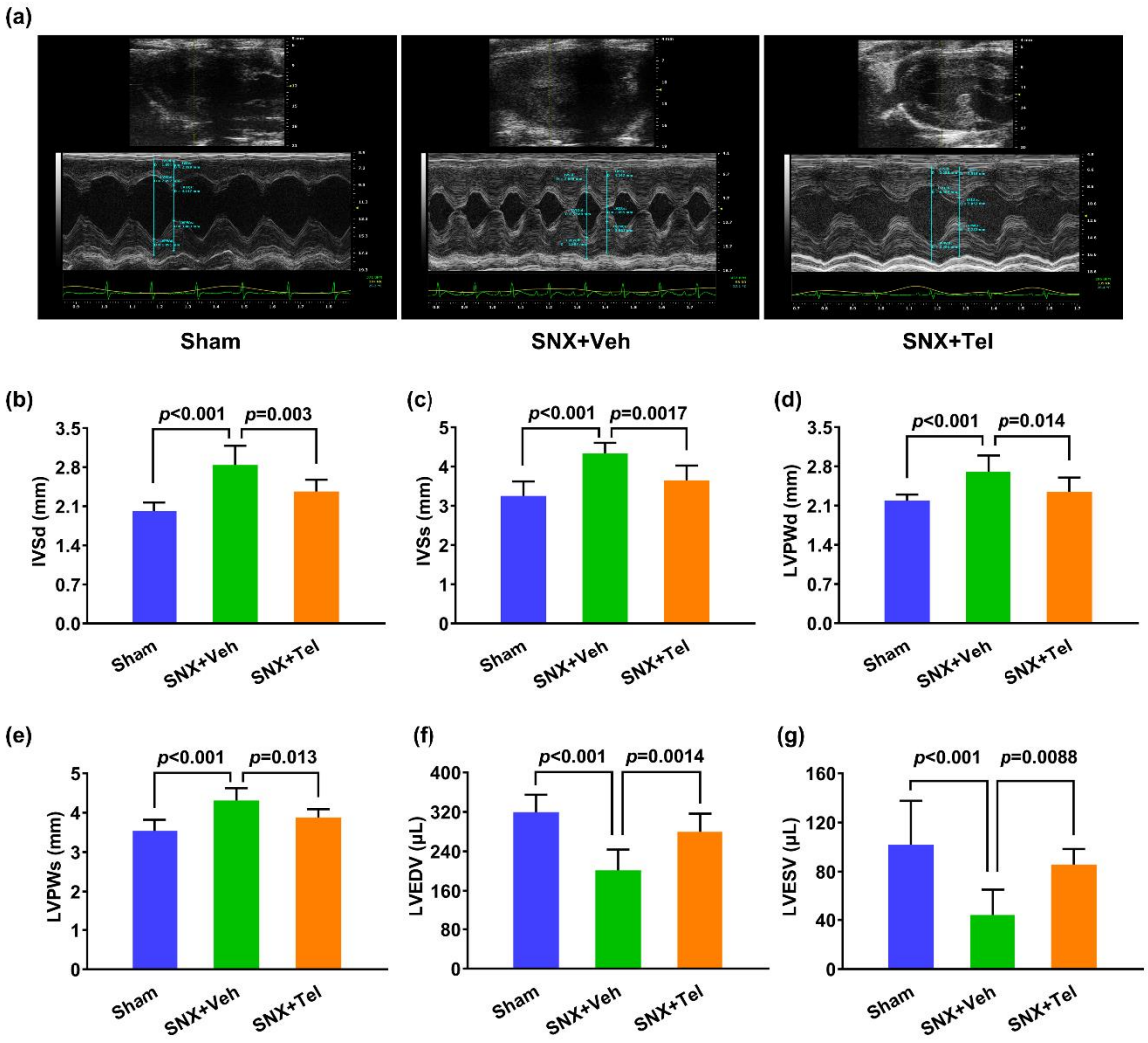
IVSd (mm)	2.05 ± 0.17	2.88 ± 0.30*	2.45 ± 0.20†
IVSs (mm)	3.08 ± 0.32	4.53 ± 0.36*	3.45 ± 0.30†
LVEDV (μl)	308.5 ± 41.8	200.4 ± 42.8*	263.5 ± 38.2†
LVESV (μl)	108.2 ± 23.7	47.9 ± 19.8*	80.9 ± 12.9†
EF	72.3 ± 10.2	78.1 ± 13.6	71.5 ± 8.6

Data are reported as the mean ± SD. MRI indicates magnetic resonance imaging; BW, body weight; HW, heart weight; Ccr, creatinine clearance; IVSd, interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVPWd, left ventricular posterior wall thickness at end-diastole; LVPWs, left ventricular posterior wall thickness at end-systole; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; EF, ejection fraction; E/A, early to late diastolic transmitral flow velocity; SNX, subtotal nephrectomy; Veh, vehicle; Tel, Telmisartan. * $p < 0.05$ for SNX+Veh vs Sham, † $p < 0.05$ for SNX+Veh vs SNX+Tel.

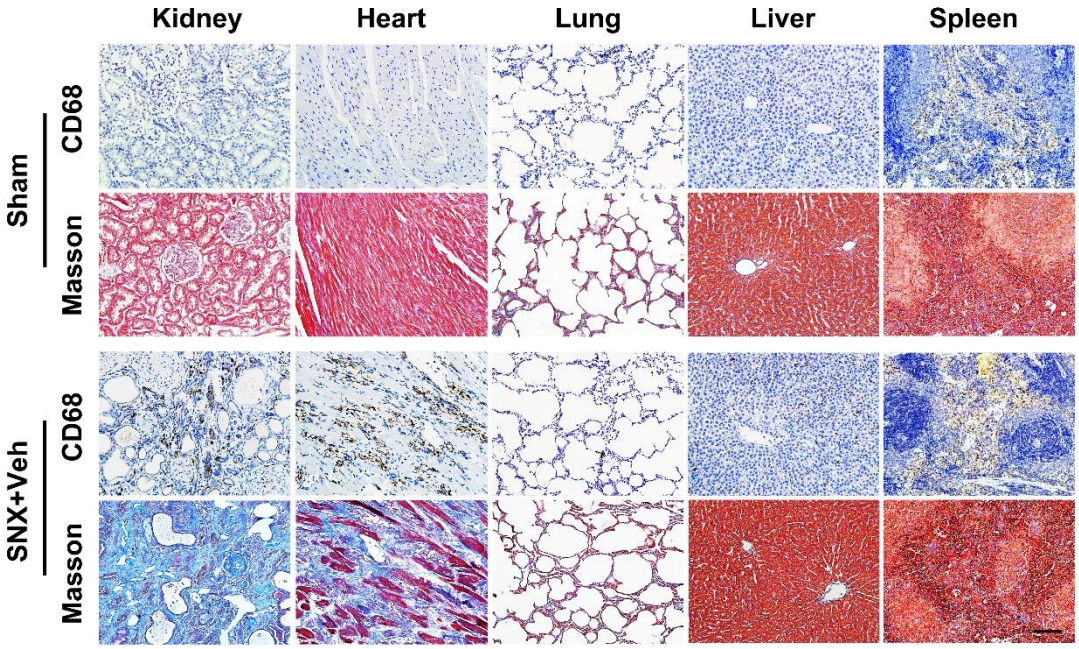
Supplementary Figures



***Supplementary Figure I*. Cardiac function analysis on T1W-Cine MRI.** For cardiac function analysis, black-blood T1W-Cine FLASH sequence was acquired and transferred to a self-developed workstation based on clinical cardiac Cine imaging analysis techniques. (a) 8-12 images of a cardiac cycle of each slice of the heart were obtained. (b) The endocardial and epicardial contours of the slice were depicted to measure IVSd and IVSs. (c) The myocardial volume-time curve of a cardiac cycle was acquired to measure LVEDV and LVESV. MRI indicates magnetic resonance imaging; IVSd, interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; SNX, subtotal nephrectomy; Veh, vehicle; Tel, Telmisartan.



Supplementary Figure II. **Left ventricular hypertrophy measurement by echocardiography and reduced hypertrophy after treatment with Telmisartan.** (a) Echocardiographic two-dimensional images (top row) and M-mode images (bottom row) of the left ventricle at week 16. The mean ± SD of IVSd (b), IVSs (c), LVPWd (d), LVPWs (e), LVEDV (f) and LVESV (g) at week 16 after the surgery of SNX. IVSd indicates interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVPWd, left ventricular posterior wall thickness at end-diastole; LVPWs, left ventricular posterior wall thickness at end-systole; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; SNX, subtotal nephrectomy; Veh, vehicle; Tel, Telmisartan.



***Supplementary Figure III*. Assessment of inflammatory macrophages and fibrosis in different organs in cardiorenal HFpEF.** CD68 immunohistological staining and Masson trichrome staining of the kidney, heart, lung, liver and spleen of rats from the Sham group and the SNX+Veh group at week 16. Increased macrophages and fibrosis were observed in all organs in the SNX+Veh group than the Sham group at week 16, which were more prominent in the kidney, heart and lung. Scale bar, 100 μm. Masson indicates Masson trichrome; SNX, subtotal nephrectomy; Veh, vehicle; Tel, Telmisartan.

Supplementary Movies

Cine images were obtained with a black-blood T1W-Cine FLASH sequence in the axial direction spanning the entire heart in the three groups (Supplementary Movies I-III).

Supplemental References

1. Haylor J, Dencausse A, Vickers M, Nutter F, Jestin G, Slater D, Idee JM, Morcos S. Nephrogenic gadolinium biodistribution and skin cellularity following a single injection of Omniscan in the rat. *Invest Radiol* 2010;**45**:507-12