

Recombinant human brain natriuretic peptide therapy combined with bone mesenchymal stem cell transplantation for treating heart failure in rats

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Abstract. This study aimed to investigate the effects of combined recombinant human brain natriuretic peptide (rhBNP) therapy and bone mesenchymal stem cell (BMSC) transplantation on cell survival in myocardial tissues and on heart function in a rat model of heart failure (HF). Rat BMSCs were isolated, amplified and adherent cultured *in vitro*. A rat model of HF was established via the intraperitoneal injection of doxorubicin (Adriamycin). The rats were randomly divided into normal, HF, BMSC, rhBNP and BMSC plus rhBNP groups. The BMSCs were administered once via tail vein injection and rhBNP was infused via the jugular vein. Echocardiography and polygraphy were used to evaluate heart function. An enzyme-linked immunosorbent assay was used to detect the changes in brain natriuretic peptide (BNP) concentration prior to and following intervention. Western blot analysis was used to detect the expression of the myocardium-specific proteins GATA-binding protein 4 (GATA-4), connexin 43 (Cx43) and cardiac troponin I (cTnI). The results of cardiac echocardiography and the hemodynamic data show that various indicators of left ventricular systolic function in the BMSC plus rhBNP group were significantly improved compared with those in the other groups ($P < 0.05$). No significant differences in the improvement of cardiac function were observed between the BMSC and rhBNP groups ($P > 0.05$). Following treatment, a significant difference in BNP levels was observed between the BMSC plus rhBNP and the BMSC groups ($P < 0.05$). The GATA-4, Cx43 and cTnI expression levels in the BMSC plus rhBNP group were higher than those in the BMSC group. Compared with rhBNP treatment, BMSC transplantation alone does not significantly improve heart function. However, combining rhBNP therapy and BMSC transplantation increases the expression levels of GATA-4 and other proteins to improve cardiac systolic and diastolic function.

Introduction

Acute myocardial infarction is one of main causes of cardiac insufficiency. When complicated by decompensated heart failure, it is one of the primary causes of mortality from coronary heart disease. Certain patients present with acute decompensated heart failure despite receiving standardized anticoagulation and anti-ischemia treatment (including thrombolysis and emergency percutaneous coronary intervention), which necessitates the use of an appropriate cardiotonic to improve the clinical symptoms and prognosis. Recombinant human brain natriuretic peptide (rhBNP) is used to treat acute decompensated heart failure (1-3).

Bone mesenchymal stem cells (BMSCs) are of particular interest to researchers due to their potential to differentiate into myocardial cells, which are convenient and have low tendency to cause immunological reactions and rejection (4-6). In recent years, animal experiments involving BMSC transplantation have been successfully conducted and transplanted BMSCs have been confirmed to improve cardiac function (7-10). However, the results of previous clinical studies using BMSCs are unsatisfactory. BMSCs for use in transplantation are not produced through pure cultures; thus, the transplanted stem cells may be reduced and variable in number, with even fewer transplanted cells surviving to undergo differentiation (11,12). Therefore, numerous studies concerning the treatment of cardiac diseases have focused on identifying non-toxic methods to enhance the survival rate of transplanted cells and to promote their differentiation into myocardial cells. In the current study, a heart failure (HF) model was established in rats. The recombinant hormone rhBNP was administered to the rats in conjunction with BMSC transplantation to investigate the effects of the combination treatment on heart function. The survival rate of the BMSCs in the myocardial tissue and the expression levels of myocardium-specific proteins were also determined to gain new insights into the treatment of heart failure with BMSCs.

Materials and methods

Animals. A total of 40 healthy male Wistar outbred rats, 6-7 weeks old with body weights of 200-220 g were selected for the study. According to the method proposed by

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Siveski-Iliskovic *et al* (13), a HF model was established in the rats. Adriamycin was administered at a dose of 2.5 mg/kg by intraperitoneal injection three times per week for one week. After a two-week interval, Adriamycin was administered for another week. These steps were conducted six times until the total dose reached 15 mg/kg. Following the last injection, the rats were continually observed for 4 weeks. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Xinxiang Medical University.

Isolation and culture of BMSCs. The rats were sacrificed and soaked in 75% ethanol for 10 min. Under sterile conditions, the femurs and tibias were bilaterally dissected to expose the epiphyseal plates. An injector containing phosphate-buffered saline (PBS) was inserted to rinse out the bone marrow completely until the bone cavity became white. Subsequently, the rinsed bone marrow was blown and beaten into a cell suspension. The suspension was centrifuged for 10 min at 1,500 rpm to remove the supernatant liquid. An appropriate amount of Dulbecco's modified Eagle's medium was added to the precipitate and the mixture was blown into a cell suspension and placed into culture flasks for cell culturing. The culture medium was replaced after 72 h, and then again every 2-3 days thereafter. When the cells reached >80% confluence, they were digested with 0.25% pancreatin and passaged to a ratio of 1:2 or 1:3. Cell morphology was observed under a phase contrast microscope daily during passage. The fourth generation of purified cells was selected for cell labeling prior to transplantation. Two days before transplantation, the cells were inoculated into 25-mm culture dishes. Upon reaching 60% confluence, bromodeoxyuridine was added to the cells at a final concentration of 10 mol/l. After incubation for 48 h, the culture medium was removed and the remaining suspension was digested and centrifuged to collect the cells. The collected cells were stored on ice for transplantation.

Grouping. The rats were divided into five experimental groups: i) the normal group (untreated normal healthy rats); ii) the HF group (HF model rats injected with an equivalent amount of normal saline via the tail vein); iii) the BMSC group (HF model rats injected with $\sim 10^7$ cultured BMSCs in 100 μ l via the tail vein); iv) the rhBNP group (HF model rats treated with 15 g/kg rhBNP via the jugular vein once daily for 4 weeks); and v) the BMSC plus rhBNP group (HF model rats injected once with an equivalent amount of BMSCs and an equivalent amount of rhBNP via the tail vein). After the corresponding treatments, the groups were observed for 4 weeks and the related indicators were measured.

Echocardiography. Following the 4-week treatment, high-frequency echocardiographic examination of the chest was conducted using a GE Vivid 7 Color Doppler Ultrasound Imaging Instrument (GE Company, Fairfield, USA). High-frequency ultrasonography was conducted with a 10S probe along the chest wall at 11.4 MHz, with an image depth of 2.0 cm. A dose of 800 mg/kg urethane (20%) was adminis-

tered by intraperitoneal injection. Using two-dimensional ultrasound, the left ventricular end-systolic diameter (LVSD), the left ventricular end-diastolic diameter (LVDD), the left ventricular end-systolic volume (LVSV) and the left ventricular end-diastolic volume (LVDDV) were detected with an M-mode ultrasound. For each parameter, three measurements were taken under a consecutive complete heartbeat cycle and the mean was obtained. The left ventricular ejection fraction (LVEF) and the left ventricular shortening fraction (LVFS) were converted according to Simpson's method.

Polygraph detection of heart function. Following the 4-week treatment, the rats were anesthetized. The right common carotid artery was isolated and a catheter was inserted into the left ventricle. A polygraph was used to record the left ventricular systolic pressure (LVSP), the left ventricular diastolic pressure (LVDP), the heart rate and the maximum variations in left ventricular pressure rise and decline ($\pm dp/dt_{max}$). The data were continuously measured 5 times at 3-min intervals. The results were expressed as the mean of at least 3 stable measurements.

Serum BNP detection. For the various treatment groups, blood was drawn via the tail vein in the morning from the fasted rats. This was performed prior to and following the 4-week treatment. The blood samples were centrifuged to collect the serum. An enzyme-linked immunosorbent assay was used to detect the serum BNP levels according to the manufacturer's instructions (Boster Company, Wuhan, China).

Western blot. Following the 4-week treatment, the rats were sacrificed and thoracotomy was immediately conducted to remove the heart. The hearts were washed with PBS and samples of the left ventricular myocardial tissue were weighed and homogenized in 10 ml cell lysis solution per 1 mg tissue. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the tissue supernatant liquid was collected for further use. Western blot analysis was used to detect the expression levels of myocardium-specific proteins in the tissue sample. Antibodies specific for cardiac troponin I (cTnI; 1:300 dilution), connexin 43 (Cx43; 1:300 dilution) and GAT-binding protein 4 (GATA-4; 1:200 dilution) were used as primary antibodies (Boster Company, Wuhan, China).

Statistical analysis. The SPSS 16.0 statistical package was used for all statistical analyses. Measured data were analyzed by the paired-samples Student's t-test. The correlation between data was analyzed by the Spearman's rank test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Animal models. In this experiment, 40 rats were used for modeling, but only 34 rats survived for further experimentation. Four of the rats suddenly died of unknown causes, whereas two other rats died of massive ascites. Echocardiography was conducted on the rats before and after modeling. The detection results show significant differences in the LVDD and LVSD of the rats before and after modeling (6.52 ± 0.76 vs. 4.18 ± 0.21 mm and 3.81 ± 0.76 vs. 1.10 ± 0.15 mm, respectively;

Table I. Cardiac functions of rats with heart failure analyzed by echocardiography 4 weeks after injection (mean \pm s).

Group	n	LVDD (mm)	LVSD (mm)	LVEF (%)	LVFS (%)
Normal	6	4.21 \pm 0.09	1.03 \pm 0.21	74.21 \pm 1.49	44.21 \pm 1.78
HF	6	6.75 \pm 0.42 ^a	4.25 \pm 0.12 ^a	47.75 \pm 2.42 ^a	20.15 \pm 1.04 ^a
BMSC	8	5.56 \pm 0.71 ^b	2.26 \pm 0.91 ^{b,c}	55.56 \pm 2.73 ^{b,c}	25.26 \pm 1.91 ^{b,c}
BMSC + rhBNP	8	5.32 \pm 0.65 ^{b,c}	2.32 \pm 0.06 ^{b,c}	65.32 \pm 4.35 ^{b,d}	29.12 \pm 1.05 ^{b,d}
rhBNP	7	6.23 \pm 0.61	3.33 \pm 0.41	56.65 \pm 3.61 ^b	24.23 \pm 1.61

LVDD, left ventricular end-diastolic diameter; LVSD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; LVFS, left ventricular shortening fraction; HF, heart failure; BMSC, bone mesenchymal stem cell; rhBNP, recombinant human brain natriuretic peptide.

^aP<0.05 vs. the normal group; ^bP<0.05 vs. the HF group; ^cP<0.05 vs. the rhBNP group; ^dP<0.05 vs. the BMSC group.

Table II. Hemodynamic data of rats with heart failure 4 weeks after injection (mean \pm s).

Group	n	LVSP (kPa)	LVDP (kPa)	+dp/dt _{max} (kPa/sec)	-dp/dt _{max} (kPa/sec)
Normal	6	21.24 \pm 1.09	-0.32 \pm 0.24	974.21 \pm 71.39	724.21 \pm 91.78
HF	6	14.35 \pm 2.62	0.85 \pm 0.36	331.45 \pm 55.82	372.05 \pm 46.84
BMSC	8	16.56 \pm 2.73 ^a	0.31 \pm 0.21 ^a	575.56 \pm 27.73 ^a	475.12 \pm 47.71 ^a
BMSC + rhBNP	8	18.02 \pm 3.85 ^a	0.22 \pm 0.46 ^a	635.52 \pm 34.85 ^{ab}	649.52 \pm 44.55 ^{a,b}
rhBNP	7	16.03 \pm 3.31 ^a	0.33 \pm 0.26 ^a	556.65 \pm 13.64 ^a	524.83 \pm 24.61 ^a

LVSP, left ventricular systolic pressure; LVDP, left ventricular diastolic pressure; dp/dt_{max}, maximum variations in left ventricular pressure; HF, heart failure; BMSC, bone mesenchymal stem cell; rhBNP, recombinant human brain natriuretic peptide. ^aP<0.05 vs. the normal or HF groups;

^bP<0.05 vs. the rhBNP or BMSC groups.

P<0.05). Significant differences were also observed in their LVEF and LVFS before and after modelling (75.34 \pm 8.45 vs. 94.51 \pm 2.61% and 41.28 \pm 1.93 vs. 73.68 \pm 1.87%, respectively; P<0.05). Using the HF model establishment criteria, the LVEF and LVFS dropped by 20-30% of the normal reference values. Five rats presented no evident expansion of their ventricular chamber and had no clear decrease in LVEF. Therefore, these animals were excluded. A total of 29 model rats that fulfilled the HF criteria survived.

Comparison of heart function. The echocardiographic findings in the HF group revealed the typical manifestations of cardiomyopathy and heart failure: the LVSD and LVDD were clearly enlarged and the LVFS and LVEF evidently reduced, which suggest a decline in left heart function. The LVSD, LVDD, LVEF and LVFS in the BMSC, the rhBNP and the BMSC plus rhBNP groups were all significantly different from those in the HF group (P<0.05), which suggests that the left heart functions of these three groups had improved. Among these three groups, the systolic function in the BMSC plus rhBNP group exhibited the most improvement. The improvement was significantly higher than those in the BMSC and rhBNP groups (P<0.05). However, the differences between the BMSC and rhBNP groups were not statistically significant (P>0.05; Table I).

Hemodynamic indicators. Following the 4-week treatment, the hemodynamic-related indicators among the various groups were significantly different (P<0.05). The LVSP and the \pm dp/dt_{max} of the BMSC, the rhBNP and the BMSC plus rhBNP

Table III. Serum BNP levels 4 weeks after injection (ng/l, mean \pm s).

Group	n	Pre-injection	Post-injection
Normal	6	75.04 \pm 7.28	72.32 \pm 6.54
HF	6	554.15 \pm 32.60	597.85 \pm 35.36
BMSC	8	566.86 \pm 19.53	376.31 \pm 27.21 ^{a,b}
BMSC + rhBNP	8	571.62 \pm 13.67	343.36 \pm 17.26 ^{a,c}
rhBNP	7	563.83 \pm 22.49	402.71 \pm 15.76 ^{a,b}

BNP, brain natriuretic peptide; HF, heart failure; BMSC, bone mesenchymal stem cell; rhBNP, recombinant human BNP. ^aP<0.05 vs. the HF group; ^bP<0.05 vs. the pre-injection group; ^cP<0.05 vs. the rhBNP or BMSC groups.

groups were significantly increased and their LVDP values were significantly decreased compared with those of the HF group (P<0.01). These results suggest that the cardiac function had improved, but was not significantly improved over that in the normal group (P<0.05). Compared with the other groups, the \pm dp/dt_{max} in the BMSC plus rhBNP group was significantly improved (P<0.05). No significant differences were observed between the BMSC and rhBNP groups (P>0.05; Table II).

Change of serum BNP levels. Compared with the normal group, the BNP levels of the various treatment groups were

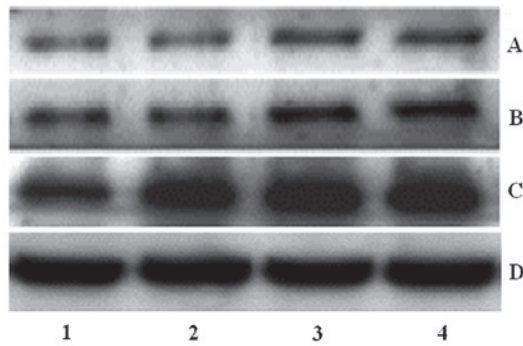


Figure 1. GATA-binding protein 4 (GATA-4), connexin 43 (Cx43) and cardiac troponin I (cTnI) protein expression in rats detected by western blot analysis. (A) GATA-4 protein; (B) Cx43 protein; (C) cTnI protein; (D) β -actin. Lane 1, heart failure (HF) group; lane 2, recombinant human brain natriuretic peptide (rhBNP) group; lane 3, bone mesenchymal stem cell (BMSC) group; lane 4, BMSC + rhBNP group.

significantly increased following treatment. No significant differences in BNP levels were observed among the BMSC, the rhBNP and the BMSC plus rhBNP groups, either before or after treatment ($P < 0.05$). However, following the intervention, the BNP levels of these groups were significantly reduced compared with those of the control group ($P < 0.05$). Following the treatment, the BNP levels in the BMSC plus rhBNP group were significantly reduced compared with those in the BMSC and rhBNP groups ($P < 0.05$). No significant difference in BNP levels was observed between the BMSC and rhBNP groups following the treatment ($P > 0.05$; Table III).

Expression of specific proteins in myocardial tissue. Following the 4-week treatment, the expression levels of specific proteins in the myocardial tissue were detected. The expression levels of GATA-4, cTnI and Cx43 proteins in the BMSC and the BMSC plus rhBNP groups were increased compared with those in the HF group ($P < 0.05$). The GATA-4, cTnI, and Cx43 protein expression levels of the BMSC plus rhBNP group were significantly higher than those of the BMSC group ($P < 0.05$). In addition, the GATA-4 expression level in the BMSC group was higher than that in the rhBNP group ($P < 0.05$; Fig.1).

Discussion

Cardiomyocytes are the most basic structural and functional units of the heart. The change in cardiac function caused by heart disease is essentially caused by the denaturation-induced necrosis of myocardial cells. The traditional drug treatments that target heart failure only relieve the symptoms of the disease and do not fundamentally stop the progression of the disease. For patients with advanced heart failure, heart transplantation is not always a viable option due to the limitations in the number of donors and the risk of transplant rejection. Unlike heart transplantation, the transplantation of functional myocardial tissues to replace injured myocardial tissues of the host to recover cardiac function has broader applications. Therefore, stem cell research to remedy, repair and replace myocardial cells has become a popular research area in the cardiovascular field (14-16).

The rhBNP protein itself has no cardiotonic effect, but it increases the intracellular cyclic GMP (cGMP) concentration, which promotes the relaxation of smooth muscle cells by interacting with the guanylate cyclase in vascular smooth muscle cells and endothelial cells. As a secondary messenger, cGMP dilates arteries and veins, which reduces the systemic arterial pressure, the right atrial pressure and the pulmonary capillary wedge pressure, ultimately reducing the cardiac preload and afterload and improving heart function. In addition, rhBNP inhibits sodium reabsorption in the proximal tubules, which generates a natriuretic effect and further reduces the cardiac preload by enhancing the glomerular filtration rate (17-19). rhBNP is also an antagonist of the renin-angiotensin-aldosterone system (RAAS) and it reduces the excessive activation of renin, aldosterone, endothelin and vasopressin to relieve their adverse effects (20,21). rhBNP is involved in the regulation of blood pressure, blood volume and the water and electrolyte balance. It reduces pulmonary resistance and the plasma volume, thereby reducing the cardiac preload and afterload and improving heart function. Considering that rhBNP has no positive inotropic action, it does not increase myocardial oxygen consumption.

By contrast, dobutamine increases cardiac contractility, quickens atrioventricular conduction and reduces peripheral vascular resistance, mainly through its agonist effect on β_1 receptors, thereby increasing the heart stroke volume and improving heart function. However, under increased cardiac contractility, with larger doses of dobutamine or in highly sensitive patients, the ventricular rate will increase and blood pressure increases abnormally. These factors increase the myocardial oxygen consumption, which is detrimental to patients with acute myocardial infarction.

We selected rats with an LVEF reduced by 20-30% as the experimental model and we selected echocardiography, hemodynamics and the serum BNP concentration as indicators of heart function. The results suggest that BMSC transplantation improves heart function, but the extent of this improvement is relatively low. Between the rhBNP and the BMSC treatments for heart failure, no significant differences were observed in terms of their effect on heart function. Compared with simple cell transplantation, the combination of BMSC transplantation and rhBNP therapy clearly improved heart function.

A question of debate in the field of cell transplantation research is whether it is possible for transplanted cells to be induced to differentiate into myocardial cells. Previous studies have suggested that bone marrow cell transplantation improves the heart function of the patients with myocardial infarction and heart failure. However, these conditions were not improved at the end of the treatment, and its long-term efficacy is controversial. At present, although a few studies have indicated that bone marrow cells have the potential to differentiate into myocardial cells, the consensus on the benefits of cell transplantation on heart function lies in the promotion of angiogenesis and resistance to myocardial apoptosis via paracrine secretion, rather than by their differentiation into myocardial cells. Based on the present study, following BMSC transplantation, the myocardial tissues expressed the transcription factor GATA-4, which is closely related to myocardial cell differentiation. In addition, the HF model rats that were treated with rhBNP expressed low levels of GATA-4, which

was possibly associated with the activation and initial division of the cardiac stem cells after myocardial injury. Following the combined BMSC transplantation and rhBNP treatment, the GATA-4 expression was significantly higher than that after BMSC transplantation or rhBNP treatment alone. The Cx43 and cTnI expression levels were also significantly higher than those in the other groups. Further studies are required to determine whether the aforementioned results are associated with enhanced BMSC differentiation into myocardial cells or the enhanced survival of BMSCs in the myocardial tissue to function in paracrine secretion. In addition, whether the combined effect of BMSCs and rhBNP is simply the sum of two effects or is synergistic should be determined.

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