ORIGINAL ARTICLE

The influence of inhibiting renal neural regeneration on the efficacy of renal denervation to chronic heart failure

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Abstract

Aims   Some studies support the occurrence of nerve regeneration in renal arteries after renal denervation (RDN). But it is unclear whether inhibiting reinnervation after RDN is beneficial to enhancing the effect of RDN on chronic heart failure (CHF).
Methods and results   Chronic heart failure Sprague Dawley rats induced by transverse aortic constriction were administered with the analogue of Nogo-B (Nogo group) or its antagonist (NEP group) respectively after RDN. Echocardiography, messenger RNA, and protein expression of calcitonin gene-related peptide (CGRP) in renal artery and nerves surrounding renal artery were detected. Relative protein expression of CGRP was significantly decreased in the Nog group compared with the RDN group (0.64 ± 0.51 vs. 1.68 ± 1.07, $P = 0.048$). The number of nerves surrounding renal artery was higher in the NEP group than in the Nog group. Left ventricular end-systolic volume and diameter (LVVs and LVDs) were greatly decreased, and left ventricular ejection fraction (LVEF) and fractional shortening (FS) increased significantly in the RDN, Nog and NEP groups when compared with the HF group (all $P < 0.05$). No significant differences were observed in left ventricular end-diastolic volume and diameter; LVDs; LVVs; FS; LVEF; and the levels of plasma renin, noradrenaline, and N-terminal pro-B-type natriuretic peptide among three groups: the RDN, Nog, and NEP groups.
Conclusions   Reinnervation of renal artery occurred in CHF rats after RDN, which had no effect on therapeutic role of RDN in CHF, and inhibiting this neural regeneration had no clinical significance and did not affect the efficacy of RDN to CHF.

Introduction

The pathophysiological role of the sympathetic nervous system in the genesis of chronic heart failure (CHF) has been well established.[1] Renal sympathetic nerve activity increased in CHF,[2] and it had detrimental roles to CHF suggesting that renal denervation (RDN) would be beneficial to heart failure (HF), because of the effect of RDN on decreasing renal sympathetic nerve activity. By now, it seems feasible that RDN does work as a treatment for hypertension[3–6] and CHF,[7–9] but neural regeneration of renal artery after RDN

was observed in some studies.[10,11] Considering the regeneration of renal nerves after injury, one of the unresolved issues is the reinnervation of the kidney's neurons after RDN in the treatment of CHF and whether this reinnervation has physiological function and clinical significance to the efficacy of RDN to CHF.

Nogo-B is a family member of reticulons that largely restricted to the tubular endoplasmic reticulum in mammalian cells.[12] As a neurite growth inhibitor, Nogo-B shows widespread expression in the central and peripheral nervous systems and can restrain the regenerative growth of the

peripheral nerves after nerve lesions.[13,14] NEP1-40 peptide is a competitive antagonist of the axonal Nogo-66 receptor (the functional domain of Nogo-B). NEP1-40 can promote the regeneration of both corticospinal, raphespinal axons, and peripheral nerves.[15] It is unclear whether the reinnervation of renal artery after RDN has effect on therapeutic role of RDN in CHF and whether inhibiting this neural regeneration by Nogo-B is beneficial to CHF and affect the efficacy of RDN to CHF. So the relationship between the regeneration of renal nerves after RDN and the efficacy of RDN to CHF was investigated in this study. The aim was to evaluate the influence of regeneration of renal nerves on the efficacy of RDN to CHF and the roles of inhibiting reinnervation on enhancing the effect of RDN on CHF.

Materials and methods

Transverse aortic constriction

Transverse aortic constriction (TAC) was performed as described previously.[16] Briefly, Sprague Dawley rats, 10 weeks of age and weighing 200–250 g, were anesthetized with 2% pentobarbital sodium (30 mg/kg, intraperitoneally injected). Abdominal aorta above left renal artery was exposed through a midline abdominal incision and gently isolated from the surrounding tissue. Aortic constriction was performed by tying a 4.0 silk string ligature around a 22-gauge needle and then removing the needle immediately. Sham-operated rats served as the control group and were subjected to the same surgeries except for ligation of the aorta. The rats were treated with Penicillin and returned to their home cages after surgery. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). The protocol was approved by the institutional ethics committee of Guangzhou First People's Hospital.

Renal denervation and the animal grouping

To perform RDN, CHF rats induced by TAC were anesthetized with 2% pentobarbital sodium (30 mg/kg, intraperitoneally injected) and placed in a supine position at 10 weeks after TAC. The abdomen was opened with a midline incision. After the intestines were reflected to the contralateral position, the right and left renal arteries were gently isolated from the surrounding tissues, respectively. Phenol (10% phenol in 95% alcohol) was applied to the surface of the left and right renal arteries, respectively, for 10 min by dripping from 1 mL syringe with 28-gauge needle, and then the surrounding tissues were washed with saline water and the skin was sutured. Rats underwent the same surgeries and applied with saline water, not phenol, used as the HF group.

The rats were then randomly divided into four groups: the HF group (*n* = 7); the RDN group (*n* = 6); the Nog group (*n* = 7), administered with Nogo-P4 (Alpha Diagnostic Intl Inc., USA), the analogue of Nogo-B, by intraperitoneal injection of 0.02 mg/day[17] for 2 weeks at 8 weeks after RDN; and the NEP group (*n* = 6), treated with NEP1-40 (ApexBio, USA), the antagonist of Nogo-B, by intraperitoneal injection of 0.025 mg/day[18] for 2 weeks also at 8 weeks after RDN. The control (*n* = 4), HF, and RDN groups were administered with the same dosage of saline. Echocardiography was performed, and blood samples and renal arteries tissues were harvested for analysis at 16 weeks after administration (*Figure 1*).

Moreover, CHF rats were divided into three groups at 8 weeks after sham-operated RDN: the HF 1 group (*n* = 5), administered with the same dosage of saline; the HF 2 group (*n* = 5), administered with Nogo-P4; and the HF 3 group (*n* = 5), treated with NEP1-40. Similarly, control rats were also divided into three subgroups at 18 weeks after sham-operated TAC: the Control 1 group (*n* = 5), administered with the same dosage of saline; the Control 2 group (*n* = 5), administered with Nogo-P4; and the Control 3 group (*n* = 5), treated with NEP1-40. Nogo-P4 and NEP1-40 were administered by the same concentration and route as Nog and NEP groups, respectively (*Figure 1*).

Biochemistry detection

The levels of plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP), Nogo-B, renin, and noradrenaline were detected by enzyme-linked immunosorbent assay according to the manufacturer's protocols (CUSABIO, China).

Quantitative real-time polymerase chain reaction

Total ribonucleic acid (RNA) was isolated from renal arteries using total RNA rapid extraction kit (BioTeke, Beijing, China) according to the manufacturer's protocol. Primer was designed to detect calcitonin gene-related peptide (CGRP) (Forward: GTCATCGCTCACCAGGGAGG, Reverse: GGGCT GCTTTCCAAGGTTGAC), nerve growth factor (β-NGF) (Forward: ACTTCCAGGCCCATGGTACA, Reverse: TGTCCGT GGCTGTGGTCTTA) and growth-associated protein 43 (GAP43) (Forward: CACTGATAACTCGCCGTCCTC, Reverse: GTTTGGCTTCATCTACAGCTTCTTT) gene expression on the basis of sequences available in NCBI database (at http://ncbi.nlm.nih.gov) using Primer software. After measuring RNA concentration, 1 μg RNA sample was reversely transcribed into complementary deoxyribonucleic acid with HiScript II Q RT SuperMix (Vazyme, Nanjing, China) and then complementary deoxyribonucleic acid was treated with Hieff TMqPCR SYBR® Green Master Mix (Yeasen, Shanghai, China).

Figure 1 The animal grouping and time line of the experimental protocol. Sprague Dawley rats, 10 weeks of age, were subjected to transverse aortic constriction (TAC). Then renal denervation (RDN) was performed at the 20th week (the 10th week after TAC) after echocardiography. At the 28th week (the 8th week after RDN), the rats underwent RDN were administered with Nogo-P4 (the analogue of Nogo-B), NEP1-40 (the antagonist of Nogo-B) or saline water by intraperitoneal injection respectively. At the 46th week, echocardiography was performed, and blood samples and renal arteries tissues were harvested for analysis.
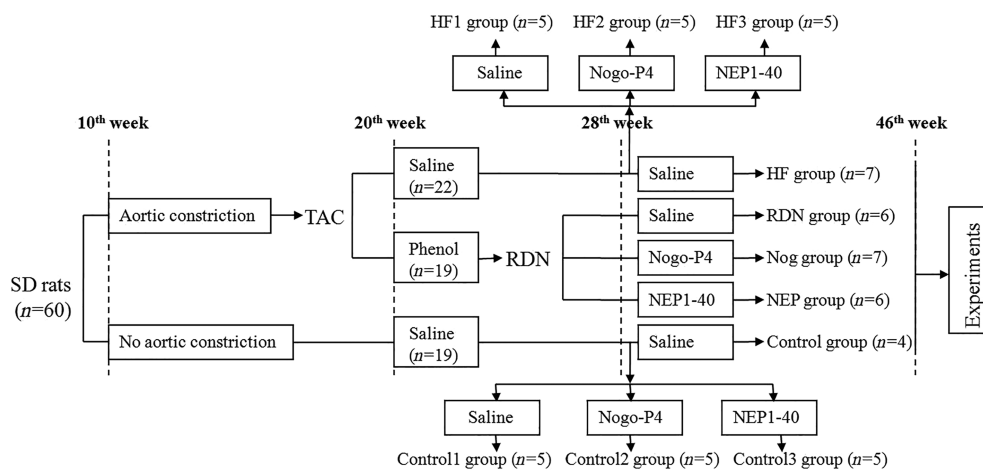
The messenger RNA (mRNA) levels were measured with quantitative real-time polymerase chain reaction system. The relative amounts of mRNA were determined based on 2-ΔΔCt calculations.

Echocardiographic detection of cardiac function

Transthoracic echocardiography was performed using Vevo 2100 imaging system equipped with a 15 Mhz to 30 Mhz linear array transducer (VisualSonics, Inc., Canada) by a single blinded observer as described previously.[19] The following parameters were measured and averaged from three cardiac cycles: left ventricular end-diastolic volume and diameter (LVVd and LVDd), left ventricular end-systolic volume and diameter (LVVs and LVDs), and left ventricular anterior and posterior wall thickness in diastole (LVAWd and LVPWd). Fractional shortening (FS, %) and left ventricular ejection fraction (LVEF, %) were calculated using the equation respectively: FS = 100 × ((LVDd − LVDs)/LVDd), LVEF = 100 × (LVVd − LVVs)/LVVd. Left ventricular mass was calculated following the previously described method of Gao *et al*.[20]: left ventricular mass = [(LVDd + LVAWd + LVPWd)^3 − LVDd^3] × 1.055, where 1.055 is the gravity of myocardium.

Western blot analysis

Renal arteries were homogenized in RIPA buffer (Beyotime Biotechnology, China) to obtain whole cell lysates and centrifuged to isolate protein. Ten micrograms of protein were run in an SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. After blocking with 5% non-fat milk, membranes were incubated with the following primary antibodies: CGRP polyclonal antibody (1:8000; Sigma-Aldrich, USA; Catalog C8198), β-NGF (1:1000; R&D Systems Inc., USA; Catalog AF-556-NA), GAP-43 (1:500; BIOSS, China; Catalog BS-0154R), or β-actin (1:1000; Boster, China; Catalog BM0627; Clone AC-15). After incubating with anti-rabbit HRP-conjugated IgG (1:2500, Boster, China) for 2 h at room temperature, immunoreactive bands were then visualized by chemiluminescence reagents (ECL; 3-Biologic, Shanghai, China). The band intensity was quantified using Image J analysis software in a blinded manner, and all bands were normalized to corresponding β-actin bands.

Immunohistochemistry

For haematoxylin and eosin (HE) staining and CGRP immunohistochemistry, formalin-fixed renal arteries tissues were embedded in paraffin and sectioned into 3-μm-thick sections. HE staining was performed following standard protocols. After dewaxed and rehydrated, the slices were incubated with haematoxylin for 2 min, followed by colour separation using 1% hydrochloric acid alcohol for 25 s. After backed to blue for 30 s with 1% ammonia and stained with eosin for 5 min, the slices were dehydrated and sealed with neutral resin. After deparaffinized and rehydrated, the sections were subjected to antigen retrieval by microwave treatment in 0.01 M sodium citrate buffer, pH 6.0, at the condition of heating for 3 min, heat preservation for 3 min, and moderate heat for 6 min. The slides were cooled at room temperature for 20 min. Then slides were performed by immersion in 3%

(w/v) H_2O_2 in methanol for 15 min to inhibit endogenous peroxidase activity. After pretreated with normal nonimmune serum, the sections were incubated with primary antibodies against CGRP (1:100; Sigma-Aldrich, USA; Catalog C8198) overnight at 4°C before being incubated with a secondary antibody, HRP-conjugated goat anti-rabbit IgG (1:200, Servicebio, China; Catalog GB23303), for 40 min at room temperature. The sections were stained with DAB (Maixin-Bio Inc., Fuzhou, China) followed by counterstaining with haematoxylin.

Statistics

Continuous variables were presented as the mean ± SD. Medians and interquartile range (IQR, 25th to 75th percentiles) were presented for skewed variables. Differences among means were analysed with a two-independent-sample *t*-test or one-way ANOVA test with S-N-K analysis, as appropriate. Pearson correlation was used to evaluate correlations between CGRP and some plasma parameters. *P* values were two-sided and considered significant when <0.05. Statistical analyses were carried out using the SPSS version 17.0 software package (SPSS Inc., Chicago, USA).

Results

The effects of renal denervation or nerve regeneration on biochemistry data

The levels of plasma renin and noradrenaline were significantly higher in the HF group than in the controls [308 ± 85 vs. 71 ± 33 mU/mL, *P* = 0.002; median, 316 (IQR 252 to 407) vs. 45 pg/mL (IQR 36 to 57), *P* = 0.021], and their levels decreased after RDN. *Figure 2* and *Table 1* showed that the levels of plasma renin and noradrenaline in the RDN [128 ± 75 vs. 308 ± 85 mU/mL, *P* = 0.034; median, 144 (IQR 106 to 219) vs. 316 pg/mL (IQR 252 to 407), *P* = 0.021] and Nog [104 ± 60 vs. 308 ± 85 mU/mL, *P* = 0.002; median, 59 (IQR 49 to 132) vs. 316 pg/mL (IQR 252 to 407), *P* = 0.011] groups decreased significantly when compared with the HF group. No statistically significant differences were observed between the RDN group and either the Nog or NEP group (*Figure 2A,B*). The levels of plasma Nog-B were significantly higher in the Nog group (4.76 ± 1.32 vs. 2.99 ± 1.36 ng/mL, *P* = 0.038) and lower in the NEP group (1.69 ± 0.72 vs. 2.99 ± 1.36 ng/mL, *P* = 0.048) when compared with the RDN group. The Nog group possessed higher levels of Nog-B than the HF group (4.76 ± 1.32 vs. 2.76 ± 1.33 ng/mL, *P* = 0.015) (*Figure 2C*).

The levels of NT-proBNP were significantly higher in HF group when compared with the control (218 ± 52 vs. 152 ± 21 pg/mL, *P* = 0.043), RDN (218 ± 52 vs. 156 ± 30 pg/ mL, *P* = 0.029), Nog (218 ± 52 vs. 131 ± 42 pg/mL, *P* = 0.014), and NEP (218 ± 52 vs. 126 ± 23 pg/mL, *P* = 0.011) groups. No differences were found among the three groups (the RDN, Nog, and NEP groups) (*Figure 2D*). The ratio of heart to body weight (heart/BW) increased significantly in the HF group than in the controls (3.84 ± 0.44 vs. 3.32 ± 0.07 mg/g, *P* = 0.047). The same tendency was seen for heart weight and body weight. There were no statistically significant differences among the four groups (the HF, RDN, Nog, and NEP groups) (*Figure 2E*).

When compared with the Control 2 group, plasma renin, noradrenaline, and NT-proBNP were significantly higher in the HF 1, HF 2, and HF 3 groups (all *P* < 0.05), but no differences were observed among the Controls 1, 2, and 3 groups (all *P* > 0.05) or among the HF 1, HF 2, ad HF 3 groups (all *P* > 0.05) (*Figure 3A,B & D*). The levels of plasma Nog-B were significantly higher in the Control 2 and HF 2 groups than those in the Control 1, Control 3, HF 1, and HF 3 groups (all *P* < 0.05) (*Figure 3C*).

The mRNA expression of indicator of nerve regeneration in the renal artery

Quantitative real-time polymerase chain reaction showed that relative CGRP mRNA levels increased several fold in the RDN group compared with the HF group [median, 6.01 (IQR 1.19 to 10.47) vs. 0.53 (IQR 0.12 to 2.12), *P* = 0.046]. Although CGRP mRNA levels decreased moderately in the Nog group when compared with the RDN [median, 0.99 (IQR 0.14 to 2.73) vs. 6.01 (IQR 1.19 to 10.47), *P* = 0.078], no statistical difference was observed (*Figure 2F*). No differences in relative mRNA levels of β-NGF and GAP-43 were seen among the four groups (the HF, RDN, Nog, and NEP groups) (*Figure 2G,H*). Pearson correlation was used to evaluate correlations between CGRP mRNA levels and plasma renin, NT-ProBNP, or logarithmically transformed skewed variable (norepinephrine). Pearson correlation showed that CGRP was not correlated with plasma renin (r = 0.234, *P* = 0.334), norepinephrine (r = 0.299, *P* = 0.200), or NT-ProBNP (r = 0.173, *P* = 0.409).

Western blot analysis of nerve regenerative protein expression in the renal artery

Relative protein expression of CGRP was significantly increased in the RDN group compared with the HF group (1.68 ± 1.07 vs. 0.36 ± 0.53, *P* = 0.017). Moreover, a decrease in levels of CGRP and β-NGF protein was found in the Nog group when compared with the RDN group (0.64 ± 0.51 vs. 1.68 ± 1.07, *P* = 0.048; 0.45 ± 0.23 vs. 0.93 ± 0.29, *P* = 0.007). However, there were no statistically significant differences in the levels of GAP-43 protein between the different groups (*Figure 4*).

Figure 2 Comparisons of plasma parameters and mRNA expression of indicator of nerve regeneration in the renal arteries among the five groups. The levels of plasma renin (A), noradrenaline (B), Nog-B (C), N-terminal pro-B-type natriuretic peptide (NT-proBNP) (D) and the ratio of heart to body weight (E) in the control, heart failure (HF), renal denervation (RDN), Nog and NEP groups. The relative mRNA levels of calcitonin gene-related peptide (CGRP) (F), nerve growth factor (β-NGF) (G), and growth-associated protein 43 (GAP-43) (H). * $P < 0.05$ vs. the HF and #$P < 0.05$ vs. the RDN group.
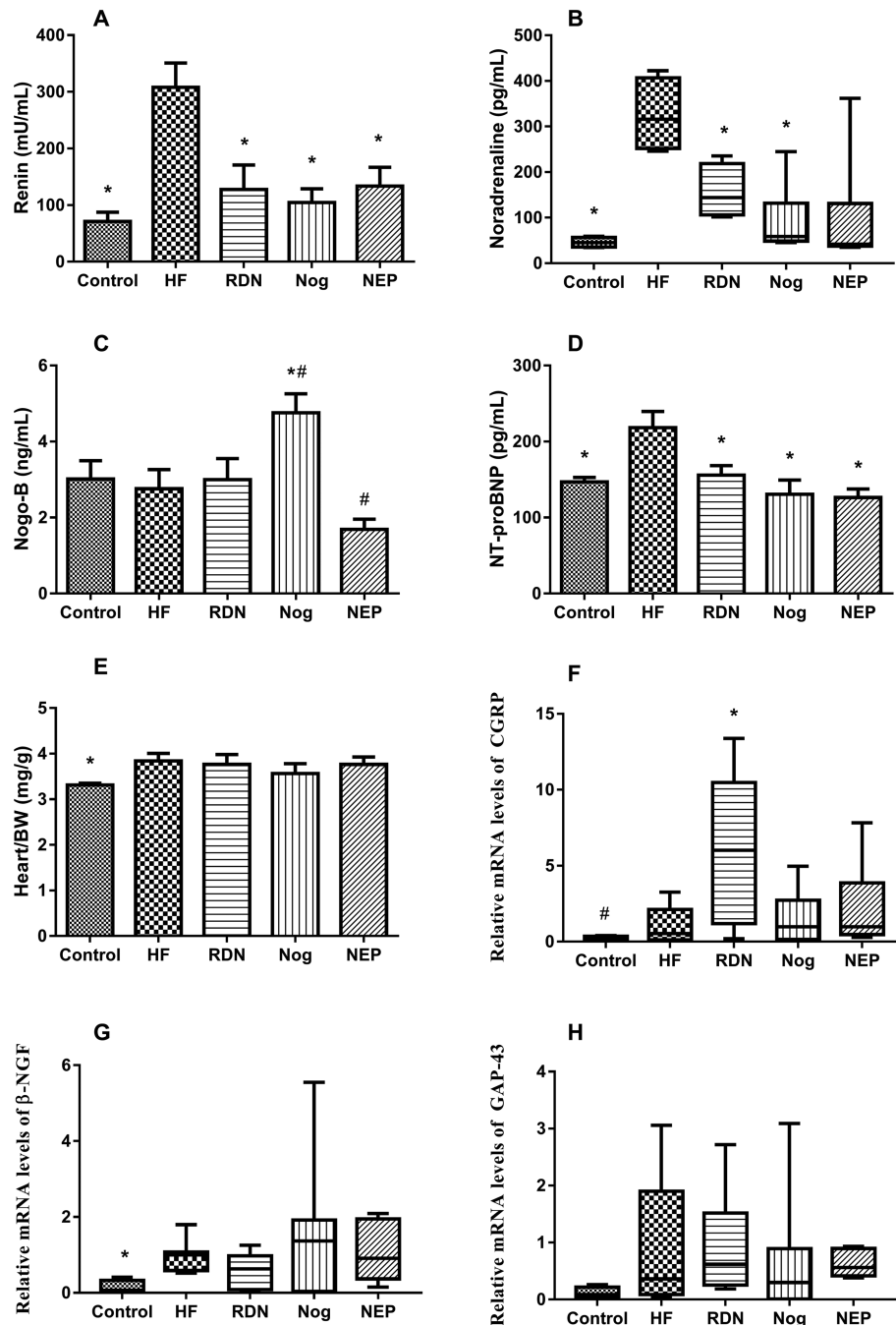
Variation of echocardiographic parameters after administration of Nog-B or its antagonist

When compared with the Control 2 group, LVDs, LVDd, LVVs, LVVd, LV mass AW, and LV mass AW (corrected) were significantly higher, and FS and LVEF lower in the HF 1, HF 2, and HF 3 groups (all $P < 0.05$), but no differences were observed among the Control 1, 2, and 3 groups (all $P > 0.05$) or among the HF 1, HF 2, and HF 3 groups (all $P > 0.05$) (*Figure 3E–H*).

Left ventricular end-systolic diameter and LVVs were greatly decreased, and FS and LVEF increased significantly in the RDN, Nog, and NEP groups when compared with the HF

Table 1 Comparisons of plasma parameters, heart weight, body weight, and relative mRNA expression of indicator of nerve regeneration in the renal arteries among the five groups

Characteristics	Control (n = 4)	HF (n = 7)	RDN (n = 6)	Nog (n = 7)	NEP (n = 6)
Renin (mU/mL)	71.20 ± 32.74*	307.98 ± 85.43	127.60 ± 75.03*	104.37 ± 60.29*	133.64 ± 81.56*
Noradrenaline (pg/mL)	45 (36–57)*	316 (252–407)	144 (106–219)*	59 (49–132)*	42 (37–131)
Nog-B (ng/mL)	3.02 ± 0.96	2.76 ± 1.33	2.99 ± 1.36	4.76 ± 1.32*#	1.69 ± 0.72#
NT-proBNP (pg/mL)	151.80 ± 21.23*	218.48 ± 51.88	155.98 ± 30.06*	130.65 ± 41.74*	126.30 ± 22.68*
Heart weight (mg)	848 ± 48*	1,457 ± 331	1367 ± 151	1443 ± 190	1186 ± 157
Body weight (g)	255.60 ± 10.22*	381.00 ± 85.48	364.67 ± 26.17	378.00 ± 55.47	341.57 ± 25.44
Heart/BW (mg/g)	3.32 ± 0.07*	3.84 ± 0.44	3.77 ± 0.52	3.56 ± 0.57	3.76 ± 0.43
Relative CGRP mRNA levels	0.23 (0.13–0.36)	0.53 (0.12–2.12)	6.01 (1.19–10.47)*	0.99 (0.14–2.73)	0.98 (0.47–3.87)
Relative β-NGF mRNA levels	0.07 (0.03–0.33)	1.00 (0.59–1.08)	0.63 (0.09–0.98)	1.37 (0.04–1.92)	0.91 (0.37–1.95)
Relative GAP-43 mRNA levels	0.09 (0.03–0.22)	0.36 (0.88–1.90)	0.62 (0.25–1.52)	0.30 (0.01–0.89)	0.56 (0.41–0.89)

β-NGF, nerve growth factor; BW, body weight; CGRP, calcitonin gene-related peptide; HF, heart failure; NT-proBNP, N-terminal pro-B-type natriuretic peptide; RDN, renal denervation.
Values presented as mean ± SD or median (interquartile range).
*$P < 0.05$ vs. the HF group.
#$P < 0.05$ vs. the RDN group.

group (all $P < 0.05$). LV mass AW and LV mass AW (corrected) were larger in the Nog group than in the RDN group (1237.50 ± 279.62 vs. 850.36 ± 177.09 mg, $P = 0.014$; 989.96 ± 223.70 vs. 680.29 ± 141.67 mg, $P = 0.014$). There were no significant differences of LV mass AW and LV mass AW (corrected) between the NEP group and the RDN group (1124.80 ± 253.82 vs. 850.36 ± 177.09 mg, $P = 0.055$; 899.85 ± 203.06 vs. 680.29 ± 141.67 mg, $P = 0.055$). However, no significant differences were observed in LVDs, LVDd, LVVs, LVVd, FS, and especially LVEF among the three groups: the RDN, Nog, and NEP groups (*Figure 5*).

Histology of the nerves surrounding renal artery

Nerves surrounding renal artery were stained with HE. The high-power field (HPF) counting of nervous cells was analysed at 200-fold magnification by two independent observers. Ten randomly selected independent microscopic fields were counted for each sample, and each value represented the mean of 10 HPF counting. *Figure 6* showed that RDN caused neuron loss or necrosis, and the number of nerves increased in the RDN (5.7 ± 1.4 vs. 3.7 ± 1.4/HPF, $P = 0.027$) and NEP (6.1 ± 1.3 vs. 3.7 ± 1.4/HPF, $P = 0.006$) groups when compared with the Nog group (*Figure 6A–D*). The protein expression of CGRP in the renal arteries was also confirmed by immunohistochemistry and semi-quantitative analysis, indicated as the values of the integrated optical density (IOD) measured by Image Pro Plus 6.0. The results showed that compared with the HF group, the protein expression of CGRP was significantly increased in the RDN group (IOD, 6.03 ± 0.54 vs. 12.79 ± 2.42, $P = 0.009$). Moreover, the levels of CGRP were significantly decreased in the Nog group compared with the RDN group (IOD, 5.13 ± 0.65 vs. 12.79 ± 2.42, $P = 0.006$) (*Figure 6E–I*).

Discussion

In the present study, RDN caused the decrease of the levels of plasma renin and noradrenaline in rats with CHF, but there were no statistically significant differences of their levels between the RDN, Nog, and NEP groups. The levels of CGRP mRNA and protein expression in renal arteries were significantly higher in the RDN group than in the HF group. Moreover, the Nog group possessed lower levels of CGRP protein than the RDN group. Immunohistochemistry results showed that the amount of nerves in renal arteries increased in the NEP group when compared with the Nog group. However, no significant differences were observed in LVDs, LVDd, LVVs, LVVd, FS, LVEF, and the levels of plasma NT-proBNP among the three groups: the RDN, Nog, and NEP groups. These findings indicated that reinnervation of renal arteries existed in CHF rats after RDN, and inhibiting this neural regeneration had no clinical significance and did not affect the efficacy of RDN to CHF.

Studies in humans observed the neural regeneration after renal transplants.[10,21,22] Mauriello et al.[10] reported that numerous neural regeneration were found in the periadventitial tissue of renal arteries, mainly in the innermost area, in patients undergoing kidney transplantation as early as 5 months after transplantation. In view of the less damage to nerves in renal arteries caused by RDN than kidney transplantation, the reinnervation of renal artery was more prone to occur after RDN than transplantation. As early as 7 days after RDN, there was evidence of regenerative activity (increased GAP43 staining) at sites of direct radiofrequency injury in renal artery.[23] After surgical RDN or chemical ablation of renal artery in rats, there was immunohistochemical evidence of anatomic reinnervation of renal arteries within 12 weeks.[24] At 5.5 and 11 months after RDN in normotensive sheep, anatomic evidence of reinnervation of the afferent and efferent nerves was indicated by the normal levels of tyrosine hydroxylase

Figure 3 Levels of plasma and echocardiographic parameters in the different groups. The levels of plasma renin (A), noradrenaline (B), Nog-B (C), N-terminal pro-B-type natriuretic peptide (NT-proBNP) (D), left ventricular end-systolic diameter (LVDs) and end-diastolic diameter (LVDd) (E), left ventricular end-systolic volume (LVVs) and end-diastolic volume (LVVd) (F), fractional shortening (FS) and left ventricular ejection fraction (LVEF) (G), and LV mass and LV mass corrected (H) in the Control 1, Control 2, Control 3, HF 1, HF 2, and HF 3 groups. *$P < 0.05$ vs. the Control 2 group.
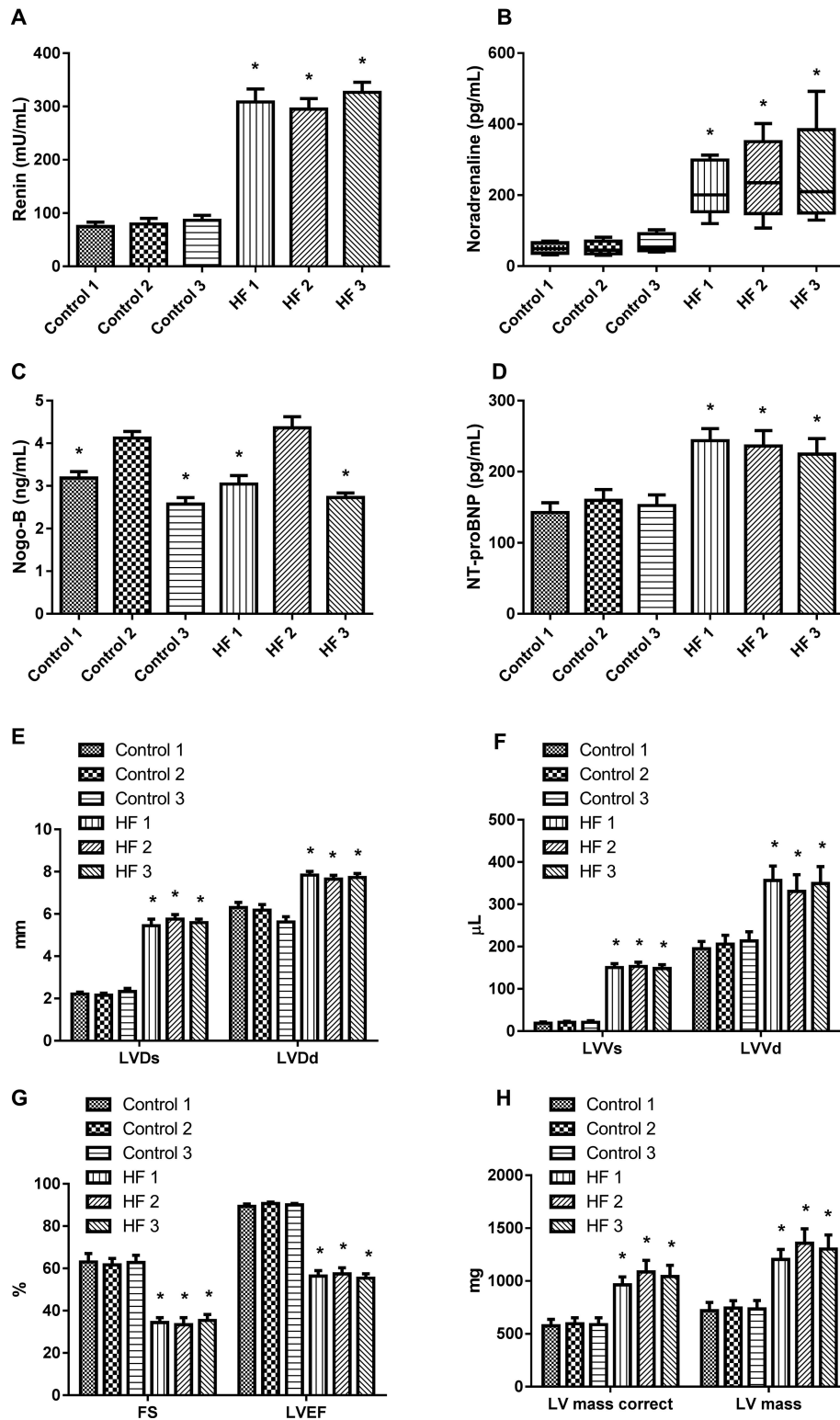
Figure 4 The protein expression of calcitonin gene-related peptide (CGRP), nerve growth factor (β-NGF) and growth-associated protein 43 (GAP-43) in the renal arteries in the different groups. Western blot analysis of CGRP, GAP-43 and β-NGF expression in the control (A), heart failure (HF) (B), renal denervation (RDN) (C), Nog (D) and NEP (E) groups and relative mRNA levels of CGRP (F), β-NGF (G), and GAP-43 (H) in the renal arteries in the different groups. *$P < 0.05$ vs. the HF and #$P < 0.05$ vs. the RDN group.
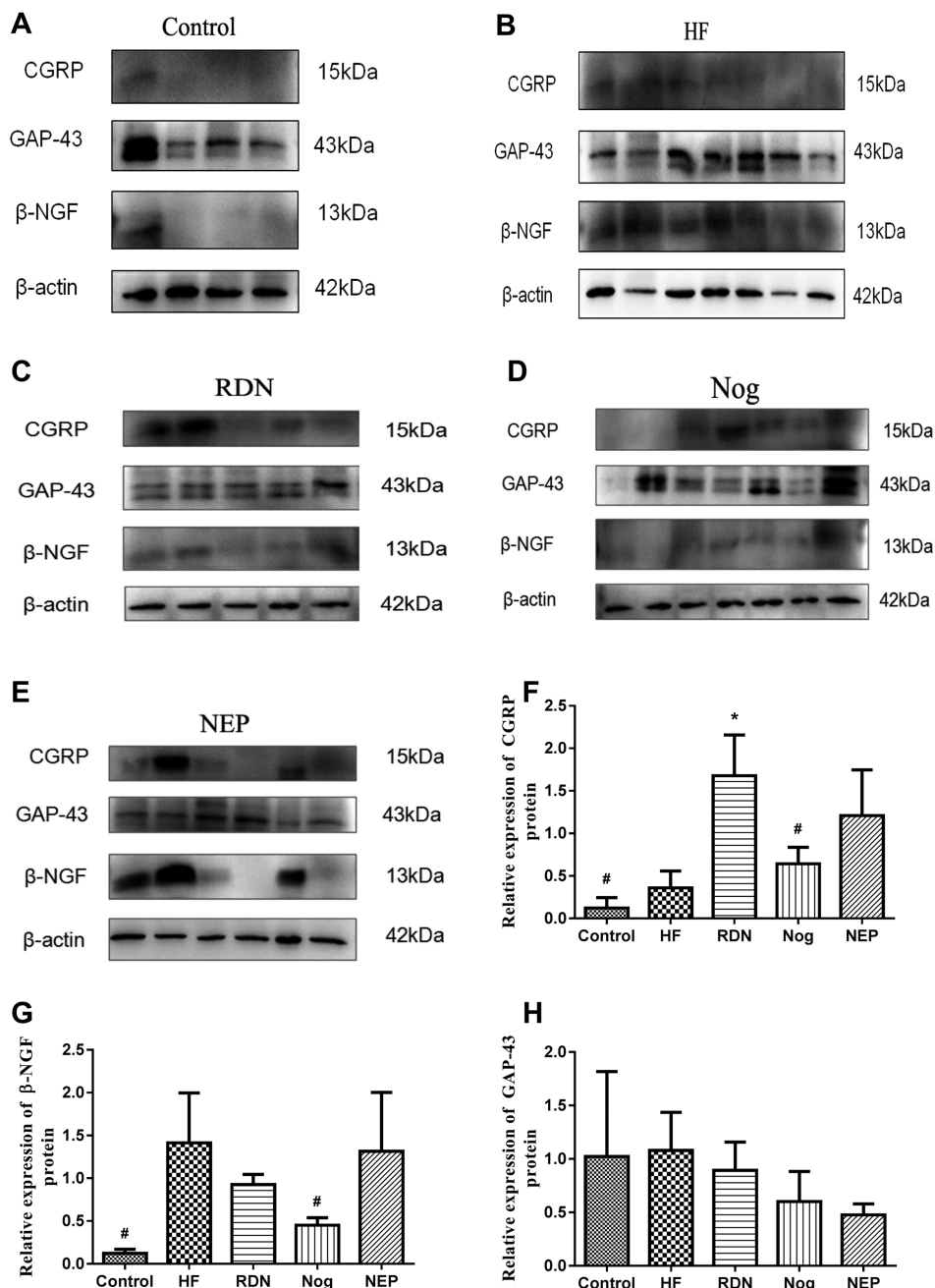

and CGRP staining.[25] All these studies supported the occurrence of nerve regeneration in renal arteries after RDN.

Unlike in the state of normotensive or hypertensive chronic kidney disease, neural regeneration in renal artery after RDN was assessed in the state of CHF in this study. Using specific markers of nerves including CGRP, GAP43, and NGF,[25] which are related to axonal growth and neuronal sprouting or create supportive environment for neural regeneration after nerve injury,[26–29] consistent with previous studies, our results showed that the levels of CGRP expression in renal artery were significantly higher in the RDN group than in the CHF rats, indicating that there existed reinnervation of renal arteries after RDN in CHF. In contrast to CGRP, no difference of the levels of GAP43 was seen between the RDN and HF group in

Figure 5 Difference of echocardiographic parameters among the control, heart failure (HF), renal denervation (RDN), Nog, and NEP groups at 4 months after administration. Comparison of left ventricular end-systolic diameter (LVDs), left ventricular end-diastolic diameter (LVDd) (A), left ventricular end-systolic volume (LVVs), left ventricular end-diastolic volume (LVVd) (B), fractional shortening (FS), left ventricular ejection fraction (LVEF) (C), and LV mass and LV mass corrected (D) among the control, HF, RDN, Nog, and NEP groups. Representative echocardiographic images in the HF (E), RDN (F), Nog (G), and NEP (H) rats. *$P < 0.05$ vs. HF and #$P < 0.05$ vs. RDN group.
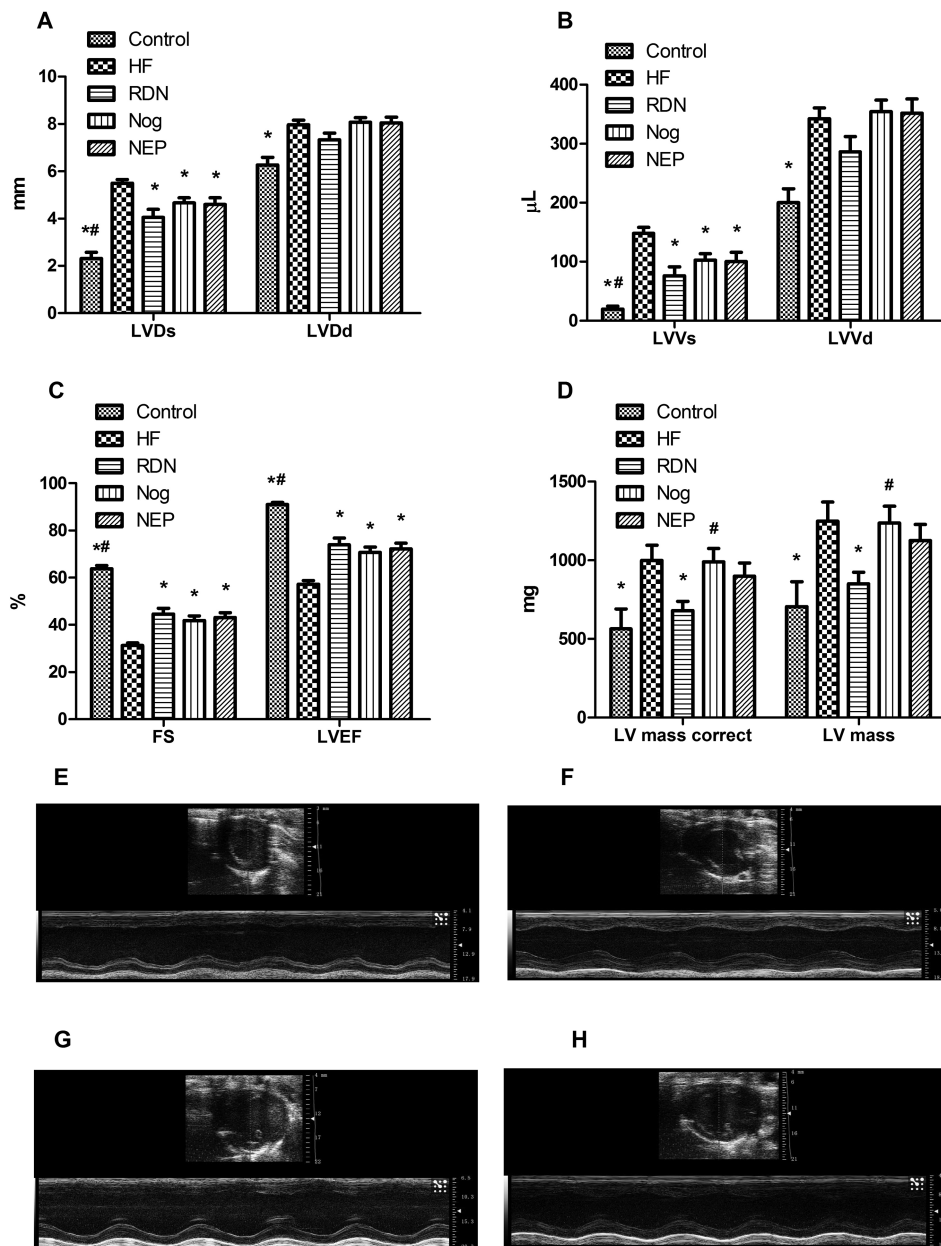

this study. Maybe the renal reinnervation process was complex, and the variation of some biological markers related to nerve repair, growth, and activity was not entirely consistent after RDN.[30] On the other hand, the comparison result of CGRP mRNA expression was inconsistent with the protein expression between the RDN and Nog groups, perhaps mRNA protein post-transcriptional regulation and translation can be responsible for the discrepancy of mRNA and protein levels in this study.

CGRP. Anyway, protein levels were closely related to its physiological function.

It was very important that whether the nerves of anatomic reinnervation after RDN had neural activity. Some researches indicated that this anatomic reinnervation was partially functional. Functional reinnervation was demonstrated by the presence of normal afferent and efferent responses to nerve stimulation after RDN.[25,31] Singh *et al.*[32] also observed the

Figure 6 Haematoxylin and eosin staining and immunohistochemistry of calcitonin gene-related peptide (CGRP) in the renal artery tissues in different groups. Haematoxylin and eosin staining of the renal artery in the heart failure (HF) *(*A), renal denervation (RDN) (B), Nog (C) and NEP (D) groups and immunohistochemistry of CGRP in the HF (E), RDN (F), Nog (G), and NEP (H) groups. Semi-quantitative analysis of CGRP levels by measuring integrated optical density (IOD) (E). $*P < 0.05$ vs. HF and $^{\#}P < 0.05$ vs. RDN group.
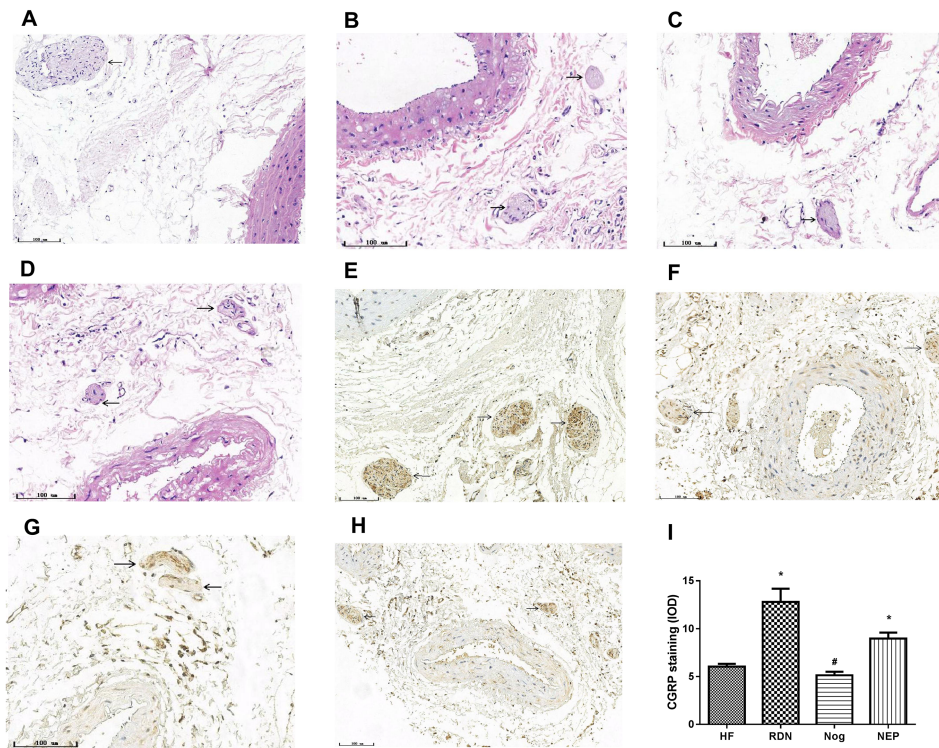
regrowth of renal nerves and return of function in hypertensive chronic kidney disease sheep after RDN by examining nerve function in isolated renal arcuate arteries and assessing renal sympathetic and sensory nerve regrowth by immunohistochemistry. But one study showed that the transplanted human kidney still remained functionally denervated and did not achieve functional reinnervation.[33] In this research, functional regeneration was assessed in the transplanted rather than RDN kidney. As mentioned above, maybe the different degree of damage to the nerves of renal artery was the cause of the distinct results.

It is crucial that whether regeneration of renal nerves after RDN has clinical significance and can affect the efficacy of RDN and whether inhibiting reinnervation is a benefit to CHF. In this study, the levels of plasma Nog-B increased in the Nog group and decreased in the NEP group, and the Nog group possessed lower levels of CGRP protein and the amount of nerves than the RDN group, indicating that regeneration of renal nerves after RDN in CHF rats was inhibited by Nogo-B. However, no significant differences were observed in LVDs; LVDd; LVVs; LVVd; FS; LVEF; and the levels of plasma renin, noradrenaline, and NT-proBNP, an indicator of CHF, among the CHF rats in the RDN, Nog, and NEP groups at 26 weeks after RDN, suggesting that either regeneration of

renal nerves or restricting regeneration after RDN in CHF rats had no clinical significance and did not affect the efficacy of RDN to CHF. In this view, microanatomical and partially functional regeneration of renal nerves did not equate its physiological functional recovery. Singh *et al*.[32] also thought that although incomplete anatomic and functional restoration of renal sympathetic and sensory nerves occurred, it did not appear to abrogate any of the beneficial effects of RDN. Although the sympathetic nerve distribution around the renal artery is a network structure that fuses or departs from each other around the renal artery, the possibility of achieving functional reinnervation is likely low in consideration of the disrupted architecture of neural circuits and networks at the ablative sites and the small amounts of anatomic reinnervation. Furthermore, a poorly organized neuromatous regeneration was another cause of not re-establishing functional reinnervation after RDN.[23]

Several limitations should be discussed. Firstly, the activities of regenerated nerves in renal artery after RDN in CHF were not assessed, and the observation time was not long enough, only 4 months after administration and 26 weeks after RDN in CHF rats. Maybe it takes a relatively long time for regenerated renal nerves to achieve physiological functional recovery and affect the cardiac function. Secondly, our study

focused on the reinnervation of the renal artery rather than the intrarenal vasculature, for example, arcuate arteries, in the kidney, perhaps the latter of which also had impact on reducing sympathetic activities effects of RDN. Finally, chemical denervation was different from catheter-based RDN. Whether our findings can be directly translated into the CHF patients subjected to catheter-based RDN remains unclear.

In conclusions, our results showed that the levels of CGRP expression in renal arteries increased after RDN in CHF rats. The Nog group possessed lower levels of CGRP protein than the RDN group and lower amount of nerves in renal arteries than the NEP group. Furthermore, neither only RDN nor the use of Nogo-B or its antagonist after RDN in CHF rats caused differences in LVDs; LVDd; LVVs; LVVd; FS; LVEF; and the levels of plasma renin, noradrenaline, and NT-proBNP. These

findings demonstrated the reinnervation of renal arteries after RDN in CHF rats, which had no effect on therapeutic role of RDN in CHF, and that inhibiting this neural regeneration had no clinical significance and did not affect the efficacy of RDN to CHF.

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