

## Original Article

# Effects of combined MCA and G-CSF treatment on myocardial fibrosis and apoptosis gene expression in rats with diastolic heart failure

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**Abstract:** Objective: To explore the therapeutic effect of combining meglumine cyclic adenosine (MCA) with granulocyte colony-stimulating factor (G-CSF) in treating rats with diastolic heart failure (DHF). Methods: A total of 40 Sprague Dawley (SD) male rats were evenly divided into 4 groups: the Sham operation group (Control), the Model group (DHF), the MCA group (MCA) and the MCA combined with G-CSF group (MCA+G-CSF). The MCA group was given 30 mg/kg/d (body weight) MCA by intragastric injection for 15 days. The MCA+G-CSF group was treated with 30 mg/kg/d MCA and 100 µg/kg/d G-CSF by intragastric and subcutaneous injection, respectively, for 15 days. The Control group and Model group were given equal volumes of saline solution. After 15 days of drug intervention, cardiac function was measured by echocardiography using a BL-420-F physiological recorder; Bcl-2 and Bax expression was measured by RT-PCR and western blotting; and serum BNP levels were determined using an ELISA. Results: Compared with fibrosis in the MCA group, myocardial fibrosis was significantly decreased in the MCA+G-CSF group. Treatment with G-CSF plus MCA significantly promoted Bcl-2 expression (mRNA,  $334.26 \pm 28.12$  vs.  $279.82 \pm 18.56$ ; protein,  $1.83 \pm 0.26$  vs.  $1.18 \pm 0.16$ ) ( $P < 0.05$ ) and reduced Bax expression (mRNA,  $103.42 \pm 7.62$  vs.  $182.46 \pm 15.83$ ; protein,  $0.81 \pm 0.06$  vs.  $1.37 \pm 0.14$ ) ( $P < 0.05$ ) and serum BNP levels ( $328.85 \pm 18.39$  vs.  $396.58 \pm 26.48$ ) ( $P < 0.05$ ). Conclusion: G-CSF may regulate cell apoptosis in DHF rats and reduce their serum BNP levels; coupling MCA with G-CSF can significantly improve cardiac diastolic function in DHF rats and is associated with a better therapeutic effect than treatment with MCA alone.

**Keywords:** Meglumine cyclic adenosine, diastolic heart failure, granulocyte colony-stimulating factor, myocardial fibrosis, myocardial apoptosis gene

## Introduction

With increases in the aging population, the prevalence rate of diastolic heart failure (DHF) has increased yearly, and hypertension is often regarded as one of the main causes of DHF. At present, there is no effective treatment for DHF [1, 2]. Cyclic adenosine monophosphate (cAMP), an important second messenger, can regulate the growth and proliferation of cells [3, 4]. Meglumine cyclic adenosine (MCA) functions as a cAMP analog and can improve the condition of patients with chronic heart failure [1, 2]. In addition, studies have shown that granulocyte colony-stimulating factor (G-CSF) can induce cardiac muscle cell proliferation and improve heart function [5]. However, combination treatment using both drugs for DHF has not yet been reported at home and abroad. The purpose of this study was to investigate the effect of MCA

combined with G-CSF on myocardial fibrosis and apoptosis gene expression in a rat cardiac pressure overload model generated by abdominal aortic constriction. This study may provide clues for the clinical treatment of DHF.

## Materials and methods

### Test materials

All procedures described in this study were approved by the Medical University of Jining Institutional Animal Care and Use Committee. A total of 40 healthy adult male Sprague Dawley (SD) rats (8-10 weeks old) weighing  $200 \pm 20$  g were provided by the animal center of Jining Medical University. They were raised in a clean animal laboratory under the following conditions: 60% relative humidity, and 12 h-light/12 h-dark photoperiod and a temperature of  $23 \pm$

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2°C. The rats were provided free access to food and water. All animal surgeries and experimental protocols were approved by the Medical University of Jining Institutional Animal Care and Use Committee (SCXK20140007).

### *Instruments and reagents*

Meglumine cyclic adenosine was purchased from Shandong Ruiyang Pharmaceutical Co Ltd., and G-CSF was purchased from Shanghai Three Biological Technology Co. Ltd. Monoclonal antibodies against Bcl-2 and Bax, as well as two anti-goat and anti-rabbit IgGs, were purchased from the Santa Cruz Corporation in the United States of America. The rat BNP enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shanghai Biological Technology Co. Ltd. The immunohistochemistry reagent kit was a product of the Zymed Company and purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. The BL-420F physiological recorder was purchased from Chengdu Taimeng Technology Co. Ltd. The Vevo2100 color Doppler ultrasonic diagnostic apparatus was purchased from VisualSonics Inc. in Canada. The LH50A inverted microscope was purchased from the Olympus Company in Tokyo.

### *Establishment and treatment of the animal model*

A rat model of DHF was established by constricting the abdominal aorta. All rats were randomly divided into 4 groups: the Sham operation group (Control), the Model group (DHF), the MCA group (MCA) and the MCA combined with G-CSF group (MCA+G-CSF). Briefly, 3% pentobarbital sodium (30 mg/kg) was injected into the abdominal cavity, and then, 1 cm of the abdominal artery was isolated, starting at the junction with the right renal artery, and a number 7 needle was placed parallel to the artery. Number 4 surgical suture was used to ligate the abdominal artery with the needle to reduce the diameter of the abdominal aorta to 35%-40% of normal. For the Sham group, rats underwent the full surgical procedure but without ligation. After the surgery, the wound was disinfected with 3% iodine and 75% alcohol, and then, the rats were injected with gentamicin for 3 consecutive days to prevent infection. Eight weeks after surgery, the rats in the MCA group were given MCA at a dose of 30 mg/kg body weight for 15 consecutive days by oral gavage.

The MCA+G-CSF group was administered 100 µg/kg/d G-CSF diluted with normal saline by subcutaneous injection for 15 days along with 30 mg/kg body weight MCA by gavage. The Control and Model groups were given the equal amounts of saline.

### *Hemodynamic detection*

Fifteen days after drug intervention, the BL-420-F recorder was used to measure the hemodynamic parameters among the 4 groups of rats. A homemade miniature right common carotid arterial and ascending aortic catheter was inserted into the left side of the heart; a miniature catheter was inserted into the other side to record the following physiological data: left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), the maximum rate of pressure rise in the left ventricle (+LVdp/dtmax) and the maximal rate of pressure drop in the left ventricle (-LVdp/dtmax).

### *Echocardiographic determination of cardiac function*

Fifteen days after drug intervention, the Vevo2100 color Doppler ultrasonic diagnostic apparatus was used to determine the following parameters in the rats: left ventricular end-systolic diameter (LVSD, mm), left ventricular end-diastolic diameter (LVDD, mm). Each parameter was measured in three consecutive complete cardiac cycles, and the average value was determined to reduce error. The computer automatically calculated the left ventricular ejection fraction (LVEF) using the following equation:  $LVEF = [(LVDD3 - LVSD3)/LVDD3] \times 100\%$ .

### *Determination of serum brain natriuretic peptide (BNP) levels by ELISA*

After 15 days of drug intervention, morning fasting venous blood was drawn (1.5 mL), left to stand for 30 min and centrifuged for 15 min at 3000 r/min. Then, the serum was collected and placed into 1.5-mL Eppendorf (EP) tubes and stored at 20°C. The double sandwich ELISA method was used to determine the serum BNP levels in the rats.

### *Preparation of pathological sections*

After 15 days of drug intervention, the rats were sacrificed, and the hearts were quickly removed and placed into heparinized saline. The blood was washed out of the heart, which

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**Table 1.** Hemodynamic Changes in the Heart Failure Rat Model ( $\bar{x} \pm s$ )

	LVSP (mmHg)	+dp/dtmax (mmHg/s)	LVEDP (mmHg)	-dp/dtmax (mmHg/s)	T (ms)
Control	126.12 ± 1.78	4,968.28 ± 432.36	3.16 ± 0.73	3,708.46 ± 392.85	592 ± 18
Model	127.32 ± 3.15	4,973.19 ± 399.12	11.72 ± 0.72**	3,768.34 ± 401.72	923 ± 31**
MCA	138.69 ± 6.72*,&	6,378.82 ± 396.28*,&	4.29 ± 0.93*,&	4,723.73 ± 429.16*,&	612.46 ± 21*,&
MCA+G-CSF	139.32 ± 5.68*,&	6,846.62 ± 426.83*,&#	3.21 ± 0.74&#	4,966.82 ± 396.86*,&#	582.38 ± 16#

\* $P < 0.05$  and \*\* $P < 0.01$ , vs. the Control group; & $P < 0.05$  and && $P < 0.01$ , vs. the Model group; and # $P < 0.05$ , vs. the MCA group.

was then placed in 4% paraformaldehyde. After ethanol dehydration, paraffin sections at a thickness of 4  $\mu\text{m}$  were acquired and stored at 4°C until use. Masson staining was performed after the sections were dewaxed, clear with xylene, and sealed. The sections were observed under an LH50A inverted phase-contrast microscope and photographed.

### Determination of specific gene expression in cardiac muscle by RT-PCR

The myocardial tissue was collected, and total RNA was extracted by TRIzol reagent. The cDNA was synthesized using a reverse transcription kit to detect the mRNA expression level of Bcl-2 and Bax. The following primers were used in this study: Bcl-2F: 5'-AGGGCTACGAGTGGGATGC-3', Bcl-2R: 5'-CCCCACGAACCTCAAAGAAGG-3'; BaxF: 5'-CCAAGAAGCTGAGCGAGTGT-C-3', BaxR: 5'-AAAGATGGTCA-CGGTCTGCCT-3'; and GAPDH-F: 5'-CGGAGTC-AACGGATTTGGTCTGAT-3', GAPDH-R: 5'-AGCCT-TCTCCATGGTGGTGAAGAC-3'. The PCR reaction conditions were as follows: pre-denaturation at 94°C for 4 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 45 s. The 2-delta CT method was used for data processing. The standard cDNA and samples were run in triplicate.

### Measuring Bcl-2 and Bax protein expression in cardiac tissue by western blotting

The myocardium of rats was collected from each group, and the protein was extracted using conventional methods. After BCA protein quantification, 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, and transfer to a membrane, the following primary antibodies were added and incubated overnight at 4°C: GAPDH antibody (1:1,000 dilution), rabbit anti-mouse Bcl-2 antibody (1:500 dilution) and rabbit anti-mouse

Bax antibody (1:500 dilution). Then, the membrane was washed, and fluorescence-labelled secondary antibodies were added (1:2,000 dilution) and incubated at room temperature for 2 h. The blots were colored by an immunofluorescence imaging system, the gray value of each band was calculated using image analysis software, and the relative expression of each protein was calculated. These experiments were repeated at least 3 times.

### Statistical methods

Quantitative data are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). All data were analyzed using SPSS 13.0, and multiple sets of data were compared by single-factor analysis of variance (one-way ANOVA), whereas group data was analyzed using least significant difference (LSD) testing.  $P < 0.05$  was regarded as statistically significant.

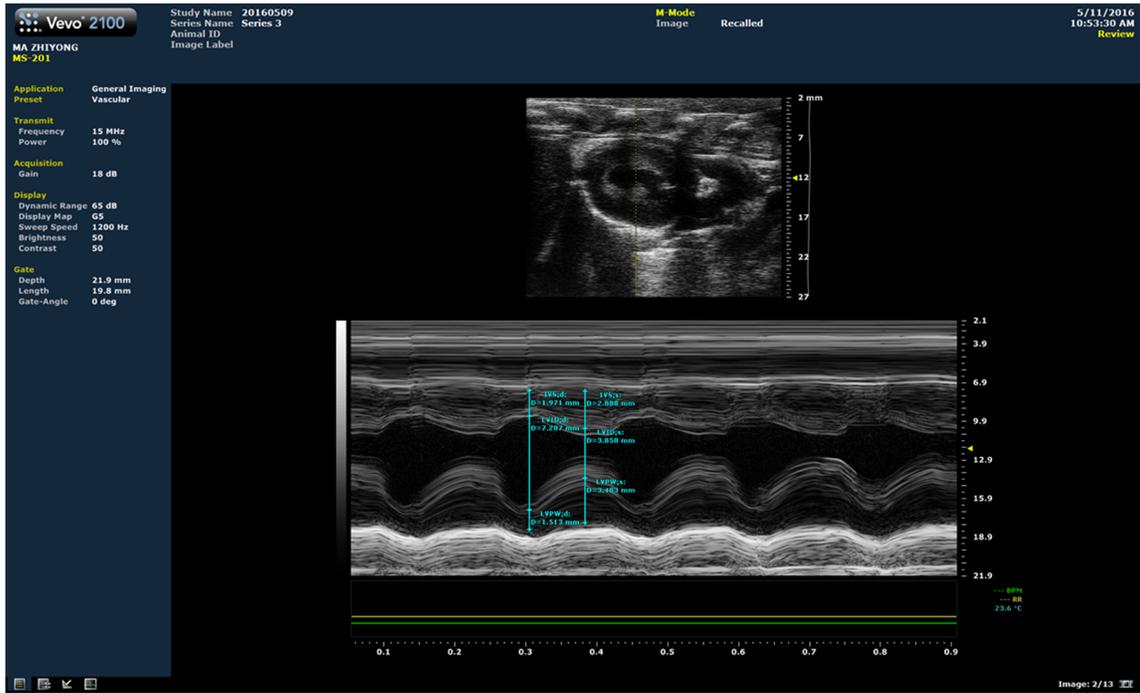
## Results

### Hemodynamic changes in the diastolic heart failure rats

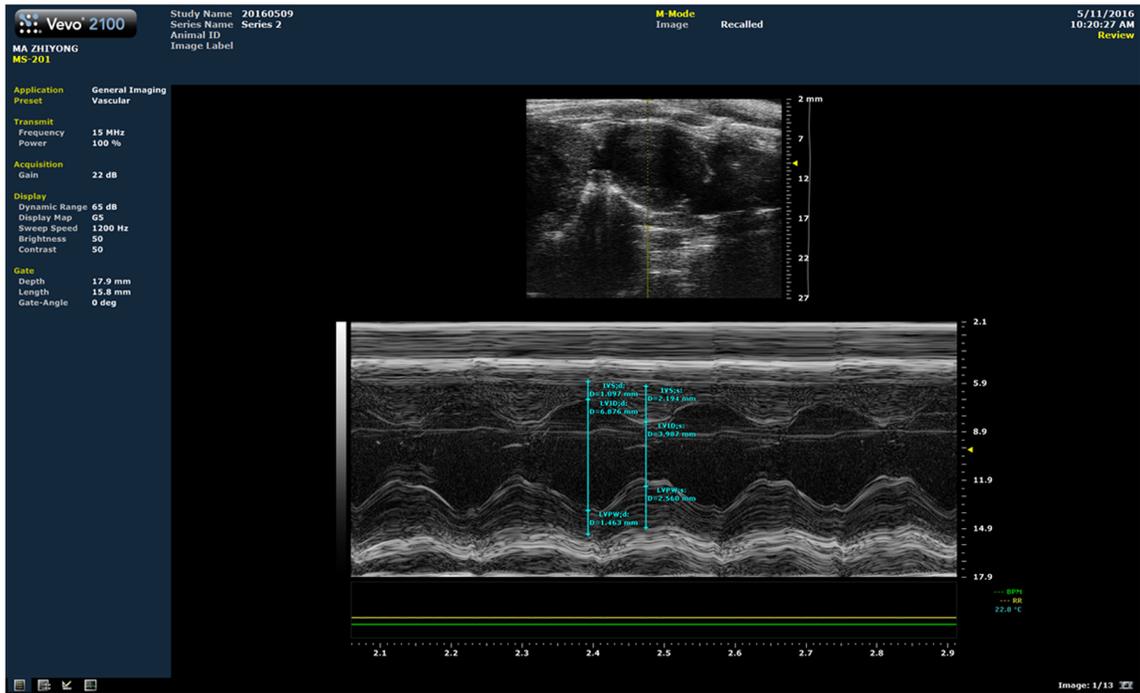
We evaluated hemodynamic changes in the rats after 15 days of drug intervention (**Table 1**). The results show that compared with the Control group, the Model group did not exhibit a statistically significant change in systolic function, LVSP and +dp/dtmax ( $P > 0.05$ ). In contrast, diastolic function was significantly decreased ( $P < 0.01$ ), LVEDP was significantly increased ( $P < 0.01$ ) and while the change in -dp/dtmax was not large, the left ventricular relaxation time constant (T) value was significantly increased ( $P < 0.01$ ). Compared with the Model group, the MCA and MCA+G-CSF groups exhibited markedly higher systolic function and LVSP; the +dp/dtmax and -dp/dtmax were significantly increased ( $P < 0.05$ ), whereas LVEDP was significantly decreased ( $P < 0.05$ ). There were no significant differences in LVEDP and T

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## Control

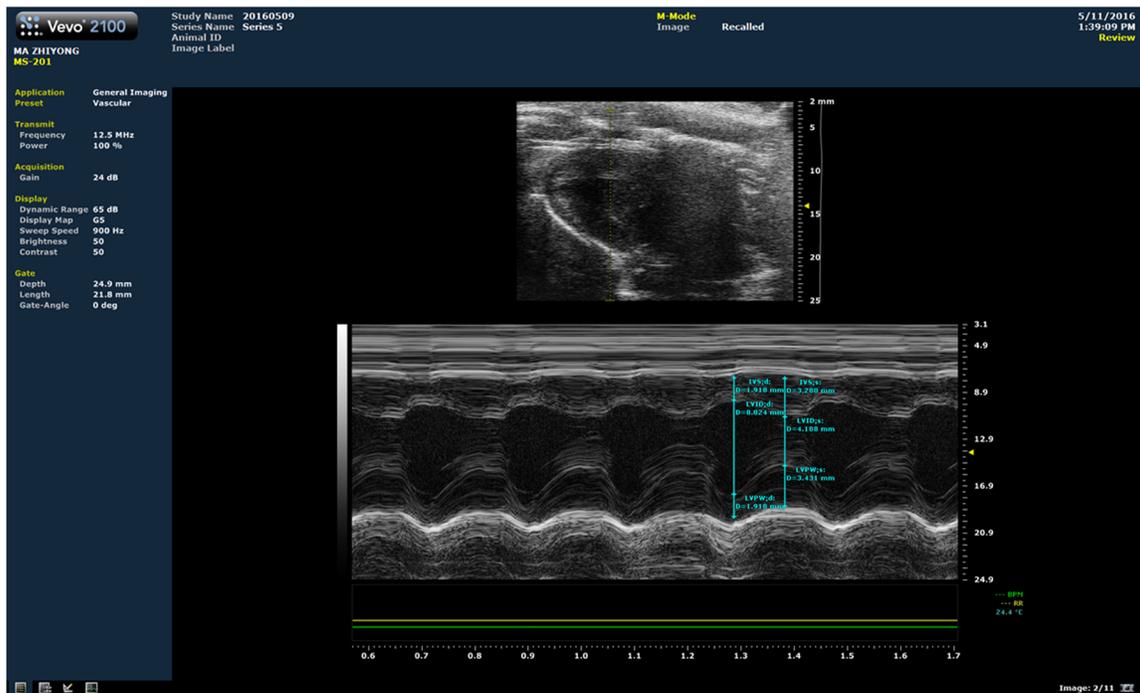


## Model



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## MCA



## MCA + G-CSF

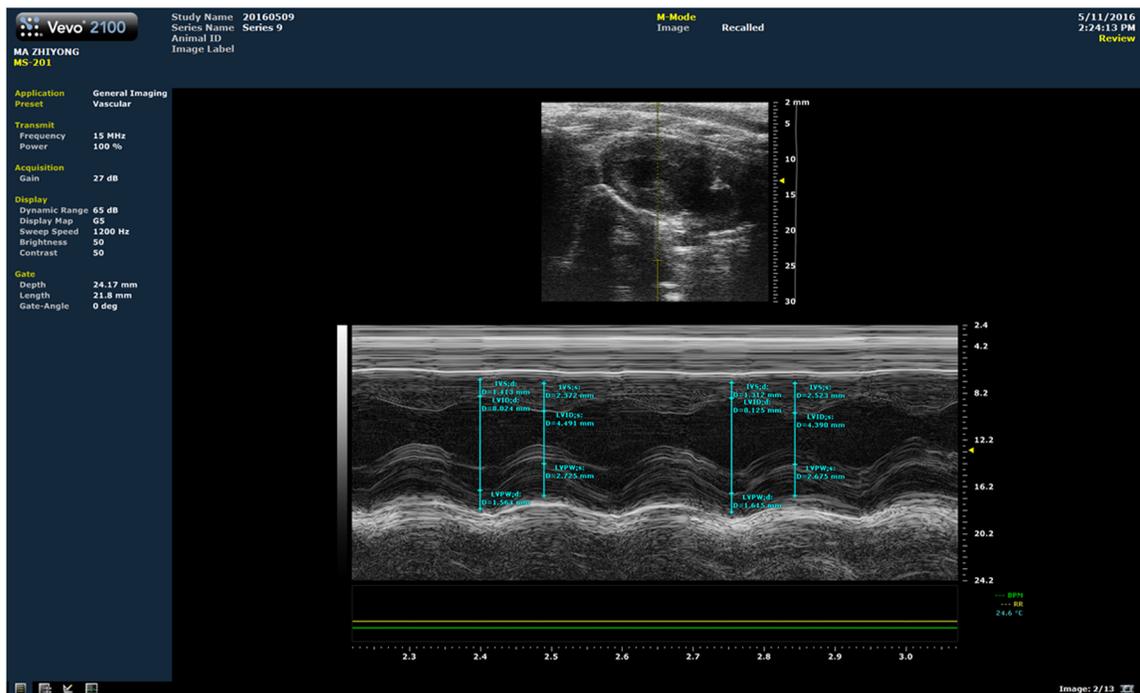


Figure 1. Diastolic heart failure rat cardiac ultrasound results.

between the MCA+G-CSF group and the Control group, but these values were significantly lower than that in the MCA rats ( $P < 0.05$ ). In addition,

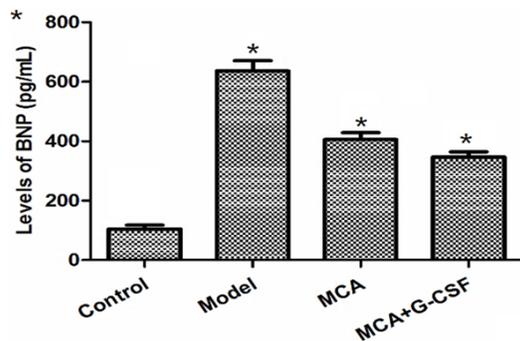
the  $+dp/dt_{max}$  and  $-dp/dt_{max}$  of the MCA+G-CSF group were significantly increased ( $P < 0.05$ ) compared to values in the MCA group.

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**Table 2.** Echocardiographic parameters in each group of rats ( $\bar{x} \pm s$ )

	LVEF (%)	LVDD (mm)	LVSD (mm)	LVM (mg)	E/A (%)
Control	80.47 ± 6.83	6.63 ± 0.82	3.30 ± 0.36	649.97 ± 54.69	1.57 ± 0.32
Model	69.05 ± 5.98*	7.03 ± 0.86*	4.23 ± 0.58*	552.93 ± 48.32*	1.03 ± 0.08*
MCA	72.09 ± 7.34*,&	7.68 ± 0.73*,&	4.40 ± 0.63*,&	831.90 ± 73.85*,&&	0.91 ± 0.15*,&
MCA+G-CSF	76.20 ± 6.83*,&&,#	8.06 ± 0.94*,&,#	4.31 ± 0.52*,&,#	877.08 ± 79.46*,&&,#	1.01 ± 0.07*,&

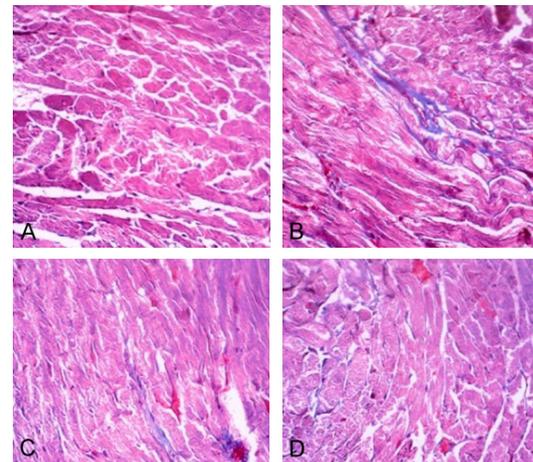
\* $P < 0.05$ , vs. the Control group; & $P < 0.05$  and && $P < 0.01$ , vs. the Model group; and # $P < 0.05$ , vs. the MCA group.



**Figure 2.** Serum BNP levels among treatment groups. The values are presented as  $\bar{x} \pm s$ , and each measurement was repeated 10 times. The asterisk above the standard error line denotes a significant difference ( $P < 0.05$ ).

### Echocardiographic parameters of heart function

To further understand the influence of MCA combined with G-CSF on heart function, echocardiography was used to examine the rats in each group (Figure 1 and Table 2). The results show that cardiac function in the Model group was obviously reduced and left ventricular wall motion was weakened compared to parameters in the Control group. The LVEF, left ventricular mass (LVM) and the maximal early diastolic flow velocity/maximal late diastolic flow velocity (E/A) ratio in the Model group were significantly lower than in the Control group ( $P < 0.05$ ), whereas LVDD and LVSD were significantly higher in the Model group than in the Control group ( $P < 0.05$ ). Fifteen days after intervention, the ventricular wall motion was significantly enhanced in the MCA and MCA+G-CSF groups relative to that in the Model group; this change was especially great in the MCA+G-CSF group ( $P < 0.05$ ). Combined treatment with MCA and G-CSF significantly improved cardiac function, as the LVEF, LVDD, LVM, and the E/A ratio in the MCA+G-CSF group were significantly higher than values in the MCA group ( $P < 0.05$ ).



**Figure 3.** Myocardial tissue morphology analysis in each group (Masson staining,  $\times 200$ ). (A) Control group; (B) Model group; (C) MCA group; and (D) MCA+G-CSF group. Collagen fibers are stained blue after Masson staining, the myocardial cells are red, and the nucleus is dark blue.

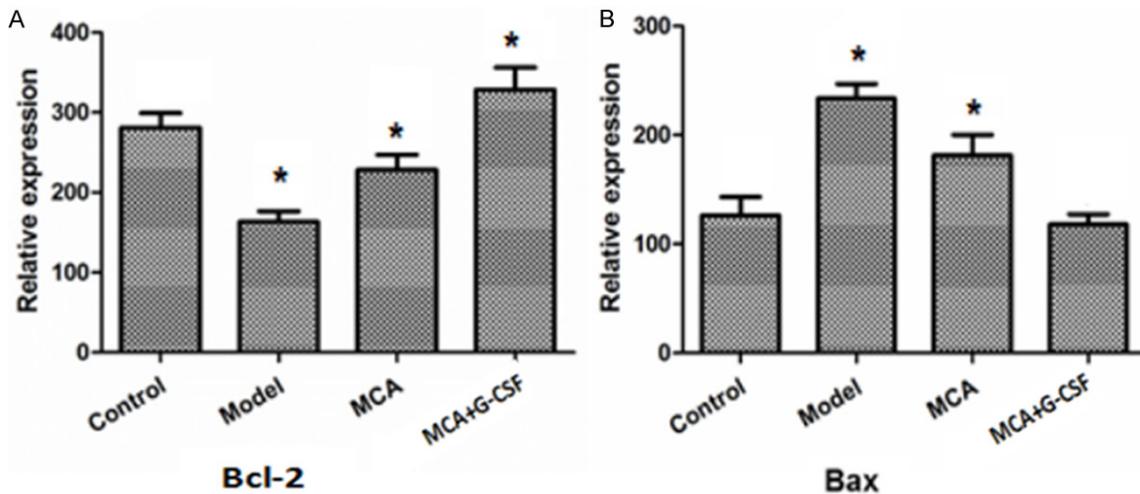
### The impact on BNP level

The serum BNP levels among the four groups of rats exhibited significant differences, as shown in Figure 2. The serum BNP level in the Model group was significantly higher than that of the Control group ( $P < 0.01$ ). MCA obviously decreased the level of serum BNP. The MCA group exhibited a lower serum BNP level than the Model group ( $P < 0.05$ ). MCA combined with G-CSF had a greater effect on BNP level, as the BNP level in the MCA+G-CSF group was significantly lower than that in the MCA group ( $P < 0.05$ ).

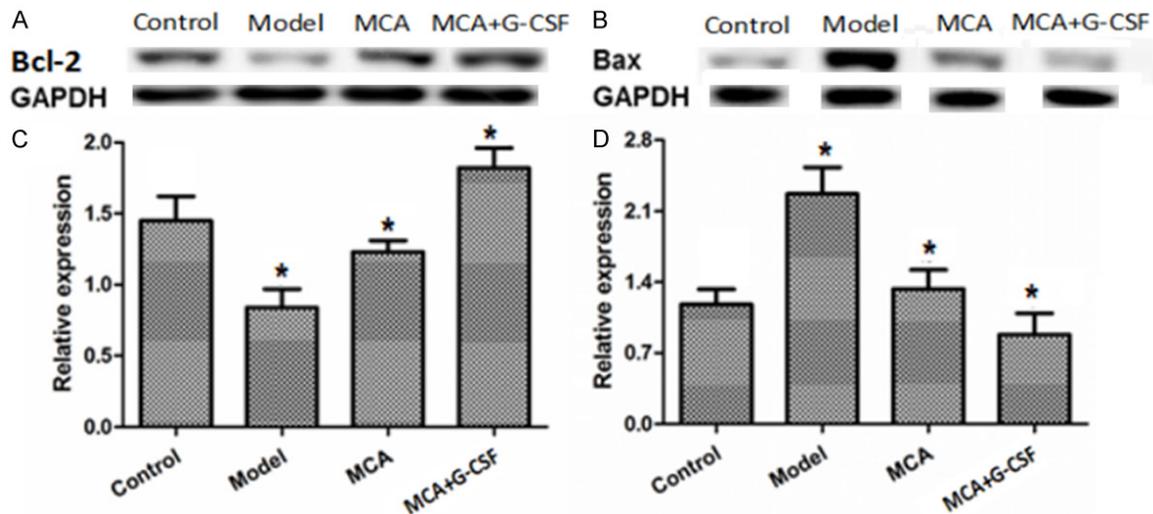
### Effect of MCA combined with G-CSF on the pathological morphology of myocardial tissue in each group

As shown in Figure 3, we carried out a pathological examination of myocardial tissue from rats in each group. Blue staining indicated fibrosis, and compared with fibrosis in the

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**Figure 4.** Effect of MCA combined with G-CSF on the expression of apoptosis genes Bcl-2 and Bax in the myocardium. GAPDH was used as the reference gene for RT-PCR. The values are presented as  $\bar{x} \pm s$ , and the asterisk above the standard error line denotes a significant difference ( $P < 0.05$ ).



**Figure 5.** Effect of MCA combined with G-CSF on apoptosis protein expression in the myocardium. Western blotting was used to determine the relative expression levels of Bcl-2 and Bax, using GAPDH as a reference gene. The values are presented as  $\bar{x} \pm s$ , and the asterisk above the standard error line denotes a significant difference ( $P < 0.05$ ).

Control group, myocardial fibrosis was more severe in the Model group and the least severe in the MCA+G-CSF group. MCA combined with G-CSF can significantly reduce DHF-induced myocardial fibrosis.

*Effect of MCA combined with G-CSF on apoptosis gene expression in the myocardium*

We determined the effect of MCA combined with G-CSF on the expression of Bcl-2 and Bax (Figure 4). The expression level of Bcl-2 mRNA

in DHF rats was significantly lower than that in normal rats, whereas the expression level of Bax mRNA was significantly higher than that in normal rats ( $P < 0.05$ ). Combined treatment with G-CSF and MCA significantly increased the expression of Bcl-2 but inhibited the expression of Bax ( $P < 0.05$ ) in heart failure rats. Compared with the MCA group, the expression level of Bcl-2 mRNA was significantly increased in the MCA+G-CSF group, whereas the expression level of Bax mRNA was significantly decreased ( $P < 0.05$ ).

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### *Effect of MCA combined with G-CSF on apoptosis protein expression in the myocardium*

In addition, we examined the effect of MCA combined with G-CSF on apoptosis gene expression in the myocardium (**Figure 5**). Bcl-2 and Bax protein levels correlated with the mRNA levels in the heart tissues, and the expression levels among the four groups were significantly different ( $P < 0.05$ ). Bcl-2 and Bax protein levels in the MCA+G-CSF group were higher than that in the other three groups ( $P < 0.05$ ). Treatment with MCA combined with G-CSF was more effective than treatment with MCA alone on myocardial apoptosis.

### **Discussion**

MCA is composed of cAMP and meglumine in a molar ratio of 1:1, and the combination of cAMP and meglumine enhances fat-soluble and intracellular cAMP concentrations. In myocardial cells, cAMP can promote the survival of myocardial cells, reduce myocardial oxygen consumption, improve myocardial cell metabolism and protect ischemic myocardial tissue [6]. Zhou et al. [4] found that MCA could enter bone marrow mesenchymal stem cells (BMSCs) to inhibit BMSC activity and promote the expression of cardiac-specific genes in BMSCs via the cAMP/PKA signaling pathway in order to differentiate the BMSCs into cardiomyocyte-like cells and improve myocardial function. In addition to playing a primary role in the proliferation, differentiation and activation of hematopoietic cells in the neutrophil lineage, G-CSF can also inhibit myocardial fibrosis, promote angiogenesis, reduce myocardial apoptosis and promote the repair of damaged myocardium [7]. This research used the abdominal aorta ligation method to establish a rat model of pressure overload, which was then used to determine the efficacy of combined MCA and G-CSF treatment on DHF. According to the results of the hemodynamic and ultrasound analyses, cardiac diastolic function in the MCA group was significantly improved compared to function in the Model group. A similar difference was observed between the MCA+G-CSF group and the Model group.

Myocardial fibrosis is one of the critical mechanisms in the occurrence and development of DHF [8]. Abnormal ventricular vascular coupling

caused by hypertension and other heart diseases will lead to hemodynamic instability, blood pressure fluctuations, severe shock and sudden death from interstitial fibrosis [9]. Interstitial fibrosis eventually leads to a reduction in diastolic compliance and diastolic dysfunction [10]. The immunohistochemical results in this study showed that the Model group exhibited the most severe myocardial fibrosis, the intervention of MCA reduced the degree of fibrosis, and the MCA combined with G-CSF treatment further reduced the degree of fibrosis. The combined treatment of MCA and G-CSF can inhibit myocardial fibrosis in rats with DHF, which has a positive effect on DHF. Studies have shown that G-CSF promotes the degradation of excess collagen by upregulating the levels of matrix metalloproteinases; additionally, it protects the myocardium by inhibiting remodeling and reducing the amount of collagen [11]. Forechi et al. [12] found that G-CSF could inhibit early remodeling of the myocardium in rats, which partly prevents collagen deposition and left ventricular enlargement. These effects may be attributed to interference with anti-apoptotic signaling, the promotion of cardiac muscle cell regeneration and the regulation of extracellular matrix degradation and synthesis. Jiang et al. [13] proved that G-CSF had a targeted therapeutic effect on pressure overload-induced myocardial fibrosis. Decreasing G-CSF activity effectively inhibits the inflammation and myocardial fibrosis induced by Ang II through the ERK1/2 and STAT3 signal transduction pathways. Geng [14] observed that MCA markedly improved the in vitro cardiac function of cardiac hypertrophy rats, and the mechanism may be related to the inhibition of NO synthesis through the NO-sGC pathway. Xu et al. [15] confirmed that MCA significantly reduced NP-proBNP levels in the peripheral blood of patients with heart failure, thereby reducing the degree of heart failure and inhibiting myocardial remodeling. Due to complex molecular interactions in organisms, the specific mechanisms underlying the inhibition of myocardial fibrosis by combined therapy with MCA and G-CSF remain to be determined.

BNP is a biomarker of chronic heart failure; its expression changes as the ventricular filling pressure changes. Additionally, an increased level of plasma BNP is positively associated with the severity of heart failure. Our results show that the BNP concentration in the MCA+G-

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CSF group was lower than that in the MCA group and that cardiac function in the MCA+G-CSF group was significantly improved compared to that in the MCA group; this may be related to the effect of G-CSF on inhibiting myocardial apoptosis and promoting survival in ischemic myocardial tissue.

Apoptosis plays an important role in the occurrence and development of DHF and other heart diseases affected by myocardial fibrosis, such as hypertension and cardiomyopathy [16]. During cell apoptosis, the Bcl-2 family plays a vital role, and its members are widely distributed in the mitochondria, endoplasmic reticulum and cytoplasm. Bcl-2 has anti-apoptotic effects, whereas Bax has pro-apoptotic effects and is mainly distributed in the cytoplasm [17]. Studies have shown that the application of benzoyl paeoniflorin significantly increases the Bcl-2/Bax ratio in rats with coronary heart disease; the expression of Bcl-2 protein inhibits the expression of Bax protein, which inhibits myocardial cell apoptosis and, consistent with the results of the present study, has a positive effect on the treatment of coronary heart disease [18]. Our study shows that treatment with MCA combined with G-CSF significantly promotes the expression of Bcl-2 and inhibits the expression of Bax both on the mRNA and protein levels.

In conclusion, this study suggests that treatment with MCA combined with G-CSF markedly improves cardiac function in DHF rats through the regulation of cell apoptosis and BNP expression, which leads to a significant reduction in the degree of myocardial fibrosis. This finding provides a novel therapeutic modality for the clinical treatment of DHF.

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### Disclosure of conflict of interest

None.

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