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Carotid baroreceptor stimulation improves cardiac performance and reverses ventricular remodelling in canines with pacing-induced heart failure

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ABSTRACT

Aims: Carotid baroreceptor stimulation (CBS) has potential protective effects on chronic heart failure (CHF). The aim of our study was to investigate the effects of CBS on more detailed aspects of ventricular remodelling and the underlying mechanisms in a CHF canine model.

Main methods: Twenty-four beagles were randomised into Con (n = 8), CHF (n = 8), and CHF-CBS (n = 8) groups. The CHF and CHF-CBS groups underwent 6 weeks of rapid ventricular pacing (RVP) at 250 beats per minute to establish a CHF model. Concomitant CBS was delivered together with RVP in the CHF-CBS group.

Key findings: RVP for 6 weeks caused typical heart failure in the CHF group. CBS significantly reversed the decrease in the high-frequency heart rate variability component and increase in low-frequency/high-frequency ratio induced by RVP. CBS significantly reduced cardiac dilation, improved left ventricle ejection fraction, and inhibited the increase in natriuretic peptide mRNA expression of LV tissue. CBS alleviated collagen volume fraction and reduced protein expression of transforming growth factor β1, matrix metallopeptidase 2, and matrix metallopeptidase 9, as well as decreased the percentage of TUNEL-positive nuclei and protein expression of Caspase-3 in LV tissue. The intracellular PKA signalling pathway and cardiac inflammation of LV tissue were upregulated in the CHF group, and markedly inhibited by CBS.

Significance: Our study found that CBS improved cardiac performance and reversed ventricular remodelling in CHF canines by rebalancing the autonomic nervous system; the suppression of the intracellular PKA signalling pathway and cardiac inflammation might underly the mechanisms.

1. Introduction

With the aggravating trend of an ageing population, the hospitalisation and mortality due to chronic heart failure (CHF) increased sharply all around the world, especially in developed countries [1]. Exaggerated neuroendocrine excitation occurs in heart failure and contributes to pathological cardiac remodelling and disease progression [2–4]. With a deeper understanding of cardiac remodelling, several classical therapeutic strategies have shown great clinical achievement, such as β blockers, renin-angiotensin-aldosterone system inhibitors, and

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BP, blood pressure; BUN, blood urea nitrogen; Caspase-3, cysteinyl aspartate specific proteinase-3; CBS, carotid baroreceptor stimulation; CHF, chronic heart failure; Cr, creatinine; CVF, collagen volume fraction; HR, heart rate; HRV, heart rate variability; IL-6, interleukin-6; LV, left ventricle; LVEDV, left ventricle end-diastolic volume; LVEF, left ventricle ejection fraction; LVESV, left ventricle end-systolic volume; LVFS, left ventricle fractional shortening; LVIDd, left ventricle end-diastolic inner dimension; LVIDs, left ventricle end-systolic inner dimension; MAP, mean arterial pressure; MMP2, matrix metallopeptidase 2; MMP9, matrix metallopeptidase 9; PKA, cAMP-dependent protein kinase; PKAC, cAMP-dependent protein kinase catalytic subunits; P-PKA, Thr-197 phosphorylated cAMP-dependent protein kinase; RVP, rapid ventricular pacing; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; TGF-β1, transforming growth factor β1; TNF-α, tumour necrosis factor α; β1-AR, β1-adrenergic receptor

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cardiac resynchronisation therapy [5–7].

New therapies of autonomic neuromodulation have developed in recent years, including carotid baroreceptor stimulation (CBS), renal denervation, and vagus nerve stimulation [2,8–10]. CBS was invented for the treatment of refractory hypertension [11,12]. Shortly thereafter, it showed promising effects on CHF [13,14]. In both ischaemic and rapid ventricular pacing (RVP) canine models of CHF, chronic CBS suppressed sympathetic over-activation, reversed left ventricular (LV) remodelling, and improved cardiac function and survival in CHF [15,16]. Studies in patients showed that CBS stabilised the muscle sympathetic nerve activity, increased the baroreflex sensitivity markedly, restored the imbalance of autonomic nervous tone in uncontrolled CHF [17,18], and significantly increased the distance walked in 6 min, quality-of-life score, LV ejection fraction (LVEF), and New York Heart Association (NYHA) class ranking [17,19,20]. Our previous study showed that low-level CBS suppressed atrial electrical remodelling and atrial fibrillation by inhibiting left stellate ganglion activity in acute rapid atrial pacing canines [21].

Ventricular remodelling in CHF relates to complicated pathophysiological alterations. The powerful evidence of CBS in CHF treatment impels us to explore its effects on more detailed aspects of ventricular remodelling and the potential mechanisms. In the present study, we hypothesised that CBS has profound effects on cardiac apoptosis, fibrosis, and inflammation in pacing-induced CHF canines.

2. Materials and methods

2.1. Animal and study protocol

Twenty-four male beagles (aged 12 months; weight 10–12 kg) were randomised into a control group (Con, $N = 8$), chronic heart failure group (CHF, $N = 8$), and chronic heart failure with carotid baroreceptor stimulation group (CHF-CBS, $N = 8$). The protocol was approved by the ethics committee of the Renmin Hospital of Wuhan University and conformed with the guidelines outlined by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

One week after settling in, all beagles underwent echocardiography and a 15-minute electrocardiography (ECG) test; blood samples were also collected. The CHF and CHF-CBS groups underwent cardiac pacemaker (HSC-20D, Harbin University of Science and Technology, Harbin, China) implantation for CHF induction, and the CHF-CBS group underwent CBS device (custom designed and made, Ensense Biomedical Technologies Co., Ltd. Shanghai, China) implantation for CBS. The Con group underwent a sham operation of both cardiac pacemaker and CBS device implantation. The CHF group underwent a sham operation of CBS device implantation. Two weeks after the operations, the pacemakers and CBS devices were switched on simultaneously, and pacing and CBS lasted for 6 weeks. At the end of the study, both the pacemaker and the CBS stimulator (in CHF-CBS group) were turned off, to permit spontaneous sinus rhythm to return. After a stabilisation period of 30 min, echocardiography and the ECG test were repeated, blood samples were collected, and LV anterior wall tissues were harvested (Fig. 1).

2.2. Cardiac pacemaker and CBS device implantation

The animals were anaesthetised with intravenous (IV) pentobarbital (3%, 30 mg/kg) and infused with saline at 50 to 100 mL/h to compensate for body fluid losses during the operation. ECG and femoral artery blood pressure (BP) were monitored continuously throughout the surgery. All recordings were displayed on a computer-based, electrophysiology system (LEAD 7000, Jinjiang, Inc., Chengdu, China), and were stored for off-line analysis.

Under fluoroscopic guidance, the tip of the pacing electrode (REF 4457, Boston Scientific, USA) was introduced to the right ventricular apex through the right external jugular vein. The end of the electrode was connected to the pacemaker, which was placed on the right thoracic wall subcutaneously. The pacemaker was set at a fixed pacing rate of 250 beats per min in both the CHF and CHF-CBS groups. Ventricular pacing was verified weekly by cardiac auscultation.

The CBS device consists of a stimulator and a bipolar electrode. The placement of the electrode tip was described previously [21,22]. Briefly, a bipolar custom-made platinum-iridium circular stimulating electrode was implanted circumferentially around the right common carotid artery adjacent to the carotid sinus. The location of the electrode was adjusted until a satisfactory response occurred, which is acute stimulation could elicit an abrupt decrease in systolic BP greater than10% or heart rate (HR) greater than15% from baseline. The end of the electrode was connected to the stimulator, which was placed on the left thoracic wall subcutaneously. The working stimulus voltage was set at the level that elicited a decrease in BP by 10% or HR by 15%. The stimulus frequency was 20 Hz; pulse duration was 2 ms; duty cycle was 9 min ON/1 min OFF.

2.3. Serum analysis, echocardiography, and ECG

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), and blood urea nitrogen (BUN) were analysed (Advia 2400 automatic biochemical analyzer, Siemens, Germany).

The LV end-diastolic inner dimension (LVIDd), LV end-systolic inner dimension (LVIDs), LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV fractional shortening (LVFS), LVEF, and the peak flow rate ratio E/A of the mitral valve in early and late diastole was measured at sinus rhythm (Vivid9 GE medical, Milwaukee, WI). The echocardiogram (at baseline and after 6 weeks of RVP) tests were performed by a physician experienced in ultrasound who was blinded to the group information of each canine and the time point of the study.

QRS complex duration and corrected QT interval were measured off-line. The corrected QT (QTc) was calculated by using the Fridericia formula: $QTc = QT/(RR^{1/3})$. The power spectrum of HRV was analysed on 15-minute ECG recording segments, and an autoregressive algorithm

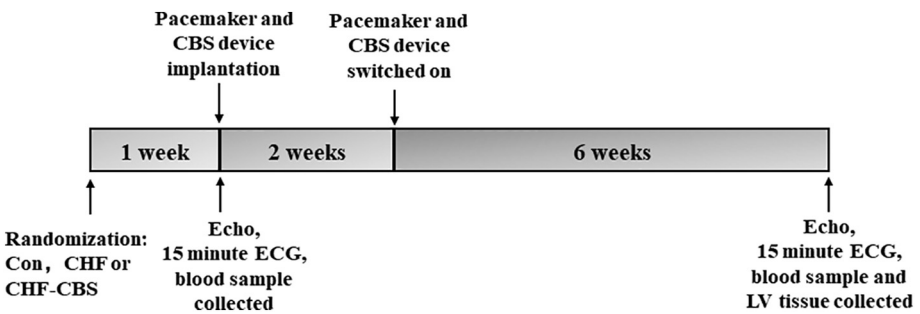

Fig. 1. Study protocol. CBS, carotid baroreceptor stimulation; Con, control group ($n = 8$); CHF, chronic heart failure group (n = 8); CHF-CBS, chronic heart failure with carotid baroreceptor stimulation group (n = 8); ECG, electrocardiograph; echo, echocardiography; LV, left ventricle.

Table 1
Effects of CBS on physiological indices.

	Con (n = 8)	CHF (n = 8)	CHF-CBS (n = 8)
HR, beat/min			
Baseline	140.6 ± 2.3	137.3 ± 5.2	140.8 ± 1.0
Endpoint	138.0 ± 1.9	148.3 ± 2.5*,††	140.8 ± 1.3$^{\#\#}$
delta	−2.6 ± 3.3	11.0 ± 4.4†	0.0 ± 1.7$^{\#}$
MAP, mm Hg			
Baseline	114.9 ± 3.8	116.5 ± 2.2	110.0 ± 1.8
Endpoint	118.3 ± 3.0	103.6 ± 3.8**,†	106.7 ± 3.7$^{£}$
delta	3.4 ± 3.0	−12.4 ± 3.2†	−3.3 ± 3.5
QRS duration, ms			
Baseline	70.1 ± 0.3	70.3 ± 0.4	71.2 ± 0.5
Endpoint	71.4 ± 0.4	75.8 ± 0.4**,††	73.7 ± 1.0$^{*,£}$
delta	1.3 ± 0.7	5.5 ± 0.8††	2.5 ± 0.9$^{\#}$
QTc interval, ms			
Baseline	307.9 ± 4.5	316.8 ± 5.3	307.4 ± 5.7
Endpoint	313.3 ± 4.1	364.5 ± 8.3**,††	331.2 ± 5.3$^{*,\#}$
delta	5.43 ± 5.1	47.7 ± 6.7††	23.9 ± 6.0$^{\#}$
ALT, U/L			
Baseline	26.9 ± 1.4	20.5 ± 2.3	24.8 ± 2.4
Endpoint	25.7 ± 1.0	30.3 ± 1.9*,†	21.4 ± 1.2$^{\#\#,££}$
delta	−1.1 ± 2.1	5.5 ± 2.5†	−3.5 ± 3.5$^{\#}$
AST, U/L			
Baseline	17.1 ± 1.5	20.5 ± 3.4	15.3 ± 2.2
Endpoint	19.7 ± 2.9	30.9 ± 2.8*,†	30.5 ± 2.8$^{**,£}$
delta	2.6 ± 4.1	10.3 ± 2.9	15.3 ± 2.8$^{£}$
BUN, mmol/L			
Baseline	3.3 ± 0.3	3.9 ± 0.3	3.2 ± 0.4
Endpoint	3.8 ± 0.3	5.1 ± 0.3**,††	3.3 ± 0.2$^{\#\#}$
delta	0.5 ± 0.5	1.3 ± 0.3	0.0 ± 0.4$^{\#}$
Cr, μmol/L			
Baseline	37.9 ± 3.5	40.2 ± 1.6	34.5 ± 4.7
Endpoint	40.9 ± 1.2	46.8 ± 2.2*,†	32.5 ± 1.7$^{\#\#,££}$
delta	3.0 ± 2.6	6.6 ± 2.3	−2.0 ± 5.8

Values are mean ± SEM. HR, heart rate; MAP, mean arterial pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine.
* $p < 0.05$ vs. baseline.
** $p < 0.01$ vs. baseline.
† $p < 0.05$ vs. Con.
†† $p < 0.01$ vs. Con.
$^{\#}$ $p < 0.05$ vs. CHF.
$^{\#\#}$ $p < 0.01$ vs. CHF.
$^{£}$ $p < 0.05$ vs. Con
$^{££}$ $p < 0.01$ vs. Con.

was used to analyse digitised signals from the ECG recordings. The power spectral variables were determined as follows: total power (TP), very low frequency power (VLF, < 0.04 Hz), LF power (0.04–0.15 Hz), HF power (0.15–0.40 Hz), and the ratio of LF power to HF power. The values of the HF and LF power were measured and expressed in normalised units (nu), which were calculated with the formula: HF nu = HF power/TP − VLF power × 100, LF nu = LF power / TP − VLF power × 100. Finally, the LF/HF ratio was calculated [22].

2.4. Histology

LV tissue was immediately fixed in 4% paraformaldehyde solution, embedded in paraffin, and sliced serially into 5-um sections. The collagen volume fraction (CVF, fibre area/the sum of fibre area and myocardial area) was calculated based on Masson trichrome staining, and the degree of myocardial fibrosis was determined. Apoptosis was evaluated by terminal deoxynucleoitidyl transferase mediated nick end labelling (TUNEL) staining. All histological images were analysed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.5. RT-PCR and western blotting

LV tissue was excised, quick-frozen in dry ice, and stored immediately at −80 °C. Total RNA was extracted and analysed using reverse transcription followed by quantitative real-time polymerase chain reaction (RT-PCR). The sequences of the RT-PCR primers are as follows: ANP (forward primer 5′-ATT TCA AGA ACT TGC TGG ACCG-3′ and reverse primer 5′-GCT TCC GCA TTC TGC TCA CT-3′); BNP (forward primer 5′-CCA CTT CTC TCC AGC GAC AT-3′ and reverse primer 5′-CAG CTC TGA AAC TGC GTC CT-3′); GAPDH (forward primer 5′-GGG TGA TGC TGG TGC TGA GTA T-3′ and reverse primer 5′-TTG CTG ACA ATC TTG AGG GAG TT-3′).

The protein concentration of LV tissue homogenate was measured by the Pierce BCA Protein Assay Kit (23,225, Thermo Scientific, MIT, USA) according to the manufacturer's instructions. The protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. The primary antibodies used in the study included polyclonal antibody for transforming growth factor β1 (TGF-β1, ab92486, Abcam), matrix metallopeptidase 2 (MMP2, ab37150, Abcam), matrix metallopeptidase 9 (MMP9, AF5228, Affinity), cysteinyl aspartate specific proteinase-3 (Caspase-3,9662, Cell Signaling Technology), β1-adrenergic receptor (β1-AR, ab77198, Abcam), cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) catalytic subunits (PKAC, AF4175-SP, R&D), Thr-197 phosphorylated PKA (P-PKA, ab75991, Abcam), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a, ab2861, Abcam), tumour necrosis factor α (TNF-α, A0277, Abclonal), interleukin-6 (IL-6, GB11117, Servicebio), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GB12002, Servicebio).

2.6. Statistical analysis

The quantitative data were expressed as mean ± standard error of the mean (SEM). Differences among the three groups were analysed by one-way ANOVA, first the Levene's test for homogeneity of variance was performed. If the Levene test had a p value > 0.05, post-hoc comparisons between groups were performed by Tukey's test; If the Levene's test had a p value < 0.05, Tamhane's T2 post-hoc test was used. Differences within a group between baseline and endpoint were assessed with a paired t-test. A P value < 0.05 was considered statistically significant. SPSS 22.0 was used for analysis.

3. Results

After 6 weeks of RVP, in the CHF group, the beagles developed clinical symptoms (tachypnoea, poor appetite, reduced physical activity), and two beagles developed moderate ascites. HR was significantly increased (148.3 ± 2.5 after RVP vs. 137.3 ± 5.2 at baseline, $P < 0.01$; 75.8 ± 0.4 after RVP vs. 70.3 ± 0.4 at baseline, $P < 0.01$, respectively), mean arterial pressure (MAP) was significantly decreased (103.6 ± 3.8 after RVP vs. 116.5 ± 2.2 at baseline, $P < 0.01$), and QRS duration and QTc interval were significantly prolonged (75.8 ± 0.4 after RVP vs. 70.3 ± 0.4 at baseline, $P < 0.01$; 364.5 ± 8.3 after RVP vs. 316.8 ± 5.3 at baseline, P < 0.01). The working stimulus voltage of CBS that induced a decrease in BP of 10% or HR by 15% was 4.8 ± 0.2 V ($N = 8$). The aforementioned symptoms were milder in the CHF-CBS group, no beagle developed ascites, and CBS significantly inhibited the increase in HR and the prolongation of QRS duration and QTc interval (Table 1).

Hepatic function was evaluated by AST and ALT concentrations. Renal function was evaluated by Cr and BUN concentrations. Six weeks of RVP resulted in a significant increase in AST, ALT, Cr, and BUN concentrations in the CHF group. CBS significantly decreased ALT, Cr, and BUN (Table 1).

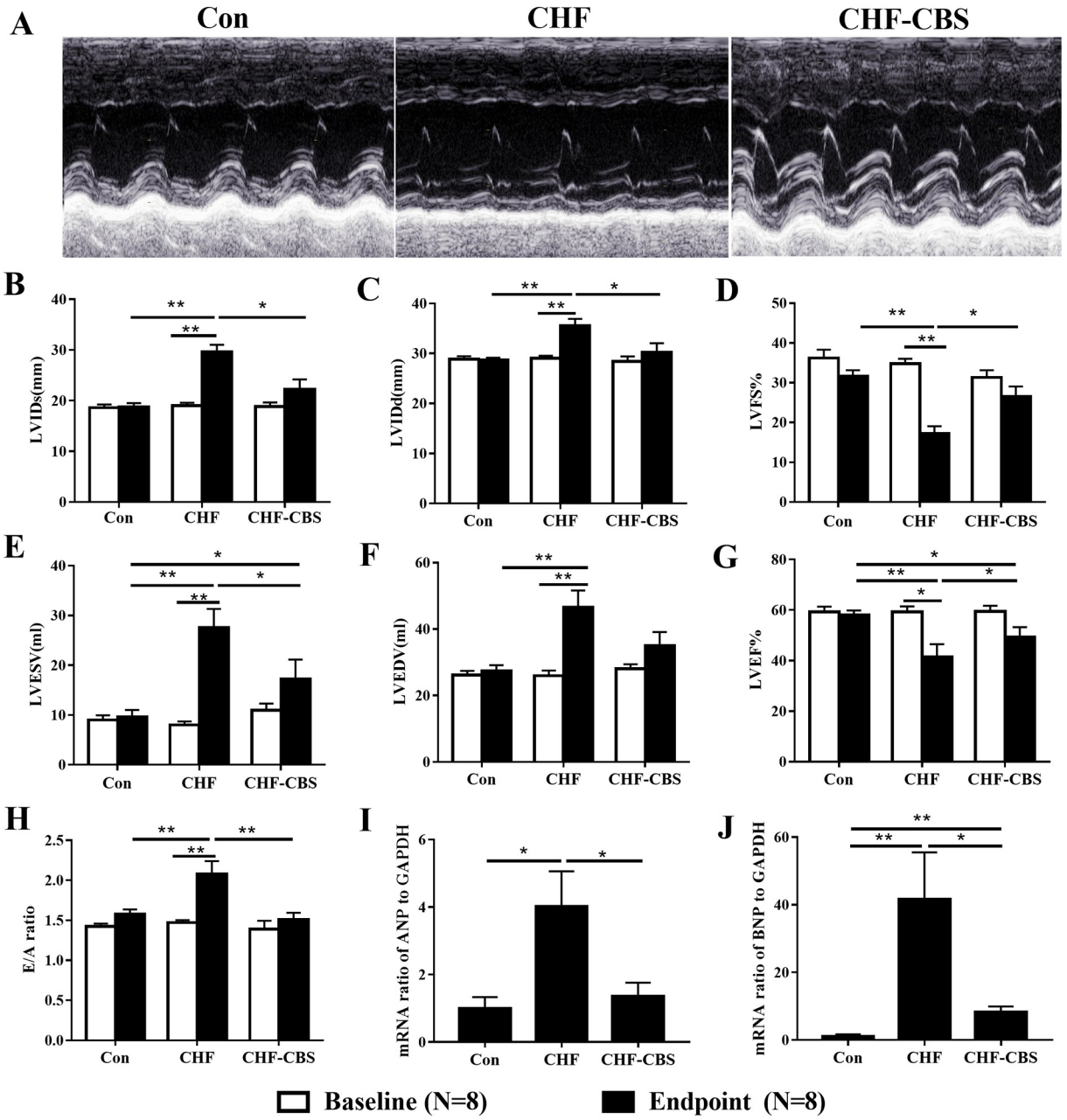

Fig. 2. Effects of CBS on cardiac structure, function, and biomarkers of CHF. Representative M-mode echocardiograms obtained after sham surgery or 6 weeks of RVP (A). The LVIDs, LVIDd, LVFS, LVESV, LVEDV, LVEF, and E/A ratio of the mitral valve in the CHF group deteriorated significantly after 6 weeks of RVP. The deterioration of LVIDs, LVIDd, LVFS, LVESV, LVEF, and E/A ratio of mitral valve were alleviated by CBS, with no significant effect on LVEDV (B–H). ANP and BNP mRNA expression of LV tissue was increased significantly in the CHF group. CBS reduced the increase in both ANP and BNP mRNA expression significantly (I, J). Values are mean ± SEM. LVIDs, LV end-systolic inner dimension; LVIDd, LV end-diastolic inner dimension; LVFS, LV fractional shortening; LVESV, LV end-systolic volume; LVEDV, LV end-diastolic volume; LVEF, LV ejection fraction; E/A ratio, the peak flow rate ratio of mitral valve in early and late of diastole. ANP, A-type natriuretic peptide; BNP, B-type natriuretic peptide $^{*}P < 0.05$, $^{**}P < 0.01$.

3.1. Effects of CBS on cardiac structure, function, and biomarkers of CHF

All echocardiographic measurements at baseline showed no significant difference among the three groups. After 6 weeks of RVP, LVIDd, LVIDs, LVEDV, LVESV, LVFS, LVEF, and E/A ratio of the mitral valve in the CHF group showed significant deterioration compared with the Con group. CBS significantly decreased LVIDd, LVIDs, LVESV, and E/A ratio of the mitral valve and improved both LVFS (26.6 ± 2.5% vs. 17.3 ± 1.8%, $P < 0.05$) and LVEF (49.3 ± 2.6% vs. 40.3 ± 3.3%, $P < 0.05$) compared with the CHF group (Fig. 2A-2H).

ANP and BNP mRNA expression in LV tissue were increased significantly in the CHF group, and CBS inhibited the increase in both ANP and BNP mRNA expression significantly (Fig. 2I-2J).

3.2. Effects of CBS on cardiac fibrosis

The CVF of LV tissue was calculated by Masson trichrome staining, as shown in the representative photomicrographs (Fig. 3A). After 6 weeks of RVP, CVF were increased significantly in the CHF group compared with the Con group (5.8 ± 0.5% vs. 2.4 ± 0.2%, $P < 0.01$). CBS significantly reduced CVF compared with the CHF group (4.5 ± 0.2% vs. 5.8 ± 0.5%, $P < 0.05$, Fig. 3B). The protein expression of TGF-β1, MMP2, and MMP9 in LV tissue was significantly increased in the CHF group. CBS suppressed the increase in protein

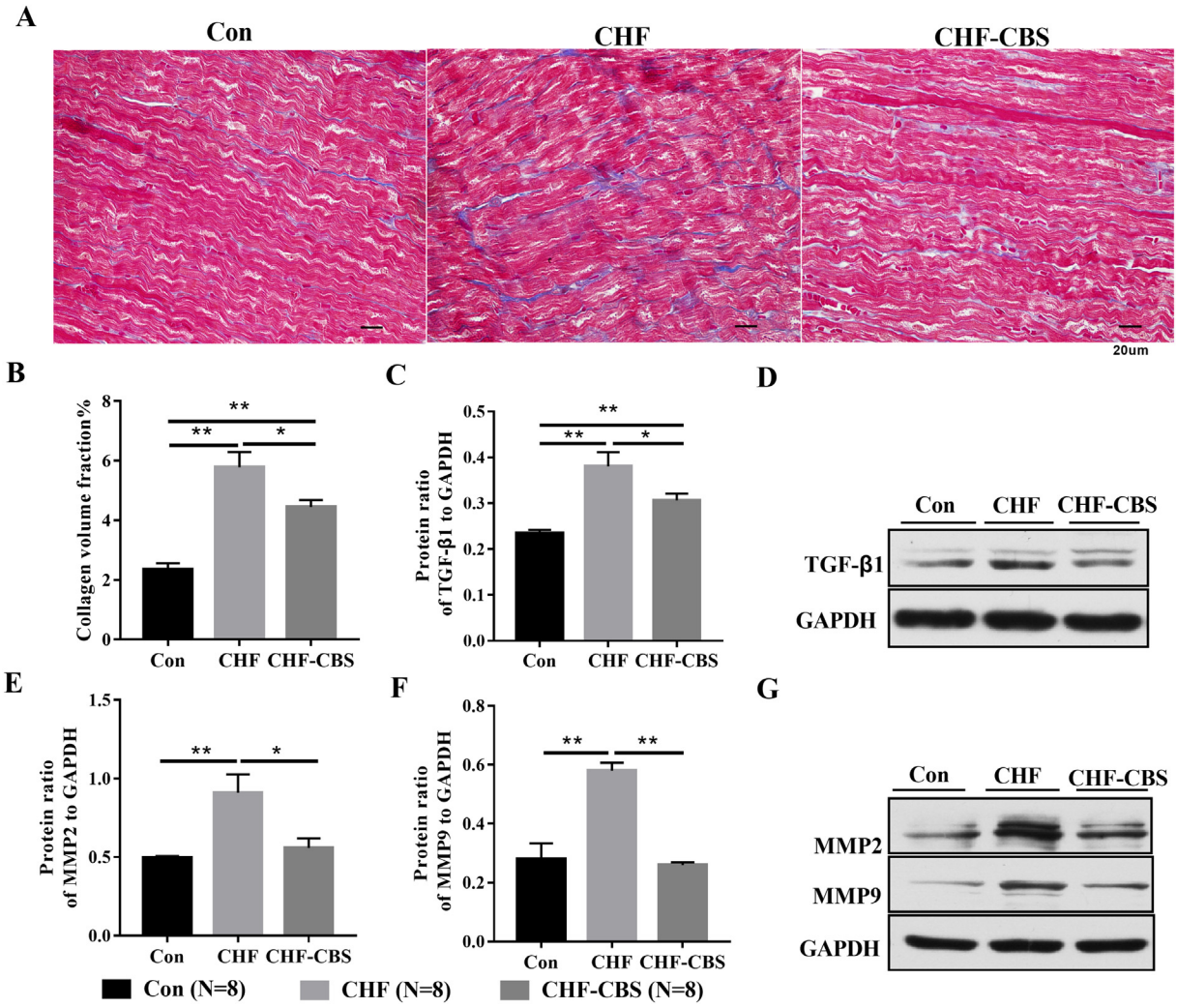

Fig. 3. Effects of CBS on cardiac fibrosis. Representative photomicrographs of LV tissue with Masson trichrome staining (A, scale bar = 20μm) and the quantitative comparison of collagen volume fraction (B) showed that CBS reduced cardiac fibrosis induced by 6 weeks of RVP. Data from western blotting and representative immunoblots (C–G) showed that the protein expression of pro-fibrotic markers (TGF-β1, MMP2, MMP9) in LV tissue increased significantly in the CHF group. CBS suppressed the increase in protein expression of all those markers significantly. Values are mean ± SEM. TGFβ1, transforming growth factor; MMP2, matrix metallopeptidase 2; MMP9, matrix metallopeptidase 9. *P < 0.05; **P < 0.01.

expression of all those pro-fibrotic factors significantly (Fig. 3C-3G).

3.3. Effects of CBS on cardiac apoptosis

As depicted in the representative photomicrographs (Fig. 4A), the percentage of TUNEL-positive nuclei to total nuclei in LV tissue increased significantly in the CHF group compared with the Con group (47.4 ± 2.1% vs. 18.1 ± 1.4%, $P < 0.01$), which was markedly blunted by CBS (34.1 ± 3.2% vs. 47.4 ± 2.1%, P < 0.01, Fig. 4B). In addition, protein expression of Caspase-3 in LV tissue increased significantly in the CHF group, which was also suppressed by CBS (Fig. 4C-4D).

3.4. Effects of CBS on HRV power spectrum

Six weeks of RVP decreased the HF components (35.6 ± 4.1 nu after RVP vs. 66.8 ± 3.7 nu at baseline, P < 0.01) and increased the LF components (50.5 ± 4.2 nu after RVP vs. 30.5 ± 3.5 nu at baseline, P < 0.01) and LF/HF ratio (11.4 ± 4.2 after RVP vs. 0.7 ± 0.2 at baseline, $P < 0.05$) of the CHF group significantly, whereas the HF, LF, and LF/HF ratio of the Con group stayed at the same level as baseline at the end of the study. The decrease of HF components and the increase of LF/HF ratio were reversed significantly (P < 0.05) when CBS was applied (49.5 ± 4.1nu and 1.4 ± 0.3, respectively) compared with the CHF group (Fig. 5A-5C).

3.5. Effects of CBS on the PKA signalling pathway and cardiac inflammation

As shown in the representative immunoblots, after 6 weeks of RVP, the protein expression of β1-AR and SERCA2a in LV tissue decreased, whereas protein expression of PKAC and P-PKA increased markedly in the CHF group compared with the Con group. CBS reversed the decrease of protein expression of β1-AR and SERCA2a and the increase of protein expression of PKAC and P-PKA significantly (Fig. 6A-6F).

The protein expression of inflammatory factor TNF-α and IL-6 in LV tissue increased significantly in the CHF group after 6 weeks of RVP, and CBS inhibited the increase in protein expression of TNF-α and IL-6 significantly (Fig. 6G-6I).

4. Discussion

In this study, we have two main findings. First, we found that 6 weeks of CBS mitigated the progression of CHF, improved the cardiac

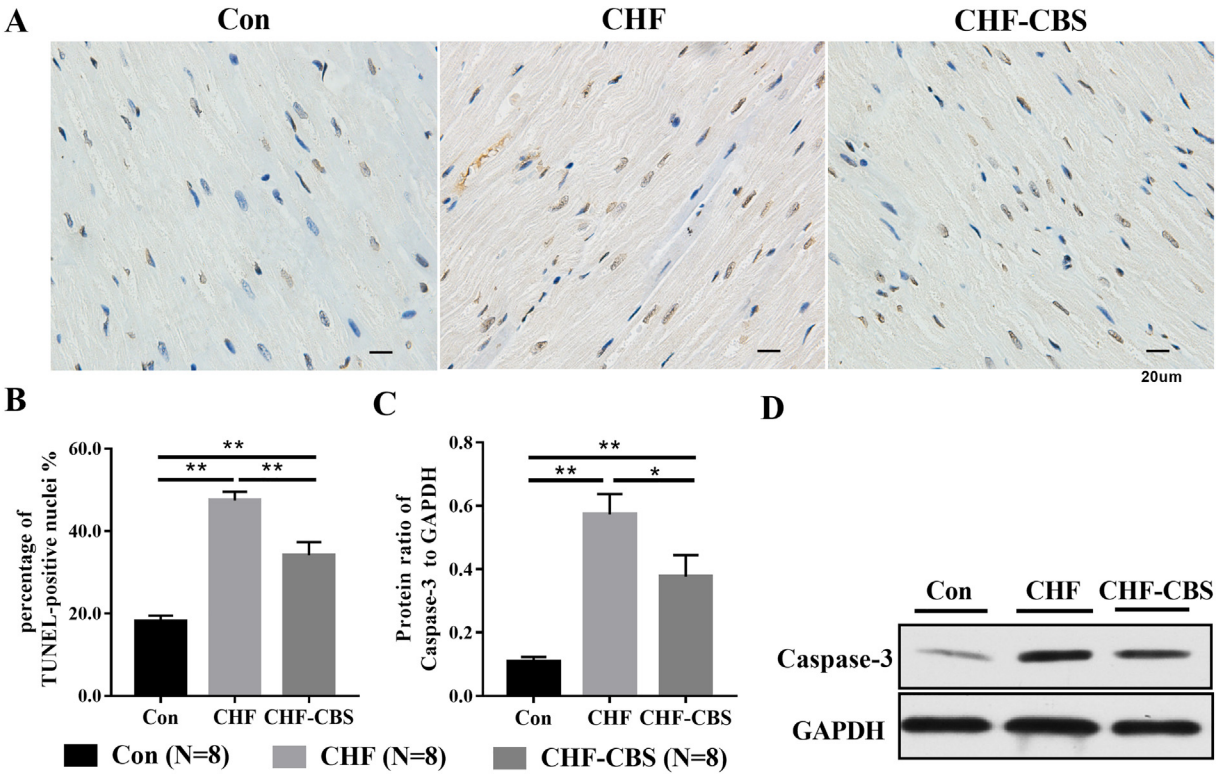

Fig. 4. Effects of CBS on cardiac apoptosis. Representative photomicrographs of LV tissue with TUNEL staining (A, scale bar = 20 μm) and quantitative comparison of the percentage of TUNEL-positive nuclei to total cell nuclei (B) showed that CBS reduced cardiac apoptosis induced by 6 weeks of RVP. Data from western blotting and representative immunoblots (C, D) showed that the protein expression of pro-apoptotic marker (Caspase-3) in LV tissue increased significantly in CHF group, and CBS reversed the increase in protein expression of Caspase 3. Values are mean ± SEM. Caspase-3, cysteinyl aspartate specific proteinase-3. *P < 0.05; **P < 0.01.

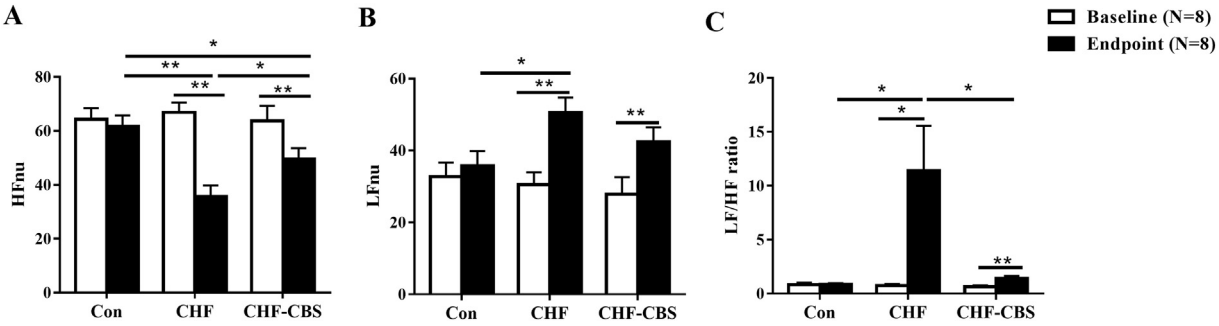

Fig. 5. Effect of CBS on short-term power spectral analysis of HRV. The HF component was diminished, whereas the LF component and LF/HF ratio were increased significantly in the CHF group after 6 weeks of RVP. The decrease of the HF component and the increase of the LF/HF ratio were reversed by CBS, with no significant effect on the LF component (A–C). HF nu = HF power / TP − VLF power × 100; LF nu = LF power/TP − VLF power × 100. TP, total power; HF, high frequency; LF, low frequency; VLF, very low frequency; nu, normalised units; RVP, rapid ventricular pacing; CBS, carotid baroreceptor stimulation. *P < 0.05; **P < 0.01.

function, reversed progressive LV dilation and chamber sphericity, and rebalanced autonomic nervous tone in CHF canines, which is coincident with previous studies [23,24]. Furthermore, we found that CBS showed profound protective effects against cardiac fibrosis and apoptosis, along with the suppression of the intracellular PKA signalling pathway and inflammation in CHF canines.

4.1. CBS restores autonomic balance in CHF

Our previous studies have indicated that low-level CBS inhibits the activity of the left stellate ganglion, decreases plasma norepinephrine and angiotensin II concentrations, and reverses the decrease in HF components and increase in LF components and LF/HF ratio in acute rapid atrial pacing models [21,22]. In this study, we did not make much effort in evaluating it, except for power spectral analysis of 15-minute ECG recordings, which provided specific evidence of autonomic disorders induced by chronic RVP. The increasing LF components and LF/HF ratio indicated the increase in cardiac sympathetic tone, whereas the decreasing HF components represented the suppression of parasympathetic tone. The increase of LF/HF ratio and the decrease of HF components were reversed significantly by CBS, which indicated that CBS rebalanced the autonomic nervous system by reducing the sympathetic tone and increasing vagal tone.

4.2. CBS improves cardiac performance in CHF

Increased HR and decreased BP are common features of CHF. In our study, CBS stabilised the haemodynamic parameters in CHF. As shown in Table 1, at the end of the study, the HR increased markedly in the CHF group, but stayed at the same level of the baseline in the CHF-CBS

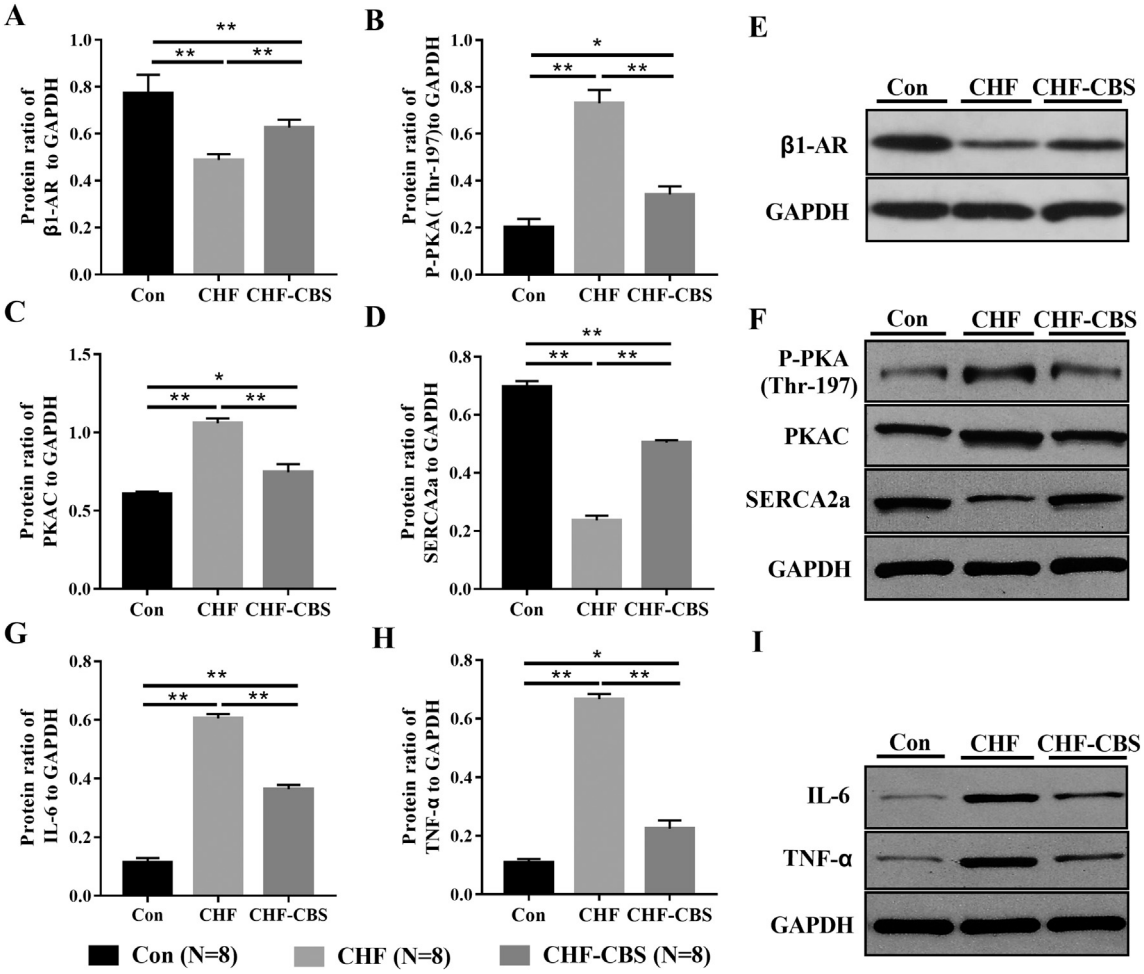

Fig. 6. Effects of CBS on the PKA signalling pathway and cardiac inflammation. Western blotting data and representative immunoblots (A–F) showed that the protein expression of β1-AR and SERCA2a in LV tissue decreased, whereas the protein expression of PKAC and P-PKA increased markedly in the CHF group after 6 weeks of RVP. CBS reversed the decrease in β1-AR and SERCA2a and the increase in PKAC and P-PKA partially. Data from western blotting and representative immunoblots (G–I) showed that the protein expression of inflammatory factors (TNF-α, IL-6) in LV tissue increased significantly in the CHF group. CBS reversed the increase in protein expression of TNF-α and IL-6 significantly. Values are mean ± SEM. β1-AR, β1-adrenergic receptor; PKAC, cAMP-dependent protein kinase catalytic subunits; P-PKA, Thr-197 phosphorylated PKA; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; TNF-α, tumour necrosis factor α; IL-6, interleukin-6. *P < 0.05; **P < 0.01.

group. The decrease in MAP of the CHF group was more overt than that of the CHF-CBS group. Generally, CBS with enough intensity has negative effects on BP and HR, especially in normal or hypertensive subjects. In our study, CBS decreased the HR only, rather than both of them, under the CHF condition. The potential reason might be that the protective effects of CBS on CHF exceed its direct effects on BP. We did not see obvious side effects of CBS on hepatic and renal function. In contrast, CBS corrected the abnormality of ALT, BUN, and Cr induced by CHF. It suggests that CBS at moderate intensity is safe in CHF.

Notably, echocardiography examination reported more exciting results in our study when compared with other studies. We found that CBS improved the cardiac structural and functional deterioration in CHF, which was consistent with the reduction of ANP and BNP expression of LV tissue. Previous studies in canines with CHF did not show a significant increase in LVEF and LVFS or a significant decrease in LV diameters and LV volumes by CBS [15]. In another study, CHF with significant decrease in LVEF was induced by coronary microembolisation. CBS lead to a significant increase in LVEF, no change in LVEDV, and a significant decrease in LVESV [16]. Clinical studies showed controversial results in echocardiography. A trial of CBS in CHF with a reduced LVEF indicated a non-significant trend toward improved LVEF in the CBS group compared with patients who were not treated by CBS [20]. A newly released study indicated that CBS improved LVEF and reduced LVEDV significantly after 43 months of treatment [18]. It is still unclear why different studies have controversial results. But, differences in several factors should not be ignored, including animal model or baseline characteristics of subjects, treatment or follow-up period, and intensity of CBS.

4.3. CBS reverse cardiac remodelling in CHF

Sympathetic hyperactivation occurs in the process of heart failure. Persistent activation of the β-adrenergic neurohormonal axis has been shown to contribute to the deterioration of cardiac function and cardiac pathological remodelling in CHF [2–4,25]. Generally, cardiac remodelling consists of myocardial hypertrophy, apoptosis, and fibrosis, and all of them are closely related to some important cellular signalling pathways, which are activated secondary to the activation of β1-AR [26].

β-AR, including three subtypes, β1, β2, and β3-AR, belongs to the superfamily of G-protein-coupled receptors. The density of β1-AR is the highest, and the density of β3-AR is the lowest in the heart [2]. Under normal conditions, the ligand-binding β1-AR activates the Gs proteins, which then activates the adenylate cyclase to produce cAMP. Then,

cAMP binds regulatory subunits of PKA and induces the release of PKAC, which then phosphorylates downstream target proteins to increase cardiac output, gene transcription, and metabolism. But prolonged overactivation of β1-AR ultimately produces cardiotoxicity effects, such as hypertrophy, fibrosis, apoptosis, dysfunction, and the reduction of β1-AR content and function in CHF [27–30]. In our study, the expression of PKAC and P-PKA increased markedly, whereas β1-AR and SERCA2a decreased, in the CHF group after 6 weeks of RVP. CBS reversed them partially, which demonstrated that the increased intracellular PKA signalling was inhibited by CBS in CHF.

β1-AR worsens TGF-β1-induced remodelling, and the overactivation of β1-AR leads to increased MMP2 and MMP9 expression and activity [31,32]. TGF-β1 is a crucial pro-fibrotic factor that induces myofibroblast transdifferentiation and interstitial fibrosis. MMP2 and MMP9 contribute to the excessive degradation of the extracellular matrix and the pathogenesis of dilative remodelling [33]. Inhibition or gene deletion of them have shown favourable effects in cardiac remodelling and dysfunction [31,34–36]. In our study, protein expression of TGF-β1, MMP2, and MMP9 in LV tissue were increased significantly of the CHF group after 6 weeks of RVP, accompanied by the increase in CVF of LV tissue, and coincided with the increase of LVESV and E/A ratio of the mitral valve. All those alterations were alleviated by CBS.

Apoptosis is a common feature of CHF. The cAMP/PKA pathway is necessary to mediate cardiomyocyte apoptosis induced by sustained β-AR agonist [37]. Impaired intracellular calcium handling, including decreased expression and activity of SERCA2a, has been shown to be an important PKA-independent mechanism in β1-AR-induced cardiomyocyte apoptosis via the primary mitochondrial death pathway [38]. The increased expression of MMP2 is another pro-apoptotic factor [39]. In our study, the reduction of TUNEL signals and Caspase-3 expression in the CHF-CBS group coincided with the reduction of PKAC, P-PKA, and MMP2 expression, and the increase in SERCA2a expression. These results demonstrated that CBS might protect against myocardial fibrosis and apoptosis by inhibiting intracellular PKA signalling in CHF.

Chronic activation of the sympathetic nervous system leads to the production of inflammatory cytokines and recruitment of immune cells [37,40,41]. Inflammatory cytokines, such as TNF-α, and IL-6, mediate the ventricular systolic dysfunction, ventricular dilation and hypertrophy, myocardial fibrosis, and apoptosis [42,43]. In our study, CBS inhibited the increased expression of TNF-α and IL-6 in CHF significantly, along with the improvement of cardiac performance and reversal of cardiac remodelling, which indicated that an anti-inflammatory effect was another possible cardioprotective mechanism of CBS.

5. Conclusion

Our results suggest that CBS exerts profound effects on canines with CHF. CBS improves the cardiac contractile performance, reverses cardiac dilation, and ameliorates cardiac remodelling by decreasing the sympathetic activity effectively. The underlying mechanisms might be the inhibition of the intracellular PKA signalling pathway and cardiac inflammation. Based on the experiment and pilot clinical studies from our and other institutions, CBS might serve as a novel approach for patients with CHF in the near future.

Conflicts of interest

None.

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